AETE

Association Européenne de Transfert Embryonnaire

European Embryo Transfer Association

31ème COLLOQUE SCIENTIFIQUE

31st SCIENTIFIC MEETING

*

* *

Dr. Michel Thibier

Special Celebration

* *

*

Ghent, Belgium, 11th and 12th September 2015

AETE

Association Européenne de Transfert Embryonnaire

European Embryo Transfer Association

Board of Governors

President	**Frank Becker, Germany**
Vice President	**Peter Vos, The Netherlands**
Treasurer	**Rainer Saner, Switzerland**
Secretary	**Urban Besenfelder, Austria**
Annual statistics	**Marja Mikkola, Finland**
Newsletter	**Dimitrios Rizos, Spain**
Responsibility students	**Leroy Jo, Belgium**
International relations	**Daniel Le Bourhis, France**
European legislation	**Ian Kippax, U.K.**
Representative French foundation	**Serge Lacaze, France**

MAIN SPONSOR

vetoquinol
ACHIEVE MORE TOGETHER

Thanks to all our sponsors!

GENERAL SPONSORS

CRV

EXHIBITORS

STIMUFOL®

ECM
Echo Control Medical

imv
TECHNOLOGIES

BCF
Technology

PETS
Professional Embryo Transfer Supply, Inc.

minitube

BODINCO B.V.
Hofdijkstraat 2
1814 EC Alkmaar / The Netherlands
ICP bio
REPRODUCTION

Vitrolife

RI | LIFE SCIENCES

STEMα

EGG TECH

SUPPORTERS

MSD
Santé Animale

NIFA
Groupe I.M.V. Technologies

DSM
BRIGHT SCIENCE. BRIGHTER LIVING.

Status 04.09.15

C O N T E N T S

Dr. Michel Thibier

A.E.T.E. Medalist 2015

Dr. Michel Thibier
A.E.T.E. Medalist 2015

Professor Michel THIBIER

Michel Thibier was born on 19 December 1944 in a rural area in Normandy, near Rouen, at Vernon (Eure, France), just across the Seine river from Giverny, the lovely place of the famous impressionist painter, C. Monnet. His birth occurred a few months after D day landings and after the allies crossed Vernon on their way to liberate Paris. Michel has two sisters, one born before the war, and the other afterwards; both are still alive and close to him.

In 1967, Michel Thibier graduated from the National Veterinary school of Maisons Alfort and defended his DVM thesis on the benefits of animal models for experimental surgery, the experimental approach already being something of interest to him.

After some years of practice as a veterinary surgeon in the Alps (at Thônes, Haute Savoie) dealing with the beautiful Abondance cows, in 1969 he began a postgraduate research career, as a young scientist and teacher at the Department of Animal Sciences at the Institut National Agronomique Paris-Grignon (INAPG). At the same time he started a PhD at the Faculty of Sciences, University Pierre & Marie Curie (Paris VI), where he had the opportunity to learn from Professor Charles Thibault. Michel was influenced by Charle's enthusiastic and intensive teaching style which attracted him to both in vivo and in vitro aspects of reproductive physiology, developing then a passion that is still evident today. At these early stages of his career, he was also fortunate to meet two pioneers of the development of reproductive technologies, Michel Parez and Jean Maurice Duplan who successively promoted the use of Artificial Insemination in France and abroad and its implementation for sanitary and genetic purposes. Since then, tackling both reproductive physiology and technologies has been a constant concern for Michel during all his working life, particularly regarding applications for the benefit of farmers and the breeding industry.

Most of his PhD (he graduated as a Docteur of Sciences in 1977) was dedicated to deciphering the endocrinology of male reproductive function while studying especially pituitary

and testis responses to GnRH in young and adult bulls. This has been achieved through successfully setting up radio-immuno assays for steroids and gonadotrophins in the "Hormonology unit" nested in the "Laboratoire de Controle des Reproducteurs" in Maisons Alfort. Part of this work was performed with the fruitful collaboration and friendly support of Marie Therese Hochereau de Reviers (INRA Nouzilly). Simultaneously, he further implemented Radio-Immunoassays to study female fertility and the response to superovulation to accompany the recent development of embryo transfer. This pioneer work in the field of embryo transfer (1975-1976) was accomplished in a national network gathering researchers from INRA such as Daniel Chupin and Jean Saumande, and Michel Nibart from the "Laboratoire de Controle des Reproducteurs".

Following his PhD he became Ass. Professor in the USA (School of Medicine, Dpt of Biochemistry - University of Miami, Fla (1978-1979). He worked intensively on the regulation of Corpus Luteum function under the leadership of late Professor John Marsh and in collaboration with other colleagues at the School of Medicine of the University of Miami (USA). When coming back from the United States he embraced the Direction of the Hormonology Unit in Maisons Alfort again and his Professor position in INAPG. During the following years both Michel Nibart and Michel Thibier played a crucial role in establishing new developments and coordinating field ET activities in the framework of the R&D unit of the National Union of Breeding companies (UNCEIA).

In 1983 he becomes Head of the "Laboratoire de Controle des Reproducteurs" and of the R&D unit of the National Union of Breeding companies (UNCEIA). This gave him the opportunity to fulfill his wishes to use reproductive technologies and especially ET as leverage for efficient genetic selection with the best sanitary guarantees. This was the start of his international career in the field of ET and associated technologies. In 1984, Michel Thibier played a crucial role in bringing AETE into existence while creating the principles and establishing its foundation stones and also as President from 1986 to 1994. His work first as an active member, then as President of the International Embryo Transfer Society (IETS) contributed to strengthen the links between AETE and IETS as well as links with the Brazilian Embryo Transfer Society. At IETS he was one of the founders, together with R. Mapletoft, A. E. Wrathall and D. S. Stringfellow, of the then so called "Import/export committee which was renamed Health And Safety Advisory Committee (HASAC) when he took over the chairmanship in 2000 until he resigned in 2010. Through his activities in scientific societies and specific research work he developed and supported unconditionally the concept, which is still true nowadays, that "ET is definitively the safest way to transfer genes within and between countries" (AETE, 1999).

To summarize, during all the time Michel Thibier was deeply involved in Research and Development work, his main areas of interest included the control of male and female reproductive function through optimization of AI results and prediction of young bulls reproductive performance, treatment of reproductive disorders and early pregnancy diagnosis in cows. He became progressively more and more involved in supporting research and development of all reproductive technologies; ET, embryo freezing, embryo sexing and In Vitro Production and in creating at the same time an official framework for their use. Both have been crucial for successful implementation of these techniques in the field and also International exchanges. During these activities, he has produced an impressive amount of scientific papers, text books, popular science papers, technical and official notes. Most of this work was accomplished through the supervision of a large number of Master students and PhD students from different countries which helped to establish international collaborations. In parallel he developed an internationally recognized expertise in the field of animal reproduction and reproductive technologies through numerous missions in developing countries.

From 1994 to 1999, he continued his activities as the General Director of the Centre National d'Etudes Veterinaires et Alimentaires (CNEVA), a multi site research center, predecessor of the current AFSSA (French Agency of Food safety). His dedication to health prospects and knowledge led him to reinforce his activities in different panels of experts in biotechnology in Inter Governmental Organizations such as the World Animal Health Organization (OIE), Food and Agricultural Organization (FAO) or Non Governmental International Organizations such as IETS. By this time, he became the deputy Permanent Representative and Scientific Counselor of France to FAO in Rome. He was elected as Vice President in 2002 and in 2006 President of the United Nations Committee for Food Security (CFS) at FAO fighting to reduce hunger across the world. His responsibilities in French central administration increased following his nomination as Director General of Education and Research at the Ministry of Agriculture and Fisheries (2002-2006). This included the supervision of agronomic and veterinary education, co administration of National Research Institutions (INRA, CEMAGREF, IFREMER and ANSES) together with extension administrations. From 2006-2009 he acted as the Senior Scientific Counselor at the Embassy of France in Australia (Canberra) before being finally called to the High Advisory Board to the French Minister of Agriculture at the end of 2009.

Professor Michel Thibier is retired from the French Public service from 1st January 2010 and he is currently acting as a private consultant.

While being involved in his administrative activities, working as an official representative, with numerous and demanding tasks Michel Thibier never forgot his main field of interest, the embryo and our society and he has been constantly active for our community. As at the beginning of his career, the same passionate glance and enthusiastic comments pops up when he sees new results from posters or presentations especially in the field of ET.

Michel is married to Catherine and together they have four children, Emmanuel, Sophie, Antoine, and Caroline and 7 grandchildren (from 1 year to 20 years old) … who benefit from their values and example to develop nicely in life. Michel and Catherine live in the lovely village of St Palais, in the Pyrénées Atlantiques not far from Biarritz. A car accident made the past year difficult for Michel. We hope that through our collective good wishes, the winds of fortune will blow more kindly on him in the future.

Professor Dr. Patrice Humblot
Department of Clinical Sciences
Division of Reproduction
Avd för reproduktion, Box 7054
75007 UPPSALA

Commercial Bovine

Embryo Transfer Activity

in Europe 2014

by Marja Mikkola, Finland

Commercial Bovine Embryo Transfer Activity in Europe 2014

General

Country	Collector	N° of approved ET teams	N° of ET teams providing data
Austria	F Führer	5	-
Belgium	P Vercauteren/I Donnay	9	8
Bosnia & Herzegovina	T Markovic	1	1
Czech Republic	P Bucek	-	3
Croatia	M Matkovic	-	-
Denmark	H Callesen	8	8
Estonia	J Kurykin	1	1
Finland	S Vahtiala	4	3
France	S Lacaze	19	15
Germany	H Cramer	39	17
Greece	F Samartzi	2	2
Hungary	F Flink	4	2
Ireland	P Lonergan	10	4
Italy	G Lazzari	-	-
Kazakhstan	A Zhanserik	2	1
Latvia	V Antane	1	1
Lithuania	G Palubinskas, V Zilaitis, J Kutra	1	1
Luxembourg	M Vaessen	2	2
The Netherlands	H Flapper	6	6
Norway	E Kummen	-	-
Poland	J Jaskowski	7	7
Portugal	J Chagas e Silva	11	6
Russian Federation	D Knurov	7	5
Slovenia	J Mrkun	1	1
Spain	S Fuentes	21	11
Sweden	C Andersson	2	2
Switzerland	R Saner	7	4
Turkey	E Emsen	2	1

Bovine *In vivo* embryo production 2014

Country	Nº of embryo collections	Nº of embryos collected	Nº of transferable embryos	Nº of transferable/ flush	% of collections with sexed semen	Distribution of breeds, % of collections (dairy/ beef / dual purpose)
Austria	212	2390	1578	7.4	1.4	14.2 / 9.9 / 75.9
Belgium	1064	6617	5138	4.8	0.9	17.5 / 82.5 / 0
Czech Republic	4	50	28	7.0	0.0	0 / 25.0 / 75.0
Denmark	642	5862	4428	6.9	0.3	91.4 / 8.6 / 0
Finland	459	5299	3617	7.9	18.5	99.8 / 0.2 / 0
France	6859	66403	37811	5.5	10.4	82.2 / 17.8 / 0
Germany	2904	27760	17877	6.2	-	78.9 / 0 / 21.1
Hungary	76	1010	675	8.9	-	17.1 / 76.3 / 6.6
Ireland	610	6161	3721	6.1	-	-
Italy	2218	26728	17726	8.0	-	97.7 / 2.3 / 0
Kazakhstan	82	509	420	5.1	0.0	47.6 / 52.4 / 0
Lithuania	85	605	447	5.3	0.0	84.7 / 3.5 / 11.8
Luxembourg	189	2031	1282	6.8	3.2	97.4 / 2.6 / 0
The Netherlands	5220	33096	32556	6.2	-	-
Norway	10	-	-	-	-	-
Poland	167	1449	1098	6.6	3.6	100.0 / 0 / 0
Portugal	71	869	494	7.0	2.8	97.2 / 2.8 / 0
Russian Federation	477	3058	2746	5.8	0.0	83.9 / 16.1 / 0
Slovenia	5	14	10	2.0	0.0	100.0 / 0 / 0
Spain	575	5237	3002	5.2	15.8	84.3 / 15.7 / 0
Sweden	100	738	395	4.0	18.0	92.0 / 8.0 / 0
Switzerland	461	5053	3369	7.3	8.2	94.1 / 2.6 / 3.3
Total	22490	200 939	138 418	6.2		

Countries reporting zero embryo collections:
Bosnia Herzegovina, Croatia, Estonia, Greece, Latvia, Turkey

Bovine *In vitro* production, OPU

Country	N° of OPU sessions	N° of oocytes collected	N° of transferable embryos
France	159	1300	610
Germany	1907	3632	2853
Italy	654	10310	1518
The Netherlands	5060	43825	5815
Portugal	15	132	1
Spain	896	13178	1898
Russian Federation	1019	11408	2998
Total	**9710**	**83785**	**15693**

Bovine *In vitro* production, "slaughtered donor"

Country	N° of oocyte donors	N° of oocytes collected	N° of transferable embryos
Czech Republic	35	526	223
France	1	5	1
Germany	28	2480	183
Italy	21	780	111
Lithuania	3	11	0
The Netherlands	106	21103	266
Portugal	1141	12389	557
Switzerland		120	28
Total	1335	37414	1369

Bovine Embryo Technologies

Country	N° of sexed embryos (via biopsy)		N° of genotyped embryos	
	In vivo	In vitro	*In vivo*	*In vitro*
France	974	20	684	-
Netherlands	537	0	537	0
Germany	251	0	251	0
Total	1762	20	1472	0

Bovine Embryo Transfer

Country	N° of transferred embryos							N° of exported embryos
	In vivo			*In vitro*			Total	
	Fresh	Frozen domestic	Frozen foreign	Fresh	Frozen domestic	Frozen foreign		
Austria	450	1003	3	0	0	0	1456	0
Belgium	1203	4368	1180	0	0	0	6751	90
Czech Republic	27	0	0	0	5	0	32	0
Denmark	2443	1269	-	-	-	-	3712	61
Estonia	0	7	24	0	0	0	31	0
Finland	942	1720	621	0	0	0	3283	8
France	18657	17249	1051	196	194	-	37347	562
Germany	19132	-	-	2765	-	-	21897	-
Hungary	220	315	101	-	-	-	636	15
Italy	6115	-	-	384	1074	-	7573	-
Ireland	834	947	-	450	-	-	2231	-
Kazakhstan	-	400	-	-	-	-	400	-
Lithuania	66	42	0	0	0	0	108	0
Luxembourg	450	950	-	-	30	-	1430	50
The Netherlands	5888	26522	-	4885	628	-	37923	1040
Norway	-	-	200	-	-	-	200	-
Poland	600	300	-	-	-	-	900	-
Portugal	56	32	31	10	34	-	163	-
Russian Federation	878	565	464	1781	483	-	4171	100
Slovenia	8	1	1	-	-	-	10	0
Spain	693	1428	141	959	479	10	3710	66
Sweden	164	231	514	0	0	0	909	0
Switzerland	720	1678	476	0	0	55	2929	151
Total	**59546**	**59027**	**4807**	**11430**	**2927**	**65**	**137802**	**2143**

Countries reporting zero embryo transfers:
Bosnia Herzegovina, Croatia, Greece, Latvia, Moldova, Turkey

Embryo Activities in other Species

Country	Sheep			
	N° embryo produced	N° embryo transfers	Fresh	Frozen
Portugal	187 (IVP)			
Turkey	90	90	40	50
France				8 (goat)
Total	277	90	40	58

Country	Horse				
	N° collections	N° embryo produced	N° embryo transfers	Fresh	Frozen
France	606	365	365	365	
Poland	18	15	11	11	
Portugal			2		2
Switzerland	30				
Total *(In vivo)*	654	380	378	376	2
	N° OPU-ICSI sessions	**N° oocytes collected**	**N° embryo produced**	**Fresh**	**Frozen**
Italy *(In vitro)*	195	2050	141	8	75

INVITED LECTURES

ESTRUS DETECTION TOOLS AND THEIR APPLICABILITY IN CATTLE: RECENT AND PERSPECTIVAL SITUATION

ROELOFS JB, VAN ERP-VAN DER KOOIJ E

HAS University of applied sciences, P.O. Box 90108, 5200 MA, Den Bosch, The Netherlands

Corresponding author:
J.B. Roelofs
Phone: 0031888903600
Fax: 0031888903699
Email: j.roelofs@has.nl
Running title: Estrus detection tools in dairy cattle

Abstract

Good reproduction is a key for successful dairy farming. Detection of estrus is the first step in getting a cow pregnant. Visual detection of estrus is a challenging job, to aid the farmer, estrus detection tools (EDT), such as pedometers, neck mounted collars to measure activity and pressure sensing devices to measure standing estrus, have been developed. EDT have proven useful in practical dairy farming, however, studies from the last five years reveal a great variation in sensitivity, specificity and positive predictive values. In research, the standard that is used to define a true estrus period can affect the performance of the EDT under investigation. Cow factors that can affect performance of EDT are number of ovulation after calving, milk production, lactation number, body condition score and lameness. The second step in getting a cow pregnant is insemination at the correct time. With EDT it is easier to determine optimal insemination time, which is 12 to 24 hours before ovulation. The optimal time interval in which to inseminate seems to be about 5 to 17 hours after an increase in activity as measured by pedometers or neck mounted collars. Novel measurements, such as rumination time, eating time, lying behavior, ultra-wide band technology to measure mounting and standing-to-be-mounted behavior and infrared thermography to measure temperature are being studied to further aid estrus detection.

Keywords: estrus, technology, insemination, dairy cattle

Introduction

Good reproduction is key for successful dairy farming. Detection of estrus is the first step in getting a cow pregnant. Visual detection of estrus is a challenging job. The expression of standing estrus is only shown by about 50% of cows in estrus and lasts for a short period of time of about 5 to 7 hours (Roelofs et al., 2005a; Sveberg et al., 2011). To aid farmers in detecting estrus and determining the optimal insemination time, many estrus detection tools (EDT) have been developed (reviewed by Roelofs et al., 2010; Saint-Dizier and Chastant-Maillard, 2012). For example, an increase in activity associated with estrus can be measured by pedometers or neck mounted collars and pressure sensing devices are on the market to detect cows expressing standing estrus.

A true estrus period can be detected by an EDT (true positive alert: TP) or not detected (false negative alert: FN). Outside a true estrus period, an EDT can give no alert (true negative alert: TN) or can give an alert (false positive alert: FP). To assess the performance of an EDT, sensitivity (TP/(TP+FN)), positive predictive value (TP/(TP+FP)) and specificity (TN/(TN+FP)) are often used (Roelofs et al., 2010). Rutten et al. (2014) concluded that an investment in activity meters for estrus detection is likely to be profitable for most dairy farms; however, this strongly depends on the increase in sensitivity that activity meters achieve, as compared with visual estrus detection.

Although automated activity monitoring systems have proven useful as EDT in practical dairy farming (Michaelis et al., 2013; Neves and LeBlanc, 2015), studies from the last five years reveal a great variation in sensitivity and positive predictive values.

The second step in getting a cow pregnant is insemination at the correct time. The optimal time for insemination is 12 to 24 hours before ovulation (Trimberger et al., 1948; Roelofs et al., 2006). Pedometers and neck mounted collars can be used to predict the time of ovulation (Roelofs et al., 2005b; Hockey et al., 2010) and therefore aid the farmer in deciding when to inseminate a cow.

In this review the performance and factors affecting the performance of different EDT will be discussed. Because this is elaborately reviewed by Saint-Dizier and Chastant-Maillard (2012), only studies performed over the last five years will be discussed in this review. The timing of insemination based on EDT and the effect on pregnancy rate will be discussed. New measurements that can aid in the detection of estrus will be reviewed.

Performance of EDT

Sensitivity and positive predictive value (PPV) varies between studies and EDT. In table 1 the performance of different EDT is presented. Sensitivity ranged from 36% to 78% and is in all studies greater than the sensitivity of visual observations (range: 20 to 59%). PPV ranged from 74% to 97% and is not consistently better or worse than visual observations (Palmer et al., 2010; Holman et al., 2011; Michaelis et al., 2014). When pedometers were compared with neck mounted collars, sensitivity was greater but PPV was less for pedometers (Holman et al., 2011; Chanvallon et al., 2014). A better sensitivity means less false negative alerts, resulting in more detected true estrus periods. A better PPV means less false positive alerts, so less alerts are given when a cow is not in estrus. The design of the study can influence the number of false positive and false negative alerts. The number of FP and FN alerts as generated by the EDT depends on the definition of a true estrus period. Measurements of milk progesterone concentration are often used as golden standard for a true estrus period. Milk samples are collected 2 to 3 times weekly and a period of low progesterone, followed by a period of high progesterone, is considered to be a true estrus period. Based on individual progesterone profiles TP, FN and FP alerts from the EDT are assigned (Palmer et al., 2010; Holman et al., 2011; Kamphuis et al., 2012; Chanvallon et al, 2014). Using this definition for true estrus, cows that do not show any estrous behavior before ovulation (silent ovulation) will have more FN alerts resulting in lower sensitivities. This is not a malfunction of the EDT, but rather a physiological issue. Factors that can play a role in these FN alerts are discussed further on in this review. Another golden standard that is used for a true estrus period is the day of an insemination which led to a pregnancy (Jónsson et al., 2011). Using this definition silent ovulations do not generate a FN alert, because a cow is not inseminated when she is not detected in estrus at all. Sensitivity is likely to be greater when this golden standard is used. Michealis et al. (2014) used 21-day cow-periods according to the cycle length. After the voluntary waiting period, every cow started into a first 21-day cow-period and cows were observed for numerous cow-periods. When a cow was reported to be in estrus by visual observation or activity as measured by a neck mounted collar, estrus was confirmed by rectal palpation, ultrasonography and a blood sample for progesterone analysis. FN alerts were assigned when no alert was generated in a 21-day cow-period. Besides silent ovulations which generates a FN alert, the luteal phase can be prolonged or a cystic ovarian follicle can develop, which means that no ovulation occurs in a 21-day period (Lamming and Darwash, 1998). Therefore, an overestimation of FN alerts will probably occur by using 21-day cow-periods as golden standard. This might explain the low sensitivity found in the study of Michaelis et al. (2014) by neck mounted collars (36%).

Factors influencing the performance

Different factors play a role in the number of false positive and false negative alerts generated by an EDT. It is clear that the threshold at which an estrus alert is generated by an EDT has a great impact on the sensitivity and PPV (Roelofs et al., 2005b; Hockey et al., 2010; Kamphuis et al.,

2012). Physiological factors might also play a role in the performance of an EDT. Factors that decrease the expression of estrus will also decrease the sensitivity of an EDT when progesterone analyses are used as golden standard for a true estrus period. The first ovulation after calving is often not accompanied by an increase in activity or standing heat. Sensitivity of neck mounted collars for first ovulations after calving was found to be 23 and 30% in two studies (Aungier et al., 2012; Chanvallon et al., 2014). Sensitivity increased to 80% for second and later ovulations after calving. The same was found for estrus detection with pedometers (Chanvallon et al., 2014), where sensitivity for first ovulations after calving was 40% compared with 86% for subsequent ovulations. Ranasinghe et al. (2010) studied sensitivity of first, second, third and fourth ovulations after calving, which resulted in sensitivities of 45%, 76%, 79%, and 89%, respectively. In normal, healthy cows, first ovulation occurs on average 28 days after calving (Johnson et al., 2012; Chanvallon et al., 2014). In practice, the voluntary waiting period is usually around 50 days. So, the low sensitivity of estrus detection found for first ovulations is not really an issue in practice. When however, many cows in a herd have an extended post partum anestrus, the performance of an EDT will be less. In interpreting and comparing research findings, it is important to take into account whether or not first ovulations were included in the calculations of sensitivity.

Lactation rank, milk protein content, body condition score, milk production, lameness and somatic cell count are studied for their effect on the performance of EDT. A high peak milk production as well as an above average daily milk yield and high production at the time of a preovulatory follicular phase were found to negatively affect sensitivity of neck mounted collars or pedometers. Sensitivity of neck mounted collars was 36% for cows with a peak milk production of more than 40kg, whereas sensitivity was 68% for cows with a peak milk production of less than 35 kg (Chanvallon et al., 2014). Another study that investigated neck mounted collars and pedometers found a sensitivity of around 37% for cows with above average daily milk yield compared with around 60% sensitivity for all cows for both EDT (Holman et al., 2011). Aungier et al. (2012) concluded that if a cow was producing 10 kg less than another cow that was also in a preovulatory follicular phase, the odds of her preovulatory phase being detected by a neck mounted collar were greater by 67%.

Body condition scores of less than two resulted in a very low sensitivity for neck mounted collars (0%) and pedometers (20%, Holman et al., 2011). Only a few cows were in this category, so firm conclusion could not be drawn. Aungier et al. (2012) found that detection of a true estrus period by a neck mounted collar increased by a factor of 1.383 for each additional 0.25 BCS unit. No effect of somatic cell count (Holman et al., 2011; Aungier et al., 2012) on sensitivity of EDT was found. Milk protein content did (Talukder et al., 2015) or did not affect (Aungier et al., 2012; Chanvallon et al, 2014) sensitivity of EDT. Aungier et al. (2012) did not find a lower sensitivity in lame cows compared with non lame cows, whereas others did find a lower sensitivity in lame cows (Holman et al., 2011; Talukder et al., 2015). Lactation number did not affect sensitivity of neck mounted collars (Aungier et al., 2012) but did affect sensitivity of pedometers (Chanvallon et al., 2014). For cows in their first lactation, a higher sensitivity (77%) was found compared with cows with higher lactation number (52%). This is in agreement with the study of Roelofs et al. (2005b) in which cows in their first lactation had a longer duration of increase in activity and higher maximum steps during the increase in activity compared with cows with higher lactation number. Depending on the threshold calculation of an estrous related activity increase, a longer period of increased activity and more steps are more likely to give an alert.

Timing of insemination

To be able to give accurate insemination advice based on oestrus detection technologies, the parameters that are measured by the EDT to indicate onset of estrus should have a strong correlation with the time of ovulation and should be consistent between animals. A few studies have looked at the time of ovulation relative to the onset of estrus. The time of ovulation relative to the onset of estrus as measured by EDT is quite consistent between different studies. Intervals of 29.3±3.9h (n=63 ovulations) and 30.2±0.6h (n=20 ovulations) between the onset of oestrus based on

pedometer measurements to ovulation were found in Holstein-Friesian and Japanese black cows, respectively (Roelofs et al., 2005, Yoshioka et al., 2010). This agrees with the interval of 28.7h (n=60 ovulations) between onset of estrus based on neck mounted collars and ovulation in synchronized dairy cows (Valenza et al., 2012). An interval of 33.4±12.4h (n=94 ovulations) was found between the onset of estrus as detected by neck collars and ovulation (Hockey et al., 2010). The interval between the first standing estrus as detected by a pressure sensing system and the time of ovulation was found to be 27.6±5.4h (n=67 ovulations, Walker et al., 1996) and 29.0±0.6h in Japanese black cows (n=20 ovulations, Yoshioka et al., 2010). The consistency in these intervals indicates that activity meters or pressure sensing systems can be used to predict time of ovulation and advise on optimal time of insemination.

The optimal time of insemination relative to ovulation was found to be 24 to 12h before ovulation (Trimberger, 1948; Pursley et al., 1998; Roelofs et al, 2006).

In 1948 the a.m. – p.m. guideline for time of insemination was established. This guideline recommends that cows observed in estrus in the morning should be inseminated in the afternoon, and cows observed in estrus during the afternoon should be inseminated the following morning (Trimberger, 1948). Since then several studies have examined the optimal time for insemination relative to the onset of estrus as detected by an EDT (table 2). Combining the optimal time of insemination relative to ovulation, with the time of ovulation after detection of the onset of estrus, will give an optimal estrus to insemination interval. Roelofs et al. (2005) calculated this interval to be 5 to 17h after the onset of increased activity as measured by pedometers. This interval is comparable to the interval found in other studies on pedometers, electric pressure sensing systems or neck mounted collars (Table 2). However, the interval that Hockey et al. (2010) found is noticeably different. Even though they found about the same interval between onset of estrus and ovulation as in other studies, the optimal time for insemination relative to ovulation was much later (16-0h before ovulation). This could explain why the optimal interval between onset of estrus and insemination is later. The reason for this discrepancy in optimal timing of insemination relative to ovulation is not clear.

Stevenson et al. (2013) found a difference in optimal interval for insemination relative to the onset of estrus based on neck mounted collars, between primiparous and multiparous cows. In primiparous cows, inseminations between 13 and 16h after onset of estrus resulted in the highest conception rates, whereas in multiparous cows insemination less than 12h after onset of estrus resulted in the highest conception rates. Primiparous cows had a longer lasting increase in activity as measured by pedometers compared with multiparous cows, but the interval between onset of increased activity and ovulation was not different (Roelofs et al., 2005b). This would mean that in primiparous cows, the interval in which an insemination results in comparable conception rates is larger than in multiparous cows. Consequently, insemination shortly before ovulation does not compromise conception rates in primiparous cows as it does in multiparous cows. A possible explanation could be a difference in quality and thereby the fertile lifespan of an oocyte. Primiparous cows have lower NEFA concentration after calving compared with multiparous cows (Wathes et al., 2007). Elevated NEFA exposure can compromise follicle growth and result in inferior quality oocytes (Van Hoeck et al., 2014). When the fertile lifespan of the oocyte is compromised, it is more important to have the sperm at the site of fertilisation ready when ovulation occurs. The difference in optimal insemination interval between primi- and multiparous cows is worth further investigation.

A study with sex-sorted semen in dairy heifers resulted in the highest conception rates for inseminations performed between 20 and 24 hours after the onset of estrus as detected by a pressure sensing device (Sá Filho et al., 2010). Further research on optimal insemination intervals for heifers as well as for the use of sex-sorted semen is needed to optimise reproductive efficiency on dairy farms.

New measurements for estrus detection

Research on increased activity associated with estrus has already been performed more than 60 years ago (Farris, 1954). In the last five years, other measurements to aid in estrus detection have been studied. Among these new measurements are lying, eating and ruminating behaviour, feed intake, water intake, temperature measurements, body weight, sound and motion measurements. Jónsson et al. (2011) automatically recorded lying behavior as well as number of steps. True estrus periods (n=18) were defined as periods around inseminations that led to confirmed pregnancy. Sensitivity was 50% when only lying behavior was used to detect estrus. A combination of number of steps and lying behavior, did not result in a higher sensitivity than using number of steps alone (89%). Specificity was high for the number of steps (99.4%), lying behaviour (99.6%) and the combination (99.8%). PPV increased by 10% when lying behaviour was combined with the number of steps, so less false positive alerts were generated compared to using number of steps alone. Silper et al. (2015) studied lying and standing behaviour in heifers. An increase in activity (as measured by the number of steps) combined with ovarian ultrasonography was used to define a true estrus period. Both lying and standing behaviour differed on the day of estrus compared with non-estrus days. A large variation was found between heifers in both standing and lying measurements. Especially the length of the longest standing bout and its relationship with the time of onset estrus (as measured by increased number of steps) seems a promising aid in estrus detection. Measurements of lying behavior, standing behavior and number of steps can be combined in a sensor. The combination of these measurements is likely to result in less false positive alerts than measurement of increase in number of steps alone. This can lead to less inseminations performed on cows not in estrus.

Changes in rumination time around estrus have been studied in the last few years (Reith and Hoy, 2012; Reith et al., 2014a; Talukder et al., 2014; Pahl et al., 2015). One study found that measurements of rumination time alone or the combination of rumination time and activity did not result in a more accurate estrus detection performance than activity alone (Talukder et al, 2014). This finding does not agree with other studies that found that measurements of rumination time could aid in estrus detection (Reith and Hoy, 2012; Reith et al., 2014a; Pahl et al., 2015). In those studies rumination time was reduced by an average 20%, on the day before insemination (Pahl et al., 2015) or on the day of estrus as defined by activity measurements or visual observation (Reith and Hoy, 2012; Reith et al., 2014a). A >10% decrease in rumination time on the estrus day was found in more than 70% of the cows, whereas about 6% of the cows showed an increased rumination time on the estrus day. A high variation in the decrease of rumination time was found (Reith and Hoy, 2012). Feeding time and roughage intake decreased around estrus with approximately 20% and 10%, respectively (Reith et al., 2014b; Halli et al., 2015; Pahl et al. 2015). Concentrate intake was not affected by estrus (Reith et al., 2014b). Rumination time and eating time can be measured automatically by neck mounted collars (e.g. SCR heatime, Nedap smarttag neck), but individual roughage intake is difficult to measure in practice. Therefore, measurements of rumination and eating time to aid in estrus detection are promising. More research on the factors affecting rumination and eating time and sensitivity, PPV and specificity, however, is needed.

Vaginal temperature increases before ovulation (Rajamahendran et al., 1989). Recently, studies were done to see whether infrared thermography could be used to detect estrus and predict time of ovulation. In one study, sensitivity of 75% was found with infrared thermography of the vulva and muzzle every four hours. This sensitivity was higher than the sensitivity with six times daily visual observations (67%). Specificity and PPV, however, were lower with infrared thermography (57% and 69%, respectively) compared with visual observations (86% and 89% respectively, Talukder et al., 2014). A study done by the same group in which eye, vulva and muzzle temperature were measured using infrared technology showed poor performance for detecting estrus (Talukder et al., 2015).

A novel approach to detect estrus is the use of ultra-wide band technology (UWB). This technology can measure 3-dimensional positioning and could be used to monitoring mounting and standing-to-be-mounted behavior. In a study, 9 out of 9 possible cows were detected in estrus

automatically by UWB technology and 6 out of 6 cows were correctly identified as not in estrus (Homer et al., 2013). Roelofs et al. (2005a) found that 90% of cows in estrus showed mounting behavior, whereas only 58% of cows in estrus showed standing-to-be-mounted behavior. The first mount was displayed on average 29 hours before ovulation. Automatic detection of mounting behavior could be a helpful tool in detection of estrus and determining optimal insemination time.

Table 1. Sensitivity and positive predictive value (PPV) of different estrus detection tools (EDT).

References	EDT	sensitivity	PPV	housing	GS
Palmer et al., 2010	pressure sensing device	69%	97%	pasture	P_4
	tail paint	65%	94%		
	VO[1] (3 times/day, 20 min.)	59%	97%		
Palmer et al., 2010	pressure sensing device	37%	77%	indoors	P_4
	tail paint	26%	92%		
	VO[1] (3 times/day, 20 min.)	20%	100%		
Holman et al., 2011	neck mounted collar	59%	94%	indoors	P_4
	pedometer	63%	74%		
	VO[2] (6 times/day, 10 min.)	57%	93%		
Kamphuis et al., 2012	neck mounted collar	78%	78%	pasture	P_4
	tail paint	91%	95%		
Chanvallon et al., 2014	neck mounted collar	62%	83%	indoors	P_4
	pedometer	71%	71%		
Michaelis et al., 2014	neck mounted collar	36%	84%	indoors	21dp
	VO[2] (2 times/day, 30 min.)	34%	75%		
Hockey et al., 2010	neck mounted collar	90%	76%	pasture	P_4
Jónsson et al., 2011	pedometer	89%	84%	indoor	preg
Aungier et al., 2012	neck mounted collar	72%	67%	pasture	P_4
Talukder et al., 2015	neck mounted collar	80%	67%	pasture	P_4

VO = visual observation
[1] Visual observation of standing to be mounted
[2] Visual observation of vulva sniffing/being sniffed, chin-resting/being chin-rested on, mounting other cows and standing to be mounted, mucoid or bloody vaginal discharge.
GS = golden standard for true estrus period
P_4 = 2 or 3 times weekly milk sampling for progesterone concentrations
21dp = 21-day cow-periods according to the cycle length
preg = confirmed pregnancy

In conclusion

Performance of estrus detection tools varies between studies, but is overall better than visual observation of estrus. Taking into account factors that affect the performance of EDT such as first ovulations after calving, high milk production, lactation rank etc. and possibly adjusting the calculations for the threshold used to generate an alert might increase the performance of pedometers and neck mounted collars. Because the beginning of estrus is detected by the EDT, inseminations can be better timed, thus increasing conception rates. An interesting area of research is optimal insemination time in heifers, and when using sex-sorted semen. Other behavioural

measurements, and measurements of physiological traits associated with estrus, are studied to aid in the detection of estrus and determining optimal insemination time. The combination of activity measurements and rumination, eating and lying time measurements seems promising.

Table 2. Optimal insemination intervals after onset of estrus as detected by different estrus detection tools (EDT)

References	EDT	Optimal insemination interval after onset of estrus (h)
Maatje et al., 1997	Pedometers	6 - 17
Roelofs et al., 2005	Pedometers	5 – 17
Yoshioka et al., 2010	Pedometers	10 -18
Stevenson et al., 2013	Neck mounted collars	13 -16[1] 9 -12[2]
Hockey et al., 2010	Neck mounted collars	24 - 40
Dransfield et al., 1998	Pressure sensing device	4 -12
Xu et al., 1998	Pressure sensing device	12 – 18
Dalton et al., 2001	Pressure sensing device	12

[1] primiparous cows
[2] multiparous cows

Reference list

1. Aungier SPM, Roche JF, Sheehy M, Crowe MA. 2012. Effects of management and health on the use of activity monitoring for estrus detection in dairy cows. J Dairy Sci, 95: 2452-2466.

2. Chanvallon A, Coyral-Castel S, Gatien J, Lamy J-M, Ribaud D, Allain C, Clément P, Salvetti P. 2014. Comparison of three devices for the automated detection of estrus in dairy cows. Theriogenology, 82:734-741.

3. Dalton JC, Nadir S, Bame JH, Noftsinger M, Nebel RL, Saacke RG. 2001. Effect of time of insemination on number of accessory sperm, fertilization rate, and embryo quality in nonlactating dairy cattle. J Dairy Sci, 84:2413-2418.

4. Dransfield MBG, Nebel RL, Pearson RE, Warnick LD. 1998. Timing of insemination for dairy cows identified in estrus by a telemetric estrus detection system. J Dairy Sci, 81:1874-1882.

5. Farris EJ. 1954. Activity of dairy cows during estrus. J Am Vet Med Ass, 125:117-120.

6. Halli K, Koch C, Romberg F-J, Hoy S. 2015. Investigations on automatically measured feed intake amount in dairy cows during the oestrus period. Arch Anim Breed, 58:93-98.

7. Hockey CD, Morton JM, Norman ST, McGowan MR. 2010. Evaluation of a neck mounted 2-hourly activity meter system for detecting cows about to ovulate in two paddock-based australian dairy herds. Reprod Dom Anim, 45:E107-117.

8. Holman A, Thompson J, Routly JE, Cameron J, Jones DN, Grove-White D, Smith RF, Dobson H. 2011. Comparison of oestrus detection methods in dairy cattle. Vet Rec, 169:47-52.

9. Homer EM, Gao Y, Meng X, Dodson A, Webb R, Garnsworthy PC. 2013. Technical note: A novel approach to the detection of estrus in dairy cows using ultra-wideband technology. J Dairy Sci, 96:6529-6534.

10. Johnson CR, Ayers MW, Ahmadzadeh A, Shafii B, Etter S, Chebel RC, Dalton JC. 2012. Short communication: characterization of early postpartum estrous behavior and ovulation in lactating dairy cows using radiotelemetry. J Dairy Sci, 95:5085-5088.

11. Jónsson R, Blanke M, Poulsen NK, Caponetti F, Hosjsgaard S. 2011. Oestrus detection in dairy cows from activity and lying data using on-line individual models. Comp Elec Agri, 76:6-15.

12. Kamphuis C, DelaRue B, Burke CR, Jago J. 2012. Field evaluation of 2 collar-mounted activity meters for detecting cows in estrus on a large pasture-grazed dairy farm. J Dairy Sci, 95:3045-3056.

13. Lamming GE, Darwash AO. 1998. The use of milk progesterone profiles to characterise components of subfertility in milked dairy cows. Anim Reprod Sci, 52:175-190.

14. Maatje K, Loeffler SH, Engel B. 1997. Predicting optimal time of insemination in cows that show visual signs of estrus by estimating onset of estrus with pedometers. J Dairy Sci, 80:1098-1105.

15. Michaelis I, Burfeind O, Heuwieser W. 2014. Evaluation of oestrous detection in dairy cattle comparing an automated activity monitoring system to visual observation. Reprod Dom Anim, 49:621-628.

16. Michaelis I, Hasenpusch E, Heuwieser W. 2013. Estrus detection in dairy cattle: changes after the introduction of an autmated activity monitoring system? Tier Prax Gross, 3:159-165.

17. Neves RC, LeBlanc SJ. 2015. Reproductive management practices and performance of Canadian dairy herds using automated activity-monitoring systems. J Dairy Sci, 98:2801-2811.

18. Pahl C, Hartung E, Mahlkow-Nerge K, Haeussermann A. 2015. Feeding characteristics and rumination time of dairy cows around estrus. J Dairy Sci, 98:148-154.

19. Palmer MA, Olmos G, Boyle LA, Mee JF. 2010. Estrus detection and estrus characteristics in housed and pastured Holstein-Friesian cows. Theriogenology, 74:255-264.

20. Pursley JR, Silcox RW, Wiltbank MC. 1998. Effect of time of artificial insemination on pregnancy rates, calving rates, pregnancy loss, and gender ratio after synchronization of ovulation in lactating dairy cows. J Dairy Sci, 81:2139-2144.

21. Rajamahendran R, Robinson J, Desbottes S, Walton JS. 1989. Temporal relationships among estrus, body temperature, milk yield, progesterone and luteinizing hormone levels, and ovulation in dairy cows. Theriogenology, 31:1173-1182.

22. Ranasinghe RMSBK, Nakao T, Yamada K, Koike K. 2010. Silent ovulation, based on walking activity and milk progesterone concentrations, in Holstein cows housed in a free-stall barn. Theriogenology, 73:942-949.

23. Reith S, Hoy S. 2012. Relationship between daily rumination time and estrus of dairy cows. J Dairy Sci, 95:6416-6420.

24. Reith S, Brandt H, Hoy S. 2014a. Simultaneous analysis of activity and rumination time, based on collar-mounted sensor technology, of dairy cows over the peri-estrus period. Livest Sci, 170:219-227.

25. Reith S, Pries M, Verhülsdonk C, Brandt H, Hoy S. 2014b. Influence of estrus on dry matter intake, water intake and BW of dairy cows. Animal, 8:748-753.

26. Roelofs JB, Van Eerdenburg FJCM, Soede NM, Kemp B. 2005a. Various behavioral signs of estrous and their relationship with time of ovulation in dairy cattle. Theriogenology, 63:1366-1377.

27. Roelofs JB, Van Eerdenburg FJCM, Soede NM, Kemp B. 2005b. Pedometer readings for estrous detection and as predictor for time of ovulation in dairy cattle. Theriogenology, 64:1690-11703.

28. Roelofs JB, Graat EAM, Mullaart E, Soede NM, Voskamp-Harkema W, Kemp B. 2006. Effects of insemination-ovulation interval on fertilization rates and embryo characteristics in dairy cattle. Theriogenology, 66:2173-2181.

29. Roelofs JB, López-Gatius F, Hunter RHF, Van Eerdenburg FJCM, Hanzen Ch. 2010. When is a cow in estrus? Clinical and practical aspects. Theriogenology, 74:327-344.

30. Rutten CJ, Steeneveld W, Inchaisri C, Hogeveen H. 2014. An ex ante analysis on the use of activity meters for automated estrus detection: To invest or not to invest? J Dairy Sci, 97:6869-6887.

31. Sá Filho MF, Ayres H, Ferreira RM, Nichi M, Fosado M, Campos Filho EP, Baruselli PS. 2010. Strategies to improve pregnancy per insemination using sex-sortedin dairy heifers detected in estrus. Theriogenology, 74:1636-1642.

32. Saint-Dizier M, Chastant-Maillard S. 2012. Towards an automated detection of oestrus in dairy cattle. Reprod Dom Anim, 47:1056-1061.

33. Silper BF, Polsky L, Luu J, Burnett TA, Rushen J, de Passillé AM, Cerri RL. 2015. Automated and visual measurements of estrous behavior and their sources of variation in Holstein heifers. II: Standing and lying patterns. Theriogenology, in press.

34. Stevenson JS, Hill SL, Nebel RL, DeJarnette JM. 2014. Ovulation time and conception risk after automated activity monitoring in lactating dairy cows. J Dairy Sci, 97:4296-4308.

35. Sveberg G, Refsdal AO, Erhard HW, Kommisrud E, Aldrin M, Tvete IF, Buckley F, Waldmann A, Ropstad E. 2011. Behavior of lactating Holstein-Friesian cows during spontaneous cycles of estrus. J Dairy Sci, 94:1289-1301.

36. Talukder S, Kerrisk KL, Ingenhoff L, Thomson PC, Garcia SC, Celi P. 2014. Infrared technology for estrus detection and as a predictor of time of ovulation in dairy cows in a pasture-based system. Theriogenology, 81:925-935.

37. Talukder S, Thomson PC, Kerrisk KL, Clark CEF, Celi P. 2015. Evaluation of infrared thermography body temperature and collar-mounted accelerometer and acoustic technology for predicting time of ovulation of cows in a pasture-based system. Theriogenology, 83:739-748.

38. Trimberger GW. 1948. Breeding efficiency in dairy cattle from artificial insemination at various intervals before and after ovulation. Nebr Agri Exp Sta Res Bul, 153:3-25.

39. Valenza A, Giordano JO, Lopes Jr. G, Vincenti L, Amundson MC, Fricke PM. 2012. Assessment of an accelerometer system for detection of estrus and treatment with gonadotropin-releasing hormone at the time of insemination in lactating dairy cows. J Dairy Sci, 95:7115-7127.

40. Van Hoeck V, Bols PEJ, Binelli M, Leroy JLMR. 2014. Reduced oocyte and embryo quality in response to elevated non-esterified fatty acid concentrations: A possiblepathway to subfertility? Anim Reprod Sci, 149:19-29.

41. Walker WL, Nebel RL, McGilliard ML. 1996. Time of ovulation relative to mounting activity in dairy cattle. J Dairy Sci, 79:1555-1561.

42. Wathes DC, Cheng Z, Bourne N, Taylor VJ, Coffey MP, Brotherstone S. 2007.Differences between primiparous and multiparous dairy cows in the inter-relationships between metabolic traits, milk yield and body condition score in the periparturient period. Domest Anim Endo, 33:203-225.

43. Xu ZZ, McKnight DJ, Vishwanath R, Pitt CJ, Burton LJ. 1998. Estrus detection using radiotelemetry or visual observation and tail painting for dairy cows on pasture. J Dairy Sci, 81:2890-2896.

44. Yoshioka H, Ito M, Tanimoto Y. 2010. Effectiveness of real-time radiotelemetric pedometer for estrus detection and insemination in Japanese Black cows. J Reprod Devel, 56:351-355.

THE DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE: IMPORTANCE FOR ANIMAL PRODUCTION

CHAVATTE-PALMER P, RICHARD C, PEUGNET P, ROBLES M,
ROUSSEAU-RALLIARD D, TARRADE A

INRA, UMR 1198 Biologie du Développement et Reproduction, Jouy en Josas, France

CORRESPONDENCE: P. Chavatte-Palmer
INRA, UMR 1198 Biologie du Développement et Reproduction, Bât 231, 78350 Jouy en Josas, France
Phone : +33 1 34652558
Email: pascale.chavatte@jouy.inra.fr

Running title: DOHAD and animal production

Abstract

The importance of management of pregnant animals during pregnancy has been long known to have effects on the quality of their offspring products. In this review, after defining and setting up the current understanding of the Developmental Origins of Health and Disease (DOHaD), effects on traits relevant to animal production, such as meat quality and lactation as well as general health are discussed, with a special interest for effects of periconceptional nutrition.

Keywords: DOHaD, fetal programming, animal production, meat, lactation

Animal management during pregnancy has been long known to have effects on the quality of offspring products. In this review, after explaining the current understanding of the Developmental Origins of Health and Disease (DOHaD), effects on traits relevant to animal production, such as meat quality and lactation as well as general health are discussed, with a particular interest for the effects of periconceptional nutrition. Since effects on reproductive function have been recently widely reviewed (Gardner et al., 2009; Chadio and Kotsampasi, 2014; Chavatte-Palmer et al., 2014; Kenyon and Blair, 2014), effects on offspring reproductive function have not been developed as the reader can refer to these reviews. Post-natal management will not be discussed thoroughly as this goes beyond the objectives of this review, but maternal environment impact on offspring's phenotype at adulthood will be.

I. PRINCIPLES OF THE DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE

In the 90's, Barker and co-workers' epidemiological studies underlined an increased risk of non-communicable metabolic diseases in people born Small-for-Gestational Age (SGA) and/or with a poor growth rate in infancy (Barker and Osmond, 1986; Hales et al., 1991; Barker, 1992). These observations, referred to as "Fetal programming", suggested that fetal and neonatal adaptations to a nutritionally poor environment induced permanent adaptations leading to a "thrifty phenotype", where the restricted individual favors energy storage and insulin resistance throughout his life. Such early and long-lasting adaptations increase the risk of developing metabolic pathologies at adulthood in the presence of excess food intake, often described as an "energy mismatch" between

early life and adulthood (Hales and Barker, 1992; Hales and Barker, 2001). Subsequently, the concept of the "Predictive Adaptive Response" stated that cues about environment delivered during pregnancy to the developing organism could also induce adaptive responses that would favor long term survival in a similar environment but may be less favorable for survival in a different environment (Bateson et al., 2004). This plasticity could provide an evolutionary advantage in the case of environments that change over a few generations but be deleterious in case of even faster changes, when the environment is very different between fetal and post-natal life (Gluckman et al., 2009). Moreover, more recent focus on obesity has shown that excess maternal body weight and adiposity also induced fetal adaptations leading to adverse outcomes at adulthood that are curiously close to that observed with growth retardation.

It is now generally admitted that components of human obesity, type-2 diabetes (T2D) and hypertension, but also bone health (Goodfellow et al., 2010), psychiatric health (Khandaker et al., 2012) and fertility (Faure et al., 2015) take root during early development, throughout gestation and lactation, as stated in the “Developmental Origins of Health and Disease” (DOHaD) hypothesis (http://www.mrc.soton.ac.uk/dohad/). Indeed, many studies in humans and animals have demonstrated that an individual's nutritional and hormonal status during fetal development and early life plays an important role for his long-term control of energy metabolism (Barker, 1995; McMillen et al., 2008). Epidemiological and experimental reports indicate that epigenetic mechanisms are the link between early life events and health later in life, with epigenetic marks being considered as long-lasting environmental cues (Gabory et al., 2011). Animals are affected by this process, which can also affect traits related to production, such as lactation, meat quality and other production traits (Wu et al., 2006; Kenyon and Blair, 2014) (Figure 1).

II. DOHAD AND THE QUALITY OF PRODUCTS

It is only recently that studies have started to explore the effects of maternal nutrition during pregnancy on livestock performance in mammals. Although considerable effort has been directed towards defining nutrient requirements of animals over the past 30 years, suboptimal nutrition during gestation remains a significant problem for many farm animal species as well worldwide (including cattle, pigs, and sheep) (Wu et al., 2004). Ruminants have been the main focus of research, but data in pigs, rabbits and horses also exist. Major observed effects, as detailed below, are summarized in Figure 2.

Growth

Growth and attainment of adult size is essential for production. Adult size is dependent on the genetics of the individual, but will be modulated by nutrition and environmental parameters at large. Already in the 1970's, McCance and Widdowson demonstrated that "critical periods" of undernutrition in the prenatal or immediate post-natal period can lead to growth retardation that cannot be recovered by subsequent catch-up growth (McCance, 1976). More studies using animal models have shown how intra-uterine growth restriction and post-natal under-nutrition can restrict offspring size as adults (Desai et al., 2005; Bieswal et al., 2006). In ruminants, undernutrition during the first half of gestation, although birth weight was usually not affected, was shown to impact metabolic function of sheep and cattle offspring, resulting in altered production and body composition later in life (Ford et al., 2007; Long et al., 2010; Long et al., 2012). Undernutrition of ~75% of recommended allowance during early stages of pregnancy compromises placental angiogenesis, cotyledon weight, and thus, fetal development (Zhu et al., 2007), with significant impact on development and function of liver and pancreas (Symonds et al., 2010). Overnutrition can also restrict placental and fetal development, resulting in decreased birth weights, post-natal growth, and altered body composition (Caton et al., 2007). Protein imbalance, with dams either nutrient restricted or supplemented with proteins, affects the development of the fetus through gestation, driving to born-too-thin or overweight neonates, respectively, both in bovine and ovine models (Funston et al., 2010).

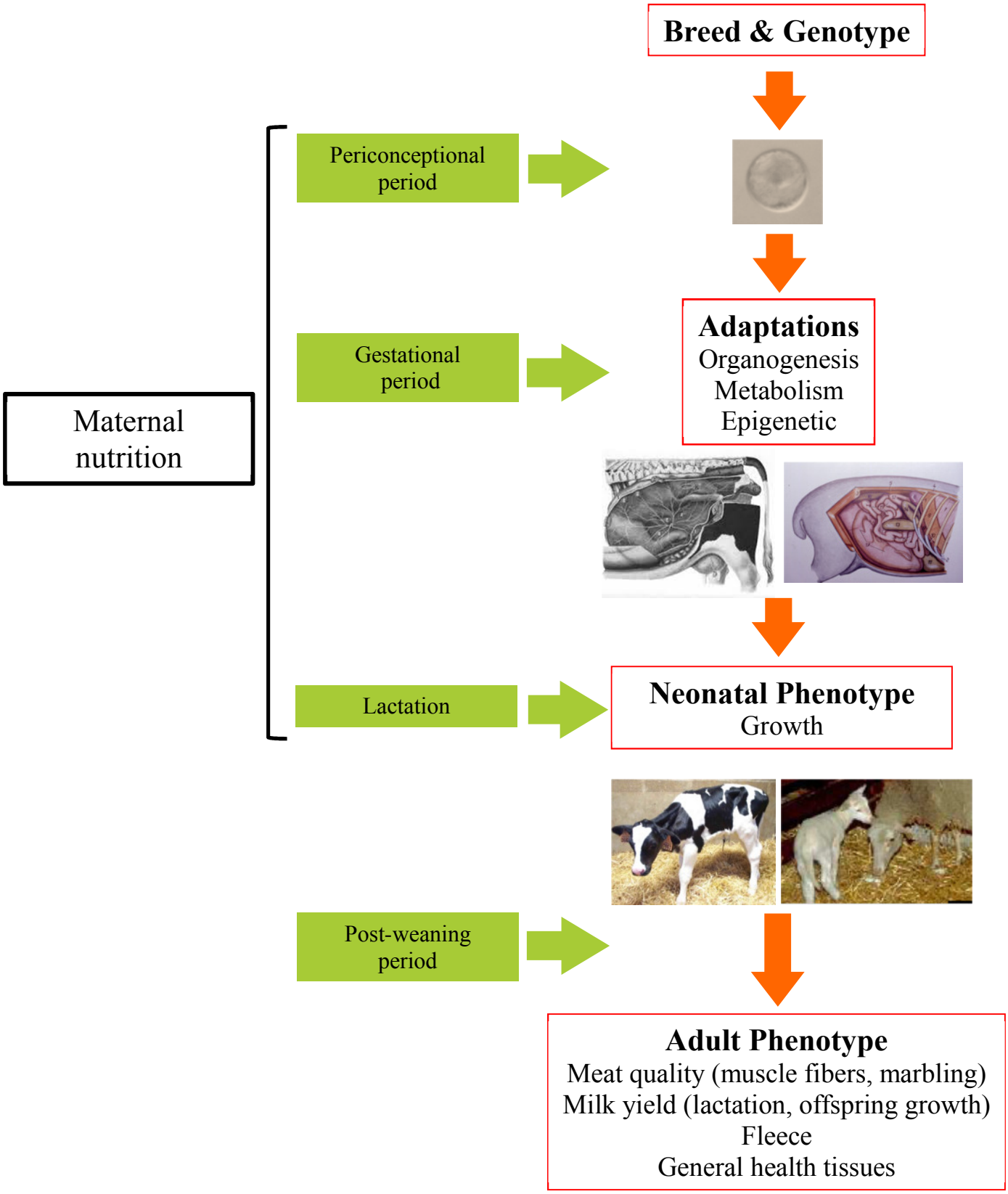

Figure 1:
Importance of the maternal nutrition during key periods on the quality of their offspring products

Beef cattle - In order to reduce fetal growth, severe maternal undernutrition is required for at least a third or half of pregnancy in beef cattle (Greenwood and Cafe, 2007; Martin et al., 2007). Fetal growth restriction, however, may also result from twinning, excess heat and is observed in heifers as opposed to multiparous animals (Greenwood and Cafe, 2007). Intra-uterine growth retardation (IUGR) due to maternal food restriction throughout gestation (difference of 10kg at birth) was shown to lead to reduced post-natal growth (Greenwood and Cafe, 2007). Moreover, undernutrition during the last third of pregnancy will decrease birth weights with a potential negative impact on long-term growth and body composition of the progeny (Underwood et al., 2010).

Dairy cattle - While growth (2 yr old cows) and milk production of the dam have clear implications for development of the fetus (Banos et al., 2007), diet during pregnancy is the primary modifiable factor that has a substantial influence on body condition and energy status of the dairy cattle as well as viability and body composition of newborn calves. Like in beef cattle, reduced post-natal growth was observed in naturally occurring low birth weight Holstein calves (Swali and Wathes, 2006).

Sheep – Effects of maternal dietary level and timing in sheep have been recently very thoroughly reviewed (Kenyon and Blair, 2014). Like in cattle, maternal undernutrition generally does not reduce birth weight, although offspring may be lighter at weaning, in particular when undernutrition has been prolonged until term (Kelly et al., 1996; Kelly et al., 2006; Field et al., 2015). Variation in the effects of undernutrition may also depend on the breed, or genetic / epigenetic background. Indeed, maternal undernutrition from 28 et 78 days of pregnancy did not reduce fetal weight in "Baggs" ewes which originated form a nomadic herd living in harsh conditions, whereas reduced fetal weight was observed in ewes bred for several generations in good conditions at the University of Wyoming (Vonnahme et al., 2006). These differences were accompanied with different placental responses to undernutrition, with earlier placental maturation in the "Baggs" ewes (Vonnahme et al., 2006). Heat stress during pregnancy also induces IUGR in sheep (Barry et al., 2008). In the field, the combination of maternal heat stress and undernutrition during pregnancy reduces lamb birth weight in ewes, which seem to better adapt to restricted feeding than to thermal stress (Sejian et al., 2011).

Overfeeding may also be harmful. As a result of dietary induced obesity in ewes, fetuses at mid-gestation were macrosomic, hyperglycemic, hyperinsulinemic with markedly increased pancreatic weight and β-cell numbers compared with fetuses of ewes fed to requirements (MacLaughlin et al., 2005; George et al., 2010; Zhang et al., 2011). Although they were still hyperglycemic at birth, the insulin secretion was blunted with decreased pancreatic growth and β-cell numbers by the end of gestation (Zhang et al., 2011).

Horse - In the horse, fetal growth is limited mainly by the uterine size of the dam, as shown by experiments using artificial insemination or embryo transfer in horses of different size (Walton and Hammond, 1938; Tischner, 2000; Allen et al., 2002; Allen et al., 2004; Peugnet et al., 2014; Peugnet et al., 2015). These works have demonstrated the importance of fetal growth for the achievement of the expected adult size, with growth retarded foals being permanently affected in terms of growth, at least for some bone segments. This is of particular importance in these species, where reduced adult size will limit access to studbooks and to some sporting events, and may affect performance. Similar experiments in sheep indicate that these effects on birth weight are not driven by changes in maternal insulin sensitivity during pregnancy (Oliver et al., 2015).

In the horse, similar to what was described in sheep, maternal overnutrition is reported to reduce glucose uptake and reduce growth rate of two thirds of pregnancy (Satterfield et al., 2010). In equidae, however, maternal undernutrition does not seem to affect birthweight nor subsequent growth, at least until weaning (Wilsher and Allen, 2006; Ousey et al., 2008; Peugnet et al., 2015), which does not preclude effects on performance (Rossdale and Ousey, 2002).

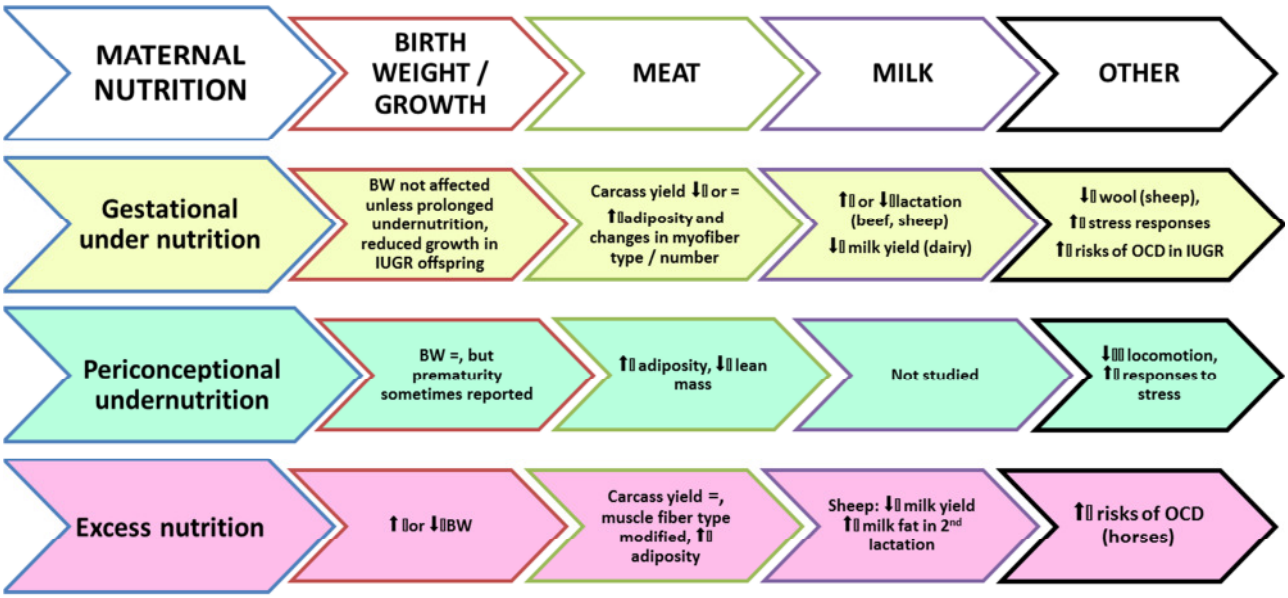

Figure 2: Main effects observed on production traits after maternal undernutrition or overnutrition during gestation, or after periconceptional undernutrition. (BW = birthweight, IUGR = intra-uterine growth retardation, OCD = osteochondrosis)

Meat quality

Meat quantity depends on the muscle mass whereas meat quality will depend on muscle fibers, which play an important role for tenderness (Guillemin et al., 2009) and the intramuscular fat, which is a major component of flavor. Different levels of intramuscular fat will be looked for according to cultures and cooking traditions.

The fetal period is crucial for skeletal muscle development, because no net increase in the number of muscle fibers occurs after birth in farm animals (Greenwood et al., 2000; Nissen et al., 2003). Myogenesis takes place during several chronologically distinct phases occurring in fetal life: proliferation of precursor cells (myoblasts), fusion of myoblasts into differentiated multinuclear cells (myotubes), and differentiation of these cells into muscle fibers. Myotube formation gives rise to primary and secondary muscle fibers, respectively. Muscle fiber generations are set up around the last third of gestation in larger species – day 180 in cattle (term= 280-284 days), around 90 days in pigs (term= 115 days) and in the early post-natal period in less mature species such as rabbits (first month after birth) and there is no increase in muscle fibers thereafter (Stickland, 1978; Picard et al., 2002; Oksbjerg et al., 2004). Therefore, a reduction in the number of muscle fibers during fetal development will lead to reduced muscle mass in the adult. Fiber type is also important for production: slow oxidative fibers (Type I) originate from primary muscle fibers, and fast, type II myofibers, which mostly develop from secondary fibers, mature into type IIA (fast-oxidative glycolytic) or IIB (fast-glycolytic) fibers. Type II fibers are more efficient in terms of growth (Du et al., 2010), but oxidative, and more so, slow oxidative fibers, are generally more likely to produce more tender meat, although there are clear discrepancies between different muscles (Guillemin et al., 2009).

Intramuscular fat (marbling) is dependent on the presence of intramuscular adipocytes. Adipocytes originate primarily from mesenchymal cells in fetal life around mid-gestation at the same time when secondary muscle fibers are produced (Du et al., 2010), but ontogenesis still needs to be completely elucidated (Boone et al., 2000; Hocquette et al., 2010), although intramuscular adipogenesis can be modified through maternal nutrition during pregnancy in sheep (Tong et al., 2008). Triglycerides are initially stored within the muscle fibers and then, in the immediate

postnatal period, intramuscular adipocytes will increase in size and volume (Hocquette et al., 2010). The hyperplasia of adipocytes is an important factor for marbling during growth (Hocquette et al., 2010).

Maternal undernutrition

Beef cattle - Severe maternal undernutrition leading to fetal growth restriction in beef cattle significantly reduces postnatal growth compared to controls but was not shown to affect muscle fiber characteristics, carcass composition and retail yield at similar carcass weight (Greenwood and Cafe, 2007). In regions where droughts are common, however, cows may experience periods of undernutrition during pregnancy, which may affect meat quality. Traditionally, they are supplemented in late gestation but not earlier (Du et al., 2010). In these harsh conditions, protein supplementation of pregnant cows in mid-gestation (day 60 to 180), or improvement of the pastures through irrigation, however, was shown to increase lean growth and reduce fat in offspring (Du et al., 2010). In a study using Angus x Gelbvieh cattle, Long et al. 2012 found no effect of maternal global undernutrition with or without protein supplementation during the first half of pregnancy on body weight or organ weight at slaughter (around 1.5 years of age). The ratio of semitendinosus muscle to carcass weight, however, tended to be reduced in the offspring from non-supplemented undernourished dams, with average adipocyte size increased in several anatomical locations (Long et al., 2012). The effect of increased or reduced maternal protein intake on offspring skeletal muscle development, however, is dependent on the timing of the supplementation during pregnancy as well as the sex of the offspring, as demonstrated in beef heifer offspring (Micke et al., 2011): as adults, males born to heifers fed a low protein diet during the first trimester of pregnancy and subsequently a high protein diet had greater *longissimus dorsi* muscle cross-sectional area compared to those whose dams were exposed to continuous high protein diet, whereas there was no effect of maternal protein intake on the female offspring. In contrast, maternal protein restriction in the second trimester was associated with higher *longissimus dorsi* muscle cross-sectional area in male and female offspring (Micke et al., 2011) although there was no effect on the *longissimus dorsi* weight/carcass weight ratio (Micke et al., 2010). These changes were associated with changes in the muscle expression of insuline-like growth factors IGF1 and IGF2 and their receptors (Micke et al., 2010; Micke et al., 2011).

Dairy cattle – In dairy cattle, conception usually takes place during early lactation, and thus lactating dams are in relative energy deficit compared to non-lactating dams (Funston and Summers, 2013). In a retrospective study of more than 1500 dairy calves, the size of the dam and its milk yield were shown to be the two most important factors influencing birth weight, with lower birth weights of calves born to high yielding cows with similar body size (Kamal et al., 2014). When heifers and cows were compared in a small number of Holstein females, maternal parity did not affect girth, birthweight nor glucose metabolism in the first month post-partum, although the birthweight and withers height of first born calves were reduced (Bossaert et al., 2014).

Sheep - In sheep, several studies indicate that maternal undernutrition in the early stages of pregnancy can affect meat quality, although birth weight is not always affected (Kenyon and Blair, 2014). Indeed, an extended period of maternal nutrient restriction during the first half of gestation results in relatively normal birth weights, but leads to increases in the length and thinness of the neonates, increased adiposity, and suppressed glucose tolerance (Whorwood et al., 2001; Ford et al., 2007). Moreover, carcass weight quality appears to be affected more by genetic background and litter size at birth than by maternal nutrition, except when undernutrition lasts most of the gestation (Kenyon and Blair, 2014). Indeed, the placenta adapts to the nutritional environment to minimize nutritional consequences to the fetus, although the extent of its adaptive capacities depend on the timing and intensity of the nutritional insult (Fowden et al., 2008; Symonds et al., 2012).

Restricted fetal nutrition throughout gestation as experienced by ewes during twin pregnancies leads to increased fetal adiposity (Edwards et al., 2005). Undernutrition of twin-bearing ewes throughout pregnancy led to low-birthweight lambs that remained smaller until adolescence and had poor energetic efficiency (Husted et al., 2007). Moreover, offspring were shown to have

disturbed responses to fasting at 6 months of age in terms of leptin (reduced), IGF1 and cortisol (increased) (Kongsted et al., 2013). When low (mean 2.29 kg) and high (mean 4.84 kg) birthweight lambs were compared, although the weight of the *semitendinosus* muscle was very significantly decreased in low birthweight lambs, the number of myofibers was similar but the fact that low birthweight lambs did not catch up in muscle growth may be due to the reduced myonuclei number (Greenwood et al., 1998; Greenwood et al., 2000).

Like in cattle, however, the timing of undernutrition matters and numerous nutritional planes have been studied, using animals of different breeds, making comparisons rather difficult (Kenyon and Blair, 2014). If most undernutrition studies do not indicate a strong effect on post-weaning liveweight, higher adiposity has been sometimes observed (Kenyon and Blair, 2014). In ewes undernourished in early gestation, term fetuses had more adipose tissue compared to controls that were fed *ad libitum* (Bispham et al., 2003), which is associated with increased glucocorticoid sensitivity (Gnanalingham et al., 2005; Mostyn and Symonds, 2009). In another study, castrated males at 8 months of age born to white face dams that were nutritionally restricted to 50% of requirements from Day 28 to 78 of pregnancy had heavier carcass weight than controls, but intra-abdominal fat deposits were also heavier and intramuscular triglyceride contents were increased as a result of the reduction of the activity of carnitine palmitoyltransferase-1, which is involved in fatty-acid oxidation (Zhu et al., 2006; Ford et al., 2007). Moreover, the total number of muscle myofibers was decreased with an increased ratio of IIB fibers (fast-glycolytic fibers) in muscle (Zhu et al., 2006). Similar findings were observed in another study where only twin animals were selected, with males and females analyzed separately (Daniel et al., 2007). This is an important point, as clear differences have been shown according to sex and litter size for many physiological parameters (MacLaughlin et al., 2010; Tarrade et al., 2015).

Pigs - In pigs, moderate IUGR is not always associated with a modification in carcass composition but very small piglets have slower growth and fatter carcasses than controls (Powell and Aberle, 1980; Mostyn and Symonds, 2009; Morise et al., 2011). Low birthweight piglets are also characterized by a reduced number of enlarged myofibers (Rehfeldt and Kuhn, 2006). Maternal undernutrition of Large White sows from mating to 50 days of pregnancy did not affect carcass weight, lean tissue and adipose tissue yield in offspring, whereas the composition of muscle in terms of myofiber types was slightly affected, with a reduced percentage of type IIB fibers (Bee, 2004). Maternal protein restriction, however, was shown to reduce the lean and increase the fat contents of offspring at 6 months of age with a tendency for reduced number of muscle myofibers associated with reduced expression of IGF2 mRNA (Rehfeldt et al., 2012).

Iberian pigs, which are genetically different form modern commercial pigs, deposit more intramuscular fat and are naturally leptin resistant (Ovilo et al., 2005; Munoz et al., 2009), presenting what resembles a "thrifty phenotype", which has been attributed to centuries of adaptation to low quality nutrition in semi-feral conditions (Lopez-Bote, 1998). In this breed that is prone to obesity, maternal undernutrition during pregnancy reduces birthweight and increases the incidence of IUGR piglets in the litter (Gonzalez-Bulnes et al., 2012). Female offspring appear to catch-up growth to controls at weaning whereas males are still growth-retarded at weaning (Gonzalez-Bulnes et al., 2012).

Excess maternal nutrition and obesity

Sheep - Extensive studies have shown that excess maternal nutrition retards placental and fetal growth, and increases fetal and neonatal mortality in sheep (Wallace et al., 2003). Excess maternal nutrition increases mid-gestation fetal weight (Ford et al., 2009). Lamb birth weight is similar to controls (Wallace et al., 2005; Zhu et al., 2009) or increased (Kenyon et al., 2011) according to the extent of the overfeeding and breed, but subsequent growth is similar (Kenyon et al., 2011). Maternal obesity, however, down-regulates myogenesis through the Wnt/□-catenin signaling pathway (Tong et al., 2009).

Pigs - In pigs, excess maternal nutrition from mating to 50 days of pregnancy increased the adipose tissue yield in the offspring carcasses, without affecting overall carcass weight and lean

yield, although muscle fiber type was modified (Bee, 2004). In another study, both reduced (50%) and excess (250%) maternal protein intake during pregnancy reduced piglet birthweight and birthweight/crown-rump length, reflecting adiposity (Rehfeldt et al., 2011). These effects, however, were not observed in fetuses at mid-pregnancy, indicating that placental insufficiency leading to IUGR had occurred in the second half of pregnancy (Rehfeldt et al., 2011). Offspring of dams fed a protein excess, however, did not differ from controls for muscle myofiber numbers and adipose tissue at 6 months of age (Rehfeldt et al., 2012).

In conclusion to this chapter, the favored fat development and reduced number in muscle myofibers mostly observed in maternal undernutrition experiments have been associated with changes in expression of insulin-like growth fators (IGFs) (Micke et al., 2011), Growth hormone (Rehfeldt and Kuhn, 2006), transcription factors involved in adipogenesis such as the Peroxisome Proliferator-Activited receptor gamma (PPAR□□ (Tong et al., 2008; Tong et al., 2009) and nutrient sensors such as mTOR. Indeed, the main regulators of adipogenesis are the peroxisome proliferator activated receptor (PPAR□) and CCAAT-enhancer binding protein (C/EBP) (Hausman et al., 2009). Moreover, fetal fat development may be favored by disturbed maternal plasma cortisol (reduced in undernourished pregnant sheep (Debus et al., 2012) or increased in pregnant sows fed a low protein diet (Otten et al., 2013)) observed during nutritional restrictions (Symonds et al., 2012).

Lactation

So far, the amount of data on the fetal programming of offspring lactation through the manipulation of maternal nutrition in domestic animals remains limited. Hence, in this part, the effects of under and over-nutrition are treated together. The organogenesis of the mammary gland begins early in pregnancy (Hovey et al., 2002; Houdebine, 2003) and may therefore be affected by maternal nutrition like other organs.

Beef cattle - In beef cattle, slower growing female calves whose dams have poor lactations tend to produce better lactation when they are adults and have offspring with faster growth, which in turn have reduced quality lactation as adults (Koch, 1972; Pala and McCraw, 2005).

Dairy cattle - In dairy cattle, the analysis of data available on the UK national fertility database and from Irish dairy cows showed that offspring from dams producing more milk before and during conception had reduced milk yields, increased somatic cell count and were culled earlier compared to those born to dams with lower milk yields (Banos et al., 2007; Berry et al., 2008). Similar observations were made in Spain, which showed that females born to dams that were lactating during early pregnancy produced significantly less milk compared to those born to dams that were not lactating and that this reduction in milk production was correlated to maternal production (Gonzalez-Recio et al., 2012).

Sheep – Kenyon and Blair (2014) have reviewed the effects of maternal nutrition on milk production in sheep. Maternal undernutrition from day 21 to 50 was shown to reduce the mammary gland weight in fetuses near term (Martín et al., 2012) and reduce milk production at first lactation (Paten et al., 2013). In contrast, fetal mammary duct density and fat production in milk in the second lactation was increased in female sheep born to dams that were fed *ad libitum* during pregnancy (1.5 times maintenance) compared to those born to control dams fed to maintenance (Blair et al., 2010). Nevertheless, the mammary mass was increased in the offspring of the maintenance group, and these offspring produced more protein and lactose, only in the first lactation at 2 years of age (Blair et al., 2010). The authors suggest that these effects limited to the first lactation may be the result of an "Adaptive Predictive Response", as defined above, where the "restricted" offspring would favor the survival of their own first offspring, with no investment in further lactations when the survival of the individual would be more hazardous. As a consequence, second generation effects were hence observed in two studies where grand-daughters of ewes fed a moderate diet during pregnancy were heavier at birth compared to the grand-daughters of dams that were fed *ad libitum* during pregnancy (van der Linden et al., 2009; Blair et al., 2010). Interestingly,

this effect was confirmed in farmed minks bred for fur, when grand-mothers were protein restricted (Matthiesen et al., 2010).

Fleece

Fleece weight in sheep is affected by body size, which induces confounding factors for the analysis of maternal effects of this parameter and may explain why a reduction in fleece has been reported in response to maternal undernutrition (Schinckel and Short, 1961; Kenyon and Blair, 2014). One study reported a change in hair follicle number, however, which could persist throughout the life of the animal (Schinckel and Short, 1961).

General health issues

Thermogenesis - In the newborn sheep, brown adipose tissue (BAT, representing 1-2% of birthweight (Symonds and Lomax, 1992)) is essential for ensuring effective adaptation to the extrauterine environment, in particular thermogenesis. Neonatal pigs and horses, although they possess BAT (Ousey, 1997; Mostyn et al., 2014), are much more dependent on shivering thermogenesis to maintain heat production during cold exposure. Gestational BAT development depends on transplacental glucose supply to the fetus (Symonds et al., 2012). In contrast to rodents, brown fat, although present at birth, is very reduced in adult large animals as most BAT is progressively replaced by white fat (Symonds et al., 2012). Moreover, it may not have the same myoblastic origin as white fat (Budge et al., 2009).

Behavior – Few studies have focused on the impact of maternal nutrition on offspring behavior in large animals, although alterations in food intake and response to stress may be important to the breeding industry.

Food restriction in late gestation in sheep was shown to decrease voluntary milk intake in lambs from 3 to 60 days of age (Geraseev et al., 2006) whereas, in another study, feed intake was not affected after weaning (Sibbald and Davidson, 1998). In dairy goats, no effects were observed in feeding behavior and stress responses in male kids before weaning (Laporte-Broux et al., 2011). Although no effects on feeding behavior in females at one year of age, at 2 years of age, the cortisol response to ACTH injection was increased in offspring from restricted dams, suggesting a higher susceptibility to stress (Laporte-Broux et al., 2012). Similarly, in pigs born to dams fed a low protein diet during pregnancy, cortisol response to weaning was increased and the medulla area within the adrenal was increased (Otten et al., 2013).

Osteoarticular pathology - In horses, although maternal undernutrition does not affect birth weight, epidemiological and experimental data indicate that IUGR due to transfer of saddle embryos into ponies (Peugnet et al., 2014), but also feeding mares with concentrates during gestation (Van der Heyden et al., 2013; Peugnet et al., 2015), may be associated with an increased risk of developing lesions of osteochondrosis in their foal, which is of strong economical importance for the horse industry. Since osteochondrosis is related to glucose/insulin metabolism, the effects may be linked to the observed trend for a reduced insulin sensitivity at 5 days of age in offspring of mares fed a high starch diet (George et al., 2009). More work is currently on-going in the authors' laboratory to explore this phenomenon.

III. PERICONCEPTIONAL PROGRAMMING

The developmental plasticity of embryos in the pre-implantation period leads to different embryo, feto-placental and post-natal responses to the environment (Laguna-Barraza et al., 2012). Specific targeting of the periconceptional period for experiments on maternal nutrition in large animals use different timing for the nutritional challenge, making it difficult to draw comparisons, although there are a lot of data available in model species and humans, which have been reviewed elsewhere (Watkins et al., 2010; Zhang et al., 2011; Fleming et al., 2012; van Montfoort et al., 2012; Steegers-Theunissen et al., 2013; Lane et al., 2015). Experiments using embryo transfer between a nutritionally challenged oocyte or embryo donor have also provided valuable insight into periconceptional effects. So far, most data on farm animals have been generated in sheep.

Using embryo transfer, it was shown that B12 vitamin and folate deficiency in embryo donor ewes and transfer of these embryos in control females induces excess weight and adiposity in sheep offspring, insulin resistance, increased blood pressure and altered response to immunological challenges, as well as differences in liver methylation (Sinclair et al., 2007). The transfer of embryos, collected from restricted or obese ewes maintained in the same nutritional plane or induced to loose or gain weight in the last month before mating and for the pre-implantation period, into control recipients, showed that maternal restriction in the periconceptional period, regardless of previous nutritional status, resulted in adrenocortical hypertrophy (Zhang et al., 2013) together with changes in the renin-angiotensin system regulation within the adrenal (Zhang et al., 2013). Periconceptional undernutrition was shown to induce an increment in the body weight and the oocyte population of the offspring, as well as an alteration of their locomotor activity (Abecia et al., 2014).

Like for general nutrition, the effects of maternal undernutrition around conception vary depending on the number of implanted embryos. First, maternal weight loss as a result of periconceptional undernutrition in sheep has been shown to reduce twinning rate (MacLaughlin et al., 2005; Debus et al., 2012; Abecia et al., 2014). In general, birth weight is not directly affected by periconceptional nutrition. Indeed, uterine blood flow is increased by 13% in ewes that were undernourished during the periconceptional period compared to controls (Rumball et al., 2008) with increased expression of growth factors (Zhu et al., 2007).

Effects of periconceptional (-60 days before to 7 days after conception) or pre-implantation (0-7 days after conception) nutrition were observed on fetal skeletal muscle insulin signaling, lipogenesis, adipose tissue and liver metabolism as well as miRNA expression, depending on twin or singletons pregnancy (Lie et al., 2012; Lie et al., 2013; Lie et al., 2014). The timing and importance of fetal pre-partum ACTH and cortisol increases as well as gestational length were shown to differ depending on embryo number, with reduced gestation duration only in singletons (Edwards et al., 2002; Edwards and McMillen, 2002; Bloomfield et al., 2003; Edwards et al., 2004). These effects may be related to the increased weight and disturbed function in the adrenal observed in singletons in these and other studies (Connor et al., 2009; Williams-Wyss et al., 2014). Responses to glucocorticoids are also affected: hypothalamic glucocorticoid receptor promoter methylation, as well as gene and protein expression, were still observed in 5 year old male and female offspring, which could explain the increased obesity observed in these animals (Begum et al., 2013). Regardless of singleton or twin status, the cardiovascular function was impaired in adult sheep (Gardner et al., 2004; Torrens et al., 2009).

Twins are generally smaller than singletons. Using an elegant approach of fetal reduction during pregnancy, Hancock et al. (2012) have shown that twins had lower lean mass and higher fat mass until 2 years of age compared to their contemporary singletons. Twin reduction to singleton pregnancy at 42-43 days of gestation did not recover the programmed twin fat and lean mass phenotype, indicating the early origin of these traits (Hancock et al., 2012).

As mentioned earlier, the genetic background of the animals induces large differences in the response to undernutrition. For example, gestational length was increased by periconceptional undernutrition only in twin-bearing Welsh mountain ewes carrying fetuses of opposite sex (Cleal et al., 2007). In another study, maternal periconceptional undernutrition did not reduce birth weight nor gestational length in the hardy Mediterranean breed Merinos d'Arles (Debus et al., 2012). Nevertheless, increased post-natal adiposity was observed in males but not in females, underlining the importance of offspring sex as well as litter size and breed (Debus et al., 2012).

In terms of response to post-natal nutritional treatment, it is interesting to observe that maternal nutrition at the time of conception was shown to directly affect lamb responses to nutritional supplementation with n-3 polyunsaturated fatty acid (PUFA)-enriched diets: offspring of dams fed a high n-6 fatty acid-riched diet for 6 weeks before mating had lower responses to algae supplementation (n-3 PUFA-enriched diet) compared to those born to control dams (Clayton et al., 2014).

In terms of behavior, maternal restriction from 60 days before to 30 days after the beginning of pregnancy was reported to be associated with decreased locomotion in 18 month old offspring (Donovan et al., 2013) whereas locomotion and attempts to escape were decreased during isolation after maternal periconceptional undernutrition, possibly reflecting decreased responses to stress (Hernandez et al., 2010; Abecia et al., 2014).

IV. CONCLUSIONS

The pre- and periconceptional periods are critical in the context of the Developmental Origins of Health and Disease (DOHaD). Maternal *in vivo* environment, in particular nutrition, can disturb the apposition of epigenetic marks throughout gametogenesis, fertilization and the first steps of embryonic development, which are times during which major epigenetic changes take place (Jammes et al., 2011). These marks will subsequently affect organ function during development, resulting in alterations in the post-natal phenotype (Watkins et al., 2008; Watkins et al., 2010). The *in vitro* environment, in the case of assisted reproduction techniques, also affects epigenetic marks. Whilst the embryo is a target of these changes, female and male gametes are both target and vector of these epigenetic changes, thus leading to multigenerational effects, so that long-term consequences on the phenotype of offspring vary according to the sex of the vector parent, the sex of the individual and the generation (Aiken and Ozanne, 2014).
More work is needed to understand how the environment modulates the genomic inheritance in order to induce a phenotype and how this may be used in agriculture to lead to more robust animals able to tackle the climatic challenges that we will be facing in the future.

V. ACKNOWLEDGEMENTS

PCP, PP, MR and AT are thankful for the funding for the Foetalim project by the Institut Français du Cheval et de l'Equitation (IFCE). PCP and PP also received funding for projects (Foetalim and sheep periconceptional undernutrition) from INRA, Physiology and Breeeding Systems incitative action. All authors are members of COST actions COST Actions FA0702 GEMINI "Maternal Interaction With Gametes and Embryo", FA1201 EPICONCEPT "Epigenetics and Periconception Environment" and BM1308 “Sharing Advances on Large Animal Models (SALAAM)”.

VI. REFERENCES

1. Abecia JA, Casao A, Pascual-Alonso M, Lobón S, Aguayo-Ulloa LA, Meikle A, Forcada F, Sosa C, Marín RH, Silva MA and Maria GA 2014 The effect of periconceptional undernutrition of sheep on the cognitive/emotional response and oocyte quality of offspring at 30 days of age. J DOHaD 5:79-87.

2. Aiken CE and Ozanne SE 2014 Transgenerational developmental programming Hum Reprod Update 20:63-75.

3. Allen WR, Wilsher S, Tiplady C and Butterfield RM 2004 The influence of maternal size on pre- and postnatal growth in the horse: III Postnatal growth Reproduction 127:67-77.

4. Allen WR, Wilsher S, Turnbull C, Stewart F, Ousey J, Rossdale PD and Fowden AL 2002 Influence of maternal size on placental, fetal and postnatal growth in the horse. I. Development in utero Reproduction 123:445-453.

5. Banos G, Brotherstone S and Coffey MP 2007 Prenatal Maternal Effects on Body Condition Score, Female Fertility, and Milk Yield of Dairy Cows J. Dairy Sci. 90:3490-3499.

6. Barker DJ 1992 The fetal origins of diseases of old age Eur J Clin Nutr 46 Suppl 3:S3-9.

7. Barker DJP 1995 Intrauterine programming of adult disease Molecular Medicine Today 1:418-423.

8. Barker DJP and Osmond C 1986 Infant mortality, childhood nutrition and ischaemic heart disease in England and Wales. The Lancet 327:1077-1081.

9. Barry JS, Rozance PJ and Anthony RV 2008 An Animal Model of Placental Insufficiency-Induced Intrauterine Growth Restriction Seminars in Perinatology 32:225-230.

10. Bateson P, Barker D, Clutton-Brock T, Deb D, D'Udine B, Foley RA, Gluckman P, Godfrey K, Kirkwood T, Lahr MM, McNamara J, Metcalfe NB, Monaghan P, Spencer HG and Sultan SE 2004 Developmental plasticity and human health Nature 430:419-421.

11. Bee G 2004 Effect of early gestation feeding, birth weight, and gender of progeny on muscle fiber characteristics of pigs at slaughter J Anim Sci 82:826-836.

12. Begum G, Davies A, Stevens A, Oliver M, Jaquiery A, Challis J, Harding J, Bloomfield F and White A 2013 Maternal Undernutrition Programs Tissue-Specific Epigenetic Changes in the Glucocorticoid Receptor in Adult Offspring Endocrinology 154:4560-4569.

13. Berry DP, Lonergan P, Butler ST, Cromie AR, Fair T, Mossa F and Evans ACO 2008 Negative Influence of High Maternal Milk Production Before and After Conception on Offspring Survival and Milk Production in Dairy Cattle Journal of Dairy Science 91:329-337.

14. Bieswal F, Ahn MT, Reusens B, Holvoet P, Raes M, Rees WD and Remacle C 2006 The importance of catch-up growth after early malnutrition for the programming of obesity in male rat. Obesity (Silver Spring) 14:1330-1343.

15. Bispham J, Gopalakrishnan GS, Dandrea J, Wilson V, Budge H, Keisler DH, Pipkin FB, Stephenson T and Symonds ME 2003 Maternal endocrine adaptation throughout pregnancy to nutritional manipulation: Consequences for maternal plasma leptin and cortisol and the programming of fetal adipose tissue development Endocrinology 144:3575-3585.

16. Blair HT, Jenkinson CMC, Peterson SW, Kenyon PR, van der Linden DS, Davenport LC, Mackenzie DDS, Morris ST and Firth EC 2010 Dam and granddam feeding during pregnancy in sheep affects milk supply in offspring and reproductive performance in grand-offspring Journal of Animal Science 88:E40-E50.

17. Bloomfield FH, Oliver MH, Hawkins P, Campbell M, Phillips DJ, Gluckman PD, Challis JRG and Harding JE 2003 A Periconceptional Nutritional Origin for Noninfectious Preterm Birth Science 300:606-.

18. Boone C, Mourot J, Grégoire F and Remacle C 2000 The adipose conversion process: regulation by extracellular and intracellular factors Reprod. Nutr. Dev. 40:325-358.

19. Bossaert P, Fransen E, Langbeen A, Stalpaert M, Vandenbroeck I, Bols PE and Leroy JL 2014 Effects of parity and periconceptional metabolic state of Holstein-Friesian dams on the glucose metabolism and conformation in their newborn calves Animal 8:975-981.

20. Budge H, Sebert S, Sharkey D and Symonds ME 2009 Session on 'Obesity'. Adipose tissue development, nutrition in early life and its impact on later obesity Proc Nutr Soc 68:321-326.

21. Caton JS, Grazul-Bilska AT, Vonnahme KA, Luther JS, Lardy GP, Hammer DA, Redmer D and Reynolds LP 2007. Nutritional management during gestation: impacts on lifelong performance? 18th Annual Florida Ruminant Nutrition Symposium, Gainesville, FL.

22. Chadio S and Kotsampasi B 2014 The role of early life nutrition in programming of reproductive function Journal of Developmental Origins of Health and Disease 5:2-15.

23. Chavatte-Palmer P, Debus N, Dupont C and Camous S 2014 Nutritional programming and the reproductive function of the offspring. Anim Prod Sci 54:1166-1176.

24. Clayton EH, Lamb TA, Refshauge G, Kerr MJ, Bailes KL, Ponnampalam EN, Friend MA and Hopkins DL 2014 Differential Response to an Algae Supplement High in DHA Mediated by Maternal Periconceptional Diet: Intergenerational Effects of n-6 Fatty Acids Lipids 49:767-775.

25. Cleal JK, Poore KR, Newman JP, Noakes DE, Hanson MA and Green LR 2007 The effect of maternal undernutrition in early gestation on gestation length and fetal and postnatal growth in sheep. Ped. Res. 62:422-427.

26. Connor KL, Bloomfield FH, Oliver MH, Harding JE and Challis JRG 2009 Effect of Periconceptional Undernutrition in Sheep on Late Gestation Expression of mRNA and Protein From Genes Involved in Fetal Adrenal Steroidogenesis and Placental Prostaglandin Production Reproductive Sciences 16:573-583.

27. Daniel ZC, Brameld JM, Craigon J, Scollan ND and Buttery PJ 2007 Effect of maternal dietary restriction during pregnancy on lamb carcass characteristics and muscle fiber composition J Anim Sci 85:1565-1576.

28. Debus N, Chavatte-Palmer P, Viudes G, Camous S, Roséfort A and Hassoun P 2012 Maternal periconceptional undernutrition in Merinos d'Arles sheep: 1. Effects on pregnancy and reproduction results of mothers and offspring growth performances. Theriogenology 77:1453-1465.

29. Desai M, Gayle D, Babu J and Ross MG 2005 Programmed obesity in intrauterine growth-restricted newborns: modulation by newborn nutrition. Am J Physiol Regul Integr Comp Physiol 288:91-96.

30. Donovan EL, Hernandez CE, Matthews LR, Oliver MH, Jaquiery AL, Bloomfield FH and Harding JE 2013 Periconceptional undernutrition in sheep leads to decreased locomotor activity in a natural environment Journal of Developmental Origins of Health and Disease 4:296-299.

31. Du M, Tong J, Zhao J, Underwood KR, Zhu M, Ford SP and Nathanielsz PW 2010 Fetal programming of skeletal muscle development in ruminant animals J Anim Sci 88:E51-60.

32. Edwards LJ, Bryce AE, Coulter CL and McMillen IC 2002 Maternal undernutrition throughout pregnancy increases adrenocorticotrophin receptor and steroidogenic acute

regulatory protein gene expression in the adrenal gland of twin fetal sheep during late gestation Mol Cell Endocrinol 196:1-10.

33. Edwards LJ, McFarlane JR, Kauter KG and McMillen IC 2004 Impact of maternal undernutrition before and during pregnancy on maternal and fetal leptin and fetal adiposity in singleton and twin pregnancies Am J Physiol Regul Integr Comp Physiol.

34. Edwards LJ, McFarlane JR, Kauter KG and McMillen IC 2005 Impact of periconceptional nutrition on maternal and fetal leptin and fetal adiposity in singleton and twin pregnancies American Journal of Physiology 288:R39-R45.

35. Edwards LJ and McMillen IC 2002 Impact of maternal undernutrition during the periconceptional period, fetal number, and fetal sex on the development of the hypothalamo-pituitary adrenal axis in sheep during late gestation Biol Reprod 66:1562-1569.

36. Faure C, Dupont C, Chavatte-Palmer P, Gautier B, Levy R and Group AC 2015 Are semen parameters related to birth weight? Fertil Steril 103:6-10.

37. Field ME, Anthony RV, Engle TE, Archibeque SL, Keisler DH and Han H 2015 Duration of maternal undernutrition differentially alters fetal growth and hormone concentrations Domestic Animal Endocrinology 51:1-7.

38. Fleming TP, Velazquez MA, Eckert JJ, Lucas ES and Watkins AJ 2012 Nutrition of females during the pen-conceptional period and effects on foetal programming and health of offspring Animal Reproduction Science 130:193-197.

39. Ford SP, Hess BW, Schwope MM, Nijland MJ, Gilbert JS, Vonnahme KA, Means WJ, Han H and Nathanielsz PW 2007 Maternal undernutrition during early to mid-gestation in the ewe results in altered growth, adiposity, and glucose tolerance in male offspring J. Anim Sci. 85:1285-1294.

40. Ford SP, Zhang LR, Zhu MJ, Miller MM, Smith DT, Hess BW, Moss GE, Nathanielsz PW and Nijland MJ 2009 Maternal obesity accelerates fetal pancreatic beta-cell but not alpha-cell development in sheep: prenatal consequences American Journal of Physiology-Regulatory Integrative and Comparative Physiology 297:R835-R843.

41. Fowden AL, Forhead AJ, Coan PM and Burton GJ 2008 The Placenta and Intrauterine Programming Journal of Neuroendocrinology 20:439-450.

42. Funston RN, Larson DM and Vonnahme KA 2010 Effects of maternal nutrition on conceptus growth and offspring performance: implications for beef cattle production J Anim Sci 88:E205-215.

43. Funston RN and Summers AF 2013 Effect of prenatal programming on heifer development Vet Clin North Am Food Anim Pract 29:517-536.

44. Gabory A, Attig L and Junien C 2011 Developmental programming and epigenetics. Am J Clin Nutr 94:1943S-1952S.

45. Gardner DS, Ozanne S and Sinclair AJ 2009 Effect of the early-life nutritional environment on fecundity and fertility of mammals. Phil Trans R Soc B 364:3419-3427.

46. Gardner DS, Pearce S, Dandrea J, Walker R, Ramsay MM, Stephenson T and Symonds ME 2004 Peri-implantation undernutrition programs blunted angiotensin II evoked baroreflex responses in young adult sheep Hypertension 43:1290-1296.

47. George LA, Staniar WB, Treiber KH, Harris PA and Geor RJ 2009 Insulin sensitivity and glucose dynamics during pre-weaning foal development and in response to maternal diet composition Domestic Animal Endocrinology In Press, Corrected Proof.

48. George LA, Uthlaut AB, Long NM, Zhang L, Ma Y, Smith DT, Nathanielsz PW and Ford SP 2010 Different levels of overnutrition and weight gain during pregnancy have differential effects on fetal growth and organ development Reprod Bio Endocrinol 8:http://www.rbej.com/content/8/1/75.

49. Geraseev LC, Perez JRO, Carvalho PA, Oliveira RPd, Quintão FA and Lima AL 2006 Efeitos das restrições pré e pós-natal sobre o crescimento e o desempenho de cordeiros Santa Inês do nascimento ao desmame Revista Brasileira de Zootecnia 35:245-251.

50. Gluckman PD, Hanson MA, Bateson P, Beedle AS, Law CM, Bhutta ZA, Anokhin KV, BougnËres P, Chandak GR, Dasgupta P, Smith GD, Ellison PT, Forrester TE, Gilbert SF, Jablonka E, Kaplan H, Prentice AM, Simpson SJ, Uauy R and West-Eberhard MJ 2009 Towards a new developmental synthesis: adaptive developmental plasticity and human disease The Lancet 373:1654-1657.

51. Gnanalingham MG, Mostyn A, Symonds ME and Stephenson T 2005 Ontogeny and nutritional programming of adiposity in sheep: potential role of glucocorticoid action and uncoupling protein-2 American Journal of Physiology-Regulatory Integrative and Comparative Physiology 289:R1407-R1415.

52. Gonzalez-Bulnes A, Ovilo C, Lopez-Bote CJ, Astiz S, Ayuso M, Perez-Solana ML, Sanchez-Sanchez R and Torres-Rovira L 2012 Gender-specific early postnatal catch-up growth after intrauterine growth retardation by food restriction in swine with obesity/leptin resistance Reproduction 144:269-278.

53. Gonzalez-Recio O, Ugarte E and Bach A 2012 Trans-generational effect of maternal lactation during pregnancy: a Holstein cow model Plos One 7:e51816.

54. Goodfellow LR, Earl S, Cooper C and Harvey NC 2010 Maternal Diet, Behaviour and Offspring Skeletal Health International Journal of Environmental Research and Public Health 7:1760-1772.

55. Greenwood PL and Cafe LM 2007 Prenatal and pre-weaning growth and nutrition of cattle: Longterm consequences for beef production Animal 1:1283-1296.

56. Greenwood PL, Hunt AS, Hermanson JW and Bell AW 1998 Effects of birth weight and postnatal nutrition on neonatal sheep: I. Body growth and composition, and some aspects of energetic efficiency J. Anim Sci. 76:2354-2367.

57. Greenwood PL, Hunt AS, Hermanson JW and Bell AW 2000 Effects of birth weight and postnatal nutrition on neonatal sheep: II. Skeletal muscle growth and development J Anim Sci 78.

58. Guillemin N, Cassar-Malek I, Hocquette JF, Jurie C, Micol D, Listrat A, Leveziel H, Renand G and Picard B 2009 La maitrise de la tendreté de la viande bovine: identification de marqueurs biologiques. INRA Productions Animales 22:331-344.

59. Hales CN and Barker DJ 1992 Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Diabetologia 35:595–601.

60. Hales CN and Barker DJ 2001 The thrifty phenotype hypothesis Br Med Bull 60:5-20.

61. Hales CN, Barker DJ, Clark PM, Cox LJ, Fall C, Osmond C and Winter PD 1991 Fetal and infant growth and impaired glucose tolerance at age 64 Bmj 303:1019-1022.

62. Hancock SN, Oliver MH, McLean C, Jaquiery AL and Bloomfield FH 2012 Size at birth and adult fat mass in twin sheep are determined in early gestation Journal of Physiology-London 590:1273-1285.

63. Hausman GJ, Dodson MV, Ajuwon K, Azain M, Barnes KM, Guan LL, Jiang Z, Poulos SP, Sainz RD, Smith S, Spurlock M, Novakofski J, Fernyhough ME and Bergen WG 2009 BOARD-INVITED REVIEW: The biology and regulation of preadipocytes and adipocytes in meat animals Journal of Animal Science 87:1218-1246.

64. Hernandez CE, Matthews LR, Oliver MH, Bloomfield FH and Harding JE 2010 Effects of sex, litter size and periconceptional ewe nutrition on offspring behavioural and physiological response to isolation Physiology & Behavior 101:588-594.

65. Hocquette JF, Gondret F, Baeza E, Medale F, Jurie C and Pethick DW 2010 Intramuscular fat content in meat-producing animals: development, genetic and nutritional control, and identification of putative markers Animal 4:303-319.

66. Houdebine LM 2003. Physiologie de la lactation. *In* pp D. Cabrol, Pons J.C. and Goffinet, F.: Obstétrique Flamarion.

67. Hovey R, Trott J and Vonderhaar B 2002 Establishing a Framework for the Functional Mammary Gland: From Endocrinology to Morphology Journal of Mammary Gland Biology and Neoplasia 7:17-38.

68. Husted SM, Nielsen MO, Tygesen MP, Kiani A, Blache D and Ingvartsen KL 2007 Programming of intermediate metabolism in young lambs affected by late gestational maternal undernourishment. Am J Physiol Endocrinol Metab 293:548-557.

69. Jammes H, Junien C and Chavatte-Palmer P 2011 Epigenetic control of development and expression of quantitative traits. Reprod Fertil Dev 23:64-74.

70. Kamal MM, Van Eetvelde M, Depreester E, Hostens M, Vandaele L and Opsomer G 2014 Age at calving in heifers and level of milk production during gestation in cows are associated with the birth size of Holstein calves Journal of Dairy Science 97:5448-5458.

71. Kelly R, Macleod I, Hynd P and Greeff J 1996 Nutrition during fetal life alters annual wool production and quality in young Merino sheep Australian Journal of Experimental Agriculture 36:259-267.

72. Kelly RW, Greeff JC and Macleod I 2006 Lifetime changes in wool production of Merino sheep following differential feeding in fetal and early life Australian Journal of Agricultural Research 57:867-876.

73. Kenyon PR and Blair HT 2014 Foetal programming in sheep - Effects on production. Small Ruminant Research 118:16-30.

74. Kenyon PR, van der Linden DS, Blair HT, Morris ST, Jenkinson CMC, Peterson SW, Mackenzie DDS and Firth EC 2011 Effects of dam size and nutritional plane during pregnancy on lamb performance to weaning Small Ruminant Research 97:21-27.

75. Khandaker GM, Dibben CRM and Jones PB 2012 Does maternal body mass index during pregnancy influence risk of schizophrenia in the adult offspring? Obesity Reviews:no-no.

76. Koch RM 1972 The Role of Maternal Effects in Animal Breeding: VI. Maternal Effects in Beef Cattle Journal of Animal Science 35:1316-1323.

77. Kongsted AH, Husted SV, Thygesen MP, Christensen VG, Blache D, Tolver A, Larsen T, Quistorff B and Nielsen MO 2013 Pre- and postnatal nutrition in sheep affects beta-cell secretion and hypothalamic control Journal of Endocrinology 219:159-171.

78. Laguna-Barraza R, Bermejo-Alvarez P, Ramos-Ibeas P, de Frutos C, Lopez-Cardona AP, Calle A, Fernandez-Gonzalez R, Pericuesta E, Ramirez MA and Gutierrez-Adan A 2012 Sex-specific embryonic origin of postnatal phenotypic variability Reprod Fertil Dev 25:38-47.

79. Lane M, Zander-Fox DL, Robker RL and McPherson NO 2015 Peri-conception parental obesity, reproductive health, and transgenerational impacts Trends Endocrinol Metab 26:84-90.

80. Laporte-Broux B, Roussel S, Ponter AA, Giger-Reverdin S, Camous S, Chavatte-Palmer P and Duvaux-Ponter C 2012 Long-term consequences of feed restriction during late pregnancy in goats on feeding behavior and emotional reactivity of female offspring. Physiol Behav 106:178-184.

81. Laporte-Broux B, Roussel-Huchette S, Ponter AA, Perault J, Chavatte-Palmer P and Duvaux-Ponter C 2011 Short term effects of maternal feed restriction during pregnancy on goat kid morphology, metabolism and behavior. J Anim Sci 89:2154-2163.

82. Lie S, Morrison JL, Williams-Wyss O, Ozanne SE, Zhang S, Walker SK, Kleemann DO, MacLaughlin SM, Roberts CT and McMillen IC 2013 Impact of embryo number and periconceptional undernutrition on factors regulating adipogenesis, lipogenesis, and metabolism in adipose tissue in the sheep fetus American Journal of Physiology-Endocrinology and Metabolism 305:E931-E941.

83. Lie S, Morrison JL, Williams-Wyss O, Suter CM, Humphreys DT, Ozanne SE, Zhang S, Maclaughlin SM, Kleemann DO, Walker SK, Roberts CT and McMillen IC 2014 Impact of embryo number and maternal undernutrition around the time of conception on insulin signaling and gluconeogenic factors and microRNAs in the liver of fetal sheep Am J Physiol Endocrinol Metab 306:E1013-1024.

84. Lie S, Morrison JL, Wyss O, Zhang S, Ozanne SE and McMillen IC 2012 Periconceptional Undernutrition Differentially Alters Insulin Signalling in Skeletal Muscle in Singleton and Twin Fetal Sheep in Late Gestation Reproductive Sciences 19:263A-263A.

85. Long NM, Prado-Cooper MJ, Krehbiel CR, DeSilva U and Wettemann RP 2010 Effects of nutrient restriction of bovine dams during early gestation on postnatal growth, carcass and organ characteristics, and gene expression in adipose tissue and muscle J Anim Sci 88:3251-3261.

86. Long NM, Tousley CB, Underwood KR, Paisley SI, Means WJ, Hess BW, Du M and Ford SP 2012 Effects of early- to mid-gestational undernutrition with or without protein supplementation on offspring growth, carcass characteristics, and adipocyte size in beef cattle J Anim Sci 90:197-206.

87. Lopez-Bote CJ 1998 Sustained utilization of the Iberian pig breed Meat Sci 49S1:S17-27.

88. MacLaughlin SM, Walker SK, Kleemann DO, Tosh DN and McMillen IC 2010 Periconceptional undernutrition and being a twin each alter kidney development in the sheep fetus during early gestation American Journal of Physiology-Regulatory Integrative and Comparative Physiology 298:R692-R699.

89. MacLaughlin SM, Walker SK, Roberts CT, Kleemann DO and McMillen IC 2005 Periconceptional nutrition and the relationship between maternal body weight changes in the periconceptional period and feto-placental growth in the sheep Journal of Physiology 565.

90. Martin JL, Vonnahme KA, Adams DC, Lardy GP and Funston RN 2007 Effects of dam nutrition on growth and reproductive performance of heifer calves Journal of Animal Science 85:841-847.

91. Martín NP, Kenyon PR, Morel PCH, Pain SJ, Jenkinson CMC, Hutton PG, Morris ST, Peterson SW, Firth EC and Blair HT 2012 Ewe nutrition in early and mid- to late pregnancy has few effects on fetal development Animal Production Science 52:533-539.

92. Matthiesen CF, Blache D, Thomsen PD and Tauson AH 2010 Feeding mink (Neovison vison) a protein-restricted diet during pregnancy induces higher birth weight and altered hepatic gene expression in the F(2) offspring Br J Nutr 104:544-553

93. McCance RA 1976 Critical periods of growth. Proc Nutr Soc 35:309-313.

94. McMillen IC, MacLaughlin SM, Muhlhausler BS, Gentili S, Duffield JL and Morrison JL 2008 Developmental origins of adult health and disease: The role of periconceptional and foetal nutrition Basic & Clinical Pharmacology & Toxicology 102:82-89.

95. Micke GC, Sullivan TM, Gatford KL, Owens JA and Perry VEA 2010 Nutrient intake in the bovine during early and mid-gestation causes sex-specific changes in progeny plasma IGF-I, liveweight, height and carcass traits Animal Reproduction Science 121:208-217.

96. Micke GC, Sullivan TM, McMillen IC, Gentili S and Perry VEA 2011 Protein intake during gestation affects postnatal bovine skeletal muscle growth and relative expression of IGF1, IGF1R, IGF2 and IGF2R Molecular and Cellular Endocrinology 332:234-241.

97. Morise A, Seve B, Mace K, Magliola C, Le Huerou-Luron I and Louveau I 2011 Growth, body composition and hormonal status of growing pigs exhibiting a normal or small weight at birth and exposed to a neonatal diet enriched in proteins Br J Nutr 105:1471-1479.

98. Mostyn A, Attig L, Larcher T, Dou S, Chavatte-Palmer P, Boukthir M, Gertler A, Djiane J, M ES and Abdennebi-Najar L 2014 UCP1 is present in porcine adipose tissue and is responsive to postnatal leptin J Endocrinol 223:M31-M38.

99. Mostyn A and Symonds ME 2009 Early programming of adipose tissue function: a large-animal perspective Proc Nutr Soc 68:393-400.

100. Munoz G, Ovilo C, Silio L, Tomas A, Noguera JL and Rodriguez MC 2009 Single- and joint-population analyses of two experimental pig crosses to confirm quantitative trait loci on Sus scrofa chromosome 6 and leptin receptor effects on fatness and growth traits J Anim Sci 87:459-468.

101. Nissen PM, Danielsen VO, Jorgensen PF and Oksbjerg N 2003 Increased maternal nutrition of sows has no beneficial effects on muscle fiber number or postnatal growth and has no impact on the meat quality of the offspring J Anim Sci 81:3018-3027.

102. Oksbjerg N, Gondret F and Vestergaard M 2004 Basic principles of muscle development and growth in meat-producing mammals as affected by the insulin-like growth factor (IGF) system Domestic Animal Endocrinology 27:219-240.

103. Oliver MH, Jaquiery AL, Kenyon PR, Pain SJ, Jenkinson CM, Blair HT, Derraik JGB and Bloomfield FH 2015 Maternal insulin sensitivity in midpregnancy does not determine birth weight after embryo transfer between large and small breed sheep Domestic Animal Endocrinology 50:50-54.

104. Otten W, Kanitz E, Tuchscherer M, Gräbner M, Nürnberg G, Bellmann O, Hennig U, Rehfeldt C and Metges CC 2013 Effects of low and high protein:carbohydrate ratios in the diet of pregnant gilts on maternal cortisol concentrations and the adrenocortical and sympathoadrenal reactivity in their offspring Journal of Animal Science 91:2680-2692.

105. Ousey JC 1997 Thermoregulation and the energy requirement of the newborn foal with reference to prematurity. Equine Vet. J. Suppl. 24:104-108.

106. Ousey JC, Fowden AL, Wilsher S and Allen WR 2008 The effects of maternal health and body condition on the endocrine responses of neonatal foals Equine Veterinary Journal 40:673-679.

107. Ovilo C, Fernandez A, Noguera JL, Barragan C, Leton R, Rodriguez C, Mercade A, Alves E, Folch JM, Varona L and Toro M 2005 Fine mapping of porcine chromosome 6 QTL and LEPR effects on body composition in multiple generations of an Iberian by Landrace intercross. Genet res 85:57-67.

108. Pala A and McCraw R 2005 Replacement Heifer Selection in A Beef Cattle Herd. Am. J. Applied Sci. 2:542-544.

109. Paten AM, Kenyon PR, Lopez-Villalobos N, Peterson SW, Jenkinson CMC, Pain SJ and Blair HT 2013 LACTATION BIOLOGY SYMPOSIUM: Maternal nutrition during early

and mid-to-late pregnancy: Comparative effects on milk production of twin-born ewe progeny during their first lactation Journal of Animal Science 91:676-684.

110. Peugnet P, Robles M, Mendoza L, Wimel L, Dubois C, Dahirel M, Guillaume D, Camous S, Berthelot V, Toquet M-P, Richard E, Sandersen C, Chaffaux S, Lejeune J-P, Tarrade A, Serteyn D and Chavatte-Palmer P 2015 Effects of Moderate Amounts of Barley in Late Pregnancy on Growth, Glucose Metabolism and Osteoarticular Status of Pre-Weaning Horses PLoS ONE 10:e0122596.

111. Peugnet P, Wimel L, Duchamp G, Sandersen C, Camous S, Guillaume D, Dahirel M, Dubois C, Jouneau A, Reigner F, Berthelot V, Chaffaux S, Tarrade A, Serteyn D and Chavatte-Palmer P 2014 Enhanced or reduced fetal growth induced by embryo transfer into smaller or larger breeds alters post-natal growth and metabolism in pre-weaning horses. Plos One 9:e102044.

112. Peugnet P, Wimel L, Tarrade A, Robles M, Dubois C, Serteyn D and Chavatte-Palmer P 2015. La santé du futur poulain se prépare dès la gestation. 41ème Journée de la Recherche Equine, Paris, France, Institut Français du Cheval et de l'Equitation.

113. Picard B, Lefaucheur L, Berri C and Duclos MJ 2002 Muscle fibre ontogenesis in farm animal species Reprod Nutr Dev 42:415 - 431.

114. Powell SE and Aberle ED 1980 Effects of birth weight on growth and carcass composition of swine J Anim Sci 50:860-868.

115. Rehfeldt C and Kuhn G 2006 Consequences of birth weight for postnatal growth performance and carcass quality in pigs as related to myogenesis Journal of Animal Science 84:E113-E123.

116. Rehfeldt C, Lang IS, Görs S, Hennig U, Kalbe C, Stabenow B, Brüssow KP, Pfuhl R, Bellmann O, Nürnberg G, Otten W and Metges CC 2011 Limited and excess dietary protein during gestation affects growth and compositional traits in gilts and impairs offspring fetal growth Journal of Animal Science 89:329-341.

117. Rehfeldt C, Stabenow B, Pfuhl R, Block J, Nürnberg G, Otten W, Metges CC and Kalbe C 2012 Effects of limited and excess protein intakes of pregnant gilts on carcass quality and cellular properties of skeletal muscle and subcutaneous adipose tissue in fattening pigs Journal of Animal Science 90:184-196.

118. Rossdale PD and Ousey JC 2002 Fetal programming for athletic performance in the horse: potential effects of IUGR Equine Veterinary Education 14:98-111.

119. Rumball CW, Bloomfield FH and Harding JE 2008 Cardiovascular adaptations to pregnancy in sheep and effects of periconceptional undernutrition Placenta 29:89-94.

120. Satterfield MC, Coverdale JA and Wu GY 2010. *In*: Review of fetal programming: implications to horse health.

121. Schinckel P and Short B 1961 Influence of nutritional level during pre-natal and early post-natal life on adult fleece and body characters Australian Journal of Agricultural Research 12:176-202.

122. Sejian V, Maurya VP and Naqvi SMK 2011 Effect of thermal stress, restricted feeding and combined stresses (thermal stress and restricted feeding) on growth and plasma reproductive hormone levels of Malpura ewes under semi-arid tropical environment Journal of Animal Physiology and Animal Nutrition 95:252-258.

123. Sibbald AM and Davidson GC 1998 The effect of nutrition during early life on voluntary food intake by lambs between weaning and 2 years of age Animal Science 66:697-703.

124. Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, Bispham J, Thurston A, Huntley JF, Rees WD, Maloney CA, Lea RG, Craigon J, McEvoy TG and Young LE 2007 DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status Proceedings of the National Academy of Sciences of the United States of America 104:19351-19356.

125. Steegers-Theunissen RP, Twigt J, Pestinger V and Sinclair KD 2013 The periconceptional period, reproduction and long-term health of offspring: the importance of one-carbon metabolism Hum Reprod Update 19:640-655.

126. Stickland NC 1978 A Quantitative Study of Muscle Development in the Bovine Foetus (Bos indicus) Anatomia, Histologia, Embryologia 7:193-205.

127. Swali A and Wathes DC 2006 Influence of the dam and sire on size at birth and subsequent growth, milk production and fertility in dairy heifers Theriogenology In Press, Corrected Proof.

128. Symonds ME and Lomax MA 1992 Maternal and environmental influences on thermoregulation in the neonate Proc Nutr Soc 51:165-172.

129. Symonds ME, Pope M, Sharkey D and Budge H 2012 Adipose tissue and fetal programming Diabetologia 55:1597-1606.

130. Symonds ME, Sebert SP and Budge H 2010 Nutritional regulation of fetal growth and implications for productive life in ruminants Animal 4:1075-1083

131. Tarrade A, Panchenko P, Junien C and Gabory A 2015 Placental contribution to nutritional programming of health and diseases: epigenetics and sexual dimorphism J Exp Biol 218:50-58.

132. Tischner M 2000 Maternal influence on pre- and postnatal growth of foals born after embryo transfer. J Reprod Fert Suppl. 56:705-708.

133. Tong J, Zhu MJ, Underwood KR, Hess BW, Ford SP and Du M 2008 AMP-activated protein kinase and adipogenesis in sheep fetal skeletal muscle and 3T3-L1 cells Journal of Animal Science 86:1296-1305.

134. Tong JF, Yan X, Zhu MJ, Ford SP, Nathanielsz PW and Du M 2009 Maternal obesity downregulates myogenesis and Œ≤-catenin signaling in fetal skeletal muscle American Journal of Physiology - Endocrinology And Metabolism 296:E917-E924.

135. Torrens C, Snelling TH, Chau R, Shanmuganathan M, Cleal JK, Poore KR, Noakes DE, Poston L, Hanson MA and Green LR 2009 Effects of pre- and periconceptional

undernutrition on arterial function in adult female sheep are vascular bed dependent Experimental Physiology 94:1024-1033.

136. Underwood KR, Tong JF, Price PL, Roberts AJ, Grings EE, Hess BW, Means WJ and Du M 2010 Nutrition during mid to late gestation affects growth, adipose tissue deposition, and tenderness in cross-bred beef steers Meat Sci 86:588-593.

137. Van der Heyden L, Lejeune JP, Caudron I, Detilleux J, Sandersen C, Chavatte P, Paris J, Deliege B and Serteyn D 2013 Association of breeding conditions with prevalence of osteochondrosis in foals. Vet Rec 172:68.

138. van der Linden DS, Kenyon PR, Blair HT, Lopez-Villalobos N, Jenkinson CM, Peterson SW and Mackenzie DD 2009 Effects of ewe size and nutrition on fetal mammary gland development and lactational performance of offspring at their first lactation J Anim Sci 87:3944-3954.

139. van Montfoort AP, Hanssen LL, de Sutter P, Viville S, Geraedts JP and de Boer P 2012 Assisted reproduction treatment and epigenetic inheritance Hum Reprod Update 18:171-197.

140. Vonnahme KA, Hess BW, Nijland MJ, Nathanielsz PW and Ford SP 2006 Placentomal differentiation may compensate for maternal nutrient restriction in ewes adapted to harsh range conditions J. Anim Sci. 84:3451-3459.

141. Wallace JM, Bourke DA, Aitken RP, Milne JS and Hay WW 2003 Placental glucose transport in growth-restricted pregnancies induced by overnourishing adolescent sheep Journal of Physiology-London 547:85-94.

142. Wallace JM, Milne JS and Aitken RP 2005 The effect of overnourishing singleton-bearing adult ewes on nutrient partitioning to the gravid uterus British Journal of Nutrition 94:533-539.

143. Walton A and Hammond J 1938 The maternal effects on growth and conformation in shire horse-shetland pony crosses. Proc. R. Soc. Lond. B. 125:311-335

144. Watkins A, Lucas ES and Fleming T 2010 Impact of the periconceptional environment on the programming of adult disease. Journal of Developmental Origins of Health and Disease, 1:1-9.

145. Watkins AJ, Papenbrock T and Fleming TP 2008 The preimplantation embryo: Handle with care Seminars in Reproductive Medicine 26:175-185.

146. Whorwood CB, Firth KM, Budge H and Symonds ME 2001 Maternal undernutrition during early to midgestation programs tissue-specific alterations in the expression of the glucocorticoid receptor, 11beta-hydroxysteroid dehydrogenase isoforms, and type 1 angiotensin ii receptor in neonatal sheep Endocrinology 142.

147. Williams-Wyss O, Zhang S, MacLaughlin SM, Kleemann D, Walker SK, Suter CM, Cropley JE, Morrison JL, Roberts CT and McMillen IC 2014 Embryo number and periconceptional undernutrition in the sheep have differential effects on adrenal epigenotype, growth, and development American Journal of Physiology-Endocrinology and Metabolism 307:E141-E150.

148. Wilsher S and Allen WR 2006 Effects of a Streptococcus equi infection--mediated nutritional insult during mid-gestation in primiparous Thoroughbred fillies. Part 1: placental and fetal development Equine Vet J 38:549-557.

149. Wu G, Bazer FW, Cudd TA, Meininger CJ and Spencer TE 2004 Maternal nutrition and fetal development J Nutr 134:2169-2172.

150. Wu G, Bazer FW, Wallace JM and Spencer TE 2006 Intrauterine growth retardation: Implications for the animal sciences Journal of Animal Science 84:2316-2337.

151. Zhang L, Long NM, Hein SM, Ma Y, Nathanielsz PW and Ford SP 2011 Maternal obesity in ewes results in reduced fetal pancreatic beta-cell numbers in late gestation and decreased circulating insulin concentration at term Domestic Animal Endocrinology 40:30-39.

152. Zhang S, Morrison JL, Gill A, Rattanatray L, MacLaughlin SM, Kleemann D, Walker SK and McMillen IC 2013 Dietary restriction in the periconceptional period in normal-weight or obese ewes results in increased abundance of angiotensin-converting enzyme (ACE) and angiotensin type 1 receptor (AT1R) in the absence of changes in ACE or AT1R methylation in the adrenal of the offspring Reproduction 146:443-454.

153. Zhang S, Morrison JL, Gill A, Rattanatray L, MacLaughlin SM, Kleemann D, Walker SK and McMillen IC 2013 Maternal Dietary Restriction During the Periconceptional Period in Normal-Weight or Obese Ewes Results in Adrenocortical Hypertrophy, an Up-Regulation of the JAK/STAT and Down-Regulation of the IGF1R Signaling Pathways in the Adrenal of the Postnatal Lamb Endocrinology 154:4650-4662.

154. Zhang S, Rattanatray L, McMillen IC, Suter CM and Morrison JL 2011 Periconceptional nutrition and the early programming of a life of obesity or adversity Progress in Biophysics & Molecular Biology 106:307-314.

155. Zhu MJ, Du M, Hess BW, Nathanielsz PW and Ford SP 2007 Periconceptional Nutrient Restriction in the Ewe Alters MAPK/ERK1/2 and PI3K/Akt Growth Signaling Pathways and Vascularity in the Placentome Placenta 28:1192-1199.

156. Zhu MJ, Du M, Nijland MJ, Nathanielsz PW, Hess BW, Moss GE and Ford SP 2009 Down-Regulation of Growth Signaling Pathways Linked to a Reduced Cotyledonary Vascularity in Placentomes of Over-Nourished, Obese Pregnant Ewes Placenta 30:405-410.

157. Zhu MJ, Ford SP, Means WJ, Hess BW, Nathanielsz PW and Du M 2006 Maternal nutrient restriction affects properties of skeletal muscle in offspring Journal of Physiology-London 575:241-250.

EMBRYO METABOLISM: WHAT DOES IT REALLY MEAN?

NIAMH LEWIS[1] & ROGER G. STURMEY[2, 3]

[1] *Institute of Aging and Chronic Disease*
University of Liverpool,
Leahurst campus,
CH64 7TE,
UK

[2] *Centre for Cardiovascular and Metabolic Research*
Hull York Medical School
University of Hull
Hull
HU67RX
UK
[3] *Correspondence: roger.sturmey@hyms.ac.uk*

Abstract

The study of early embryo metabolism has fascinated researchers in the field for nearly a century. Herein, we give a brief account of the general features of embryo metabolism and some consideration of the research performed to reach such conclusions. It is becoming increasingly obvious that metabolism informs many fate decisions and outcomes beyond ATP generation, such as DNA methylation, Reactive Oxygen Species generation and cell signaling. We discuss the reasons for studying metabolism in the face of our current knowledge of the effect that the culture environment on the developing embryo and the downstream effects that can cause. The study of *in vitro* embryo metabolism can also give us insight into developmental perturbations *in vivo*. The strengths and limitations of the methods we use to study metabolism are reviewed with reference to species-specific fundamental biology and plasticity and we discuss what the future holds for metabolic studies and the unanswered questions that remain.

Introduction

The study of mammalian early embryo metabolism has a rich history (Leese, 2012). Whilst work in the period of the 1940s-1960s focused on the effect of adding energy substrates to embryos in culture, real progress in understanding embryo metabolism was made in the 1970s by the likes of Biggers and Stern (1973), Brinster (1973) and Gwatkin and Haidri (1974) who examined the fate of radiolabeled compounds added to the medium. From experiments such as these, a picture of early embryo metabolism began to emerge. Like so much of our knowledge of early mammalian embryo development, the first data came from the classical laboratory model species; mouse and rabbit, as well as the hamster. Interest grew, and embryo metabolism was soon examined in the large domestic animals; pigs, cattle, sheep and, to a lesser extent, the horse, dog and cat. Underpinning research were studies on early human development with the aim of clinical translation for the treatment of infertility; a feat first achieved in 1978 by Steptoe and Edwards. Alongside this feat was the development of assisted conception techniques for use in farm animals. It is not the intention of this article to re-describe the history of the research that lead to successful embryo culture or the contribution that studies on metabolism. For expert insight, the reader is encouraged to read (Leese, 2012; Chronopoulou and Harper, 2014).

Embryo metabolism: what do we know?

The description of carbohydrate metabolism during preimplantation development is largely accepted and will be familiar to anyone who has an interest in the early embryo. In almost all

species studied, the cleavage stage embryo, from fertilisation through to formation of the morula, is relatively metabolically quiescent. Oxygen consumption at this time remains comparatively low, and the dominant substrate depleted from the culture environment is pyruvate. Pyruvate is consumed at an almost steady rate during cleavage, with a proportion of the carbon (depending on the species) appearing in the medium as lactate with the generation of metabolic energy. The source of the pyruvate involved in such reactions is generally either glycolytic conversion of glucose or that taken up directly from the external environment. Pyruvate may also enter the Tricarboxylic Acid (TCA; Krebs) cycle, where it can be oxidised completely generating electron donors for the electron transport chain which occurs in the matrix of mitochondria and relies on oxygen acting as the terminal electron acceptor. For this reason, oxygen consumption provides a good marker of overall oxidative metabolic activity (for review, see Smith and Sturmey, 2013).

As the cleavage stage embryo progresses to a blastocyst, there is a sharp and characteristic rise in the amount of glucose consumed in all species studied, and a concomitant rise in lactate release into the medium. Coincident with this is a fall in pyruvate consumption. This general pattern of "blastocyst glycolysis" appears to be conserved across all species studied. There are a range of explanations for this, however, as glycolysis is a comparatively inefficient means of generating ATP therefore energy production is unlikely to be the prime reason. Moreover, as the blastocyst forms, oxygen consumption also rises (Fridhandler *et al.*, 1957, Houghton *et al.*, 1996, Sturmey and Leese, 2003, Thompson *et al.*, 1996, Trimarchi *et al.* 2000) further supporting the notion that glycolytic production of lactate is of minor consequence in contributing ATP for the blastocyst. It is much more likely that glycolysis rises to meet the need for carbon for biosynthetic processes. A description of glycolysis in the early embryo can be found in Smith and Sturmey (2013). This general picture of embryo metabolism was summarized with great prescience by Brinster in 1973; in the intervening years many laboratories across the world have generated evidence to support such a description, illustrating the robustness with which these findings can be considered.

While early work focused on carbohydrate metabolism, it is now clear that the metabolism of amino acids, lipids and vitamins such as folate all also act in an interdependent manner to produce a viable embryo. Amino acids are crucial components of the culture environment *in vitro (*reviewed by Sturmey *et al*., 2010). Their addition to simple culture medium either singly (Rieger *et al.*, 1992) or in combinations (Chatot *et al*., 1989, Gardner and Lane, 1993) permitted mouse embryos to be cultured past the so-called 2-cell block (Chatot *et al.*, 1989) and their widespread inclusion lead to improved blastocyst rates in almost every species studied. The addition of amino acids has had such a positive effect on the efficacy of *in vitro* embryo culture, that their inclusion is often described as having a primary role in the formulation of "next generation medium" (Leese, 2012). The precise mechanism for the positive effect of amino acid provision is still to be defined, however it is well established that addition of amino acids to *in vitro* medium can alleviate culture associated stress in flushed murine embryos (Lane and Gardner, 1998). The contribution that amino acid metabolism makes to ATP production remains unclear, however the turnover of amino acids (that is, the sum of their depletion or accumulation into the culture droplet) has been linked to embryo blastocyst rates (Houghton, 2002), human embryo live birth rates (Brison *et al*., 2004), DNA damage (Sturmey, 2009), aneuploidy (Picton *et al.*, 2010) embryo sex (Sturmey *et al.*, 2009a), maternal age (Picton *et al.*, 2010) and embryonic stress (Wale and Gardner, 2012).

When considering energy metabolism of early embryos, it is vital that the contribution made by endogenous triglyceride is not overlooked. Fatty acid β-oxidation was studied in detail in the 1970s by Kane and colleagues (1979) but then largely ignored, with the notable exception of the work by Downs (see Downs 2015). However, interest in fatty acid metabolism has re-awakened, partly in response to the report from Dunning *et al*., (2010) who elegantly demonstrated that mouse oocytes require fatty acid oxidation in order to develop. A similar conclusion was drawn by

Sturmey and Leese (2003) in the pig, underlining the importance of fatty acid β-oxidation during oocyte maturation, development and in the preimplantation stages. Species differences in the importance of fatty acid oxidation during oocyte and embryo development have also been identified. For example, where a mouse zygote will arrest after 15 hours in media lacking nutrients (cited in Leese, 2012) a rabbit embryo can complete up to 3 cleavage divisions in the absence of energy substrates (Kane, 1987) and sheep embryos can also develop to the blastocyst stage in the absence of glucose (Thompson *et al.*, 1992). This can be explained by the differences in intracellular triglyceride content, acting in a buffering capacity by providing an alternate energy source (Ferguson and Leese, 2006; Sturmey *et al.*, 2009). Recently, a number of laboratories have described altered fatty acid metabolism by embryos from overweight and obese mice (Pantasri *et al.*, 2015; Reynolds *et al.*, 2015) and the human (Leary *et al.*, 2014). After receiving comparatively little attention since the work of Kane, interest in fatty acid metabolism by oocytes and embryos has been intense, and has been widely reviewed in recent years (Downs, 2015, Dunning *et al*., 2014, Leroy *et al.*, 2012, McKeegan and Sturmey, 2012, Sturmey *et al*., 2009).

This very brief overview is intended to remind the reader of the basic features of early embryo energy metabolism. However, 'metabolism' refers to significantly more functions than ATP generation. For example, there is an extensive literature describing the role of the pentose phosphate pathway (Downs *et al.*, 1998; Sutton-McDowall *et al.*, 2010) in mammalian oocytes and early embryos. Moreover, metabolic processes link to signaling mechanisms (Manser and Houghton, 2006), generation of Reactive Oxygen Species (Agarwal *et al.*, 2005) and gene expression in terms of establishment of epigenetic marks such as methylation and acetylation and post-translational modifications of proteins (DeBerardinis and Thompson, 2012). For example, defects in folate metabolism have been linked to methylation and epigenetic modifications affecting developmental competence (Xu and Sinclair, 2015). However, reviewing all of the literature on embryo metabolism in it's broadest sense would require several articles and so in the remainder of this article, we will consider some more fundamental aspects.

Why do we study embryo metabolism?

Understanding the basic physiology and metabolism of the early embryo is a noble quest in itself that has fascinated researchers over the past decades. However, a major gap in our knowledge is the metabolism of the *in vivo* produced embryo, as well as the embryo *in situ*, which remain an elusive goal. We aim to gain information that can, and has been, translated into clinical practice in many ways; to design appropriate species specific culture media with the aim of producing viable healthy offspring; to design non-invasive methods for embryo selection for transfer and shed light on metabolic perturbations occurring *in vivo.* Moreover, as our understanding of somatic cell nuclear transfer (SCNT; Wilmut *et al.*, 2002) grows and becomes linked inextricably to stem cell physiology and regenerative medicine, we must also accept that we know comparatively little about the impact of such techniques may have on embryo physiology. Furthermore, we are on the brink of many new and exciting developments in Assisted Conception, including mitochondrial transfer for the treatment of debilitating hereditary conditions as well as the replenishment of mitochondria in aged oocytes with the aim of improving pregnancy rates in older women (Craven *et al.*, 2010; Smeets, 2013). Such techniques may be considered 'beyond experimental'; mitochondrial transfer was licensed for treatment in the UK in 2014 and autologous mitochondrial transfer for infertility is already commercially available in some countries. However, since each of the approaches described above involve, in some way, altering the mitochondrial content of embryos, the need for detailed understanding of metabolic regulation of individual preimplantation mammalian embryo has never been greater.

A further drive to study embryo metabolism comes from the need to identify biomarkers of embryo health and viability. This relies on the inherent variability in metabolism between different embryos and has been used in an attempt to select viable embryos for transfer, with the end goal

being clinical IVF in humans. There have been several observations that have yielded promising results. The 'quiet embryo hypothesis' proposed by Leese in 2002, stated that those embryos that are viable have a decreased metabolic rate; a proposition that has been supported by several studies showing embryos with an upregulated metabolism of both carbohydrates and amino acids to have decreased viability post transfer (Guerif *et al*., 2013, Lane and Gardner, 1996, Sturmey *et al.*, 2009). However, the notion is contested, and there are recent studies suggesting that elevated metabolism, particularly with respect to glucose consumption is associated with embryo viability (Gardner *et al.*, 2011). Clearly, this is an area in which more work is needed.

Since pioneering observations linking human birth weight to cardiovascular events in later life by David Barker (1989) it has now been shown unequivocally in many species that the periconceptual environment can have downstream effects which can impact on the viability of the developing embryo and on the future health of the resulting offspring (Ceelen *et al*., 2008, Fleming *et al.*, 2012, Frank *et al*., 2014, Leroy *et al.*, 2009, Watkins *et al*., 2008). It is also clear that certain embryonic stages are more susceptible to damage (Rieger, 1992), such as the early cleavage embryo during embryonic genome activation, suggesting that progeny may have a 'memory' of their origins.

With the rising obesity epidemic both in humans and companion animals, in addition to metabolic disease in farm animal species due to increased production pressures, the study of embryo metabolism *in vitro* can provide insight into the mechanisms of resultant suppressed fertility and potentially identify therapeutic interventions.

These are important reasons for studying embryo metabolism, and it is clear that metabolic processes can directly influence gene expression (Van Hoeck *et al*., 2013, Van Hoeck *et al.*, 2011), and patterning of the embryo (Leary *et al.*, 2014). However, it is also of fundamental importance to be aware of what is measured when studying embryo metabolism. In the final part of this review, we will describe the strengths and limitations of embryo metabolic studies.

What are we actually measuring?

The measurement of embryo metabolism is faced with many technical challenges. Critically, the *in vivo* environment is still largely unknown for most species, meaning that the extrapolation of knowledge to an embryo *in vivo* is of questionable validity. The data available on embryo metabolism inform us of the strategy of substrate depletion and appearance in a given milieu. *In vitro*, this milieu is constrained by the addition of a limited number of substrates at static levels; supply and ratio of substrates varies only in response to an embryo's own activity. This is in stark contrast to the situation *in vivo*, which is dynamic and responsive (Leese *et al.,* 2008). Even in species for which the *in vivo* embryo environment has been described, the method used to define it should be noted. Often *post mortem* changes and/or inflammatory changes due to catheterization can influence results thus making samples non-representative (Leese *et al.*, 2008). Moreover, the embryo *in situ* likely exists in a microenvironment within the oviduct, thus any subtle, specific composition features will be lost in flushing of the tube.

Given the heterogeneity in developmental potential, measures pertaining to single embryos are key and thus highly sensitive assays are needed. Both the use of radiolabelled substrates (Rieger *et al.*, 1992) and enzyme-linked fluorescence assays to detect the appearance and disappearance of a substrate from culture media have been described (Leese and Barton, 1984; Guerif *et al.*, 2013). The relative metabolic quiescence of single embryos means that 'analysis media' (that is a medium in which the concentrations of substrates is reduced to enable measurement of change) is often used in order to permit detection of changes in substrate concentration (Hardy *et al.*, 1989; Sturmey and Leese, 2003). This 'analysis medium' is often different to the *in vitro* culture media known to support development for most species, which, in turn differs vastly to the *in vivo* environment. Of

course, it also must be realized that there are many complex cell transport and metabolic pathways involved, and notions of influx and efflux leads' us to make what are essentially educated guesses about what occurs in the cell. Despite these limitations, these assays have greatly advanced our knowledge of metabolic pathways involved and have yielded highly repeatable results across different laboratories. Further methods that have been used to detect metabolic activity of embryos include culturing individually in micro-droplets or in large groups of embryos. However, the resolution of data from group culture is reduced since individual embryo heterogeneity is lost by 'averaging'.

New promising studies using NMR metabolomic technology, where substrate flux can be measured in situ have been recently described (Krisher *et al.,* 2015), however the subsequent interpretation and analysis of the complex data acquired presents new challenges.

Inferences about the contribution of oxidative metabolism are usually derived from measuring oxygen consumption. Methods vary, the most widely used being pyrene fluorescence (Houghton *et al.*, 1996) and nanorespirometry (Lopes *et al.*, 2010). Again while allowing accurate measurement of oxygen depletion in single embryos and seemingly not affecting development (Lopes *et al*., 2005), the methods represents a significant 'alien' environment for the embryo.

Studies involving metabolic inhibitors and enzymatic co-factors have also added to our knowledge of embryo metabolism and in some cases provided the initial proof of certain pathways occurring and either being essential or non-essential for development. Among these, Brison and Leese (1994) showed that oxidative phosphorylation was not an absolute requirement for blastocoele formation in the rat by culturing embryos in the presence of cyanide, while Macháty and colleagues (2001) indicated that suppression of oxidative phosphorylation at the morula stage improved development to the blastocyst in the pig. Moreover, Dunning *et al.*, (2010) have shown that β-oxidation is essential for optimal development in the mouse by culturing in the presence of etomoxir. In some cases, inhibition of certain metabolic pathways has been shown to improve developmental potential; for example the addition of EDTA to embryo culture medium (Gardner *et al.*, 2000). Although the mechanism is not confirmed, one possible role of EDTA in embryo culture medium is the suppression of glycolysis (Gardner *et al.*, 2000). However, it is equally likely that EDTA acts as an antioxidant by sequesteration of metal ions which would otherwise catalyse the formation of Reactive Oxygen Species (Orsi and Leese, 2001). Studies such as these illustrate the importance of appropriate regulation of metabolic pathways during development and also indicate why it is necessary for pathways to be correctly orchestrated to match needs at a given stage of development.

It all depends on the environment

It could be argued that measuring embryo metabolism *in vitro* (by necessity) amounts to measuring a stress response. This issue must be considered given the extremely adaptable nature of embryos of all species. Metabolism is necessarily dynamic, enabling rapid changes in needs to be met to maintain development. However, such dynamism means that the metabolic profile of an embryo can respond quickly in response to a change in external environment, shown clearly in mice, where perturbations occur within 3 hours of *in vitro* culture in flushed *in vivo* blastocysts (Lane and Gardner, 1998). Both the presence and relative quantities of metabolic substrates in the environment in which experiments are conducted will significantly affect the results. While not attempting to provide a detailed discussion on the controversial aspects of *in vitro* culture systems, which still vary widely across laboratories, this point can be further illustrated by the differential metabolism that results from the presence or absence of serum and the atmospheric oxygen concentration (Wale and Gardner 2010).

While the human IVF industry has moved towards defined culture media using macromolecular sources such as recombinant albumin, serum is still used in many production animal systems. Culture with serum has been shown to increase blastocyst development rates in the horse (Choi *et al.*, 2004) and the kinetics of blastocyst development in the cow (Rizos *et al.*, 2003). However, its presence has also been associated with increased intracellular lipid content (Ferguson and Leese, 2006) and altered metabolism (Reis *et al.*, 2003), up-regulation of oxidative stress and inflammatory pathways (Cagnone & Sirard, 2014) and decreased survival after vitrification (Gómez *et al.*, 2008). In addition, the oxygen tension of the reproductive tract in all species studied has been found to be below 10% (Fischer and Bavister, 1993), In terms of the environmental gas profile, there is now unequivocal evidence to support the notion that 20% oxygen reduces embryo development (Thompson *et al.*, 1990, Wale and Gardner, 2010) and that culture in low oxygen (5%) results in metabolic and proteomic profiles more closely matching *in vivo* counterparts (Thomson *et al.*, 1990; Katz-Jaffe *et al.*, 2005). Clearly, these factors will influence the results of any metabolic study and must be kept in mind when comparing studies.

In addition to the embryo adapting to its environment, the culture environment itself is not static. Depletion and accumulation of excreted of substrates such as lactate and amino acids will change the local environment. Spontaneous de-amination will occur at 37°C, especially of glutamine, resulting in ammonium build up (Gardner and Lane, 1993), lactate build up may overwhelm pH buffering system of the media and depletion of energy substrates can lead to alternative ATP generating pathways being used (Kane, 1987).

It is also important to note that the manner in which an embryo responds to its environment is species specific. This can be seen in differences in response to hyperglycaemia. While species such as rodents and humans, will have significant diminished development in the presence of high glucose (Moley *et al.*, 1998; Frank *et al.*, 2014), others such as the horse and pig are apparently unaffected (Choi *et al.,* 2015, Sturmey and Leese, 2003). Qualitative testing of equine embryos produced in hyperglycaemic conditions however, highlights subtle differences not reflected in the blastocyst development rate such as a decrease in ICM cell number allocation (also observed in the rat) and known to be mediated through apoptosis (Moley *et al.*, 1998; Choi *et al.*, 2015).

It is thus vital to consider that studies on embryo metabolism provide us a snapshot of physiology *in a given set of conditions*. Whilst such data are of fundamental importance, care must be taken when extrapolating and comparing such information. It is thus much more desirable that studies on the depletion and appearance of embryo metabolism are reinforced by consideration of mechanisms of metabolic regulation of early development.

Embryo metabolism: some unanswered questions

As the emphasis in human IVF is increasingly on single embryo transfer, the identification of reliable non-invasive methods of determining embryo quality to maximize pregnancy rate per transfer remains the Holy Grail. Moreover, in species such as the horse where *in vitro* embryo production is rapidly generating interest, a specific tailored culture media has yet to be formulated. Whilst acceptable blastocyst rates (41%) and pregnancy rates after transfer (66%) can be achieved by some laboratories in the horse using cell culture media such as DMEM-F12, (Jacobson *et al.*, 2010; Hinrichs *et al.*, 2014) the more subtle effects of potentially inappropriate culture conditions leading to decreased viability remain to be seen. Identifying optimal species-specific culture systems presents an exciting challenge for those involved in studying embryo metabolism.

Sex selection is another lively area of embryo metabolism. Ethical considerations preclude the implementation of sex selection in the human, but in the production animal industry, and in dairy cattle in particular, appropriate non-invasive identification of sex before transfer would be an application with many uses. Promising results have been presented so far showing that both glucose

metabolism and amino acid metabolism varies with sex (Sturmey *et al.,* 2010; for review see Gardner *et al.*, 2010), however more work will need performed to increase specificity in order for the technology to make the transition to commercial practice.

New information is emerging all the time on the far-reaching downstream effects of aberrations in early embryo metabolism (Harrison and Langley-Evans, 2009). Given the clear links between the periconceptual environment and sub-optimal health outcomes in the human (Barker *et al.*, 2002) and production species such as the bovine (for example, the so-called Large Offspring Syndrome; Young *et al.*, 1998), understanding and attempting to mitigate the negative effects on suboptimal embryo development and life-long health of the offspring is an important area for future study (Leese 2014).

Conclusions

It is acknowledged "that metabolism pervades every aspect of cell physiology" (DeBerardinis and Thompson, 2012) and this is especially pertinent to the developmentally plastic early mammalian embryo. As genomic, transcriptomic and imaging techniques advance we will be able to expand our understanding of embryo metabolism and how it links inextricably with developmental pathways through subsequent stages of gestation leading to the birth of a healthy offspring. It is the responsibility of us all working in the earliest stages of this process to understand the periconceptual environmental challenges faced by the embryo and to optimize the conditions under which it is grown to ensure the best start in life. Metabolic studies allow us to gain vital information on the requirements of a competent embryo and identify when things go wrong, but the reader is cautioned towards careful interpretation of measures of metabolism especially between laboratories and to consider the environment as a whole under which they have been taken.

Acknowledgements

NL is funded by the UK BBSRC. The authors are grateful to Professor Henry Leese for critical comments in the drafting of this article.

References

1. Agarwal, A., Gupta, S. & Sharma, R.K. 2005. Role of oxidative stress in female reproduction. Reprod. Biol. Endocrinol. 3: 28.

2. Barker, D., Eriksson, J., Forsen, T. & Osmond, C. 2002. Fetal origins of adult disease: strength of effects and biological basis. Int J Epidemiol 31: 1235–1239.

3. Barker, D.J., Osmond, C., Golding, J., Kuh, D. & Wadsworth, M.E. 1989. Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. Bmj 298: 564–567.

4. Biggers JD & Stern S. 1973. Metabolism of preimplantation mammalian embryos. Adv. Reprod. Physiol 6: 1–59.

5. Brinster R.L. 1973. Nutrition and metabolism of the ovum, zygote and blastocyst. In: Handbook of Physiology, pp. 165–185.

6. Brison, D.R., Houghton, F.D., Falconer, D., Roberts, S.A., Hawkhead, J., Humpherson, P.G., et al. 2004. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. 19: 2319–2324.

7. Brison, D.R. & J, L.H. 1994. Blastocoel cavity formation by preimplantation rat embryos in the presence of cyanide and other inhibitors of oxidative phosphorylation. Reproduction.

8. Cagnone, G. & Sirard, M.A. 2014. The impact of exposure to serum lipids during in vitro culture on the transcriptome of bovine blastocysts. Theriogenology 81: 712–722. Elsevier Inc.

9. Ceelen, M., Van Weissenbruch, M.M., Vermeiden, J.P.W., Van Leeuwen, F.E. & Delemarre-Van De Waal, H. a. 2008. Cardiometabolic differences in children born after in vitro fertilization: Follow-up study. J. Clin. Endocrinol. Metab. 93: 1682–1688.

10. Chatot, C.L., Ziomek, C. a, Bavister, B.D., Lewis, J.L. & Torres, I. 1989. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J. Reprod. Fertil. 86: 679–688.

11. Choi, Y., Ross, P., Velez, I.C., Riera, F.L., Hinrichs, K. & Sciences, B. 2015. Cell lineage allocation in equine blastocysts produced in vitro under varying glucose concentrations. 1–40.

12. Choi, Y.H., Roasa, L.M., Love, C.C., Varner, D.D., Brinsko, S.P. & Hinrichs, K. 2004. Blastocyst formation rates in vivo and in vitro of in vitro-matured equine oocytes fertilized by intracytoplasmic sperm injection. Biol. Reprod. 70: 1231–8.

13. Chronopoulou, E. & Harper, J.C. 2014. IVF culture media: past, present and future. Hum. Reprod. Update 0: 1–17.

14. Craven, L., Tuppen, H. a, Greggains, G.D., Harbottle, S.J., Murphy, J.L., Cree, L.M., et al. 2010. Pronuclear transfer in human embryos to prevent transmission of mitochondrial DNA disease. Nature 465: 82–85.

15. DeBerardinis, R.J. & Thompson, C.B. 2012. Cellular Metabolism and disease: what do metabolic outliers teach us? Cell 29: 997–1003.

16. Downs, S.M. 2015. Nutrient pathways regulating the nuclear maturation of mammalian oocytes. 572–582.

17. Downs, S.M., Humpherson, P.G. & Leese, H.J. 1998. Meiotic induction in cumulus cell-enclosed mouse oocytes: involvement of the pentose phosphate pathway. Biol. Reprod. 58: 1084–1094.

18. Dunning, K.R., Cashman, K., Russell, D.L., Thompson, J.G., Norman, R.J. & Robker, R.L. 2010. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. Biol. Reprod. 83: 909–18.

19. Dunning, K.R., Russell, D.L. & Robker, R.L. 2014. Lipids and oocyte developmental competence: the role of fatty acids and β-oxidation. Reproduction 148: R15–R27.

20. Ferguson, E.M. & Leese, H.J. 2006. A Potential Role for Triglyceride as an Energy Source During Bovine Oocyte Maturation and Early Embryo Development. 1201: 1195–1201.

21. Fischer, B. & Bavister, B.D. 1993. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. J. Reprod. Fertil. 99: 673–679.

22. Fleming, T.P., Velazquez, M. a., Eckert, J.J., Lucas, E.S. & Watkins, a. J. 2012. Nutrition of females during the peri-conceptional period and effects on foetal programming and health of offspring. Anim. Reprod. Sci. 130: 193–197. Elsevier B.V.

23. Frank, L.A., Sutton-mcdowall, M.L., Gilchrist, R.B. & Thompson, J.G. 2014. The Effect of Peri-Conception Hyperglycaemia and the Involvement of the Hexosamine Biosynthesis Pathway in Mediating Oocyte and Embryo Developmental Competence. 408: 391–408.

24. Fridhandler, L., Hafez, E.S.E. & Pincys, G. 1957. Developmental changes in the respiratory activity of rabbit ova. Expl. Cell Res. 13: 132.

25. Gardner, D.K. & Lane, M. 1993. Amino acids and ammonium regulate mouse embryo development in culture. Biol. Reprod. 48: 377–385.

26. Gardner, D.K., Lane, M.W. & Lane, M. 2000. EDTA stimulates cleavage stage bovine embryo development in culture but inhibits blastocyst development and differentiation. Mol. Reprod. Dev. 57: 256–261.

27. Gardner, D.K., Larman, M.G. & Thouas, G. a. 2010. Sex-related physiology of the preimplantation embryo. Mol. Hum. Reprod. 16: 539–547.

28. Gardner, D.K., Wale, P.L., Collins, R. & Lane, M. 2011. Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. Hum. Reprod. 26: 1981–1986.

29. Gómez, E., Rodríguez, a, Muñoz, M., Caamaño, J.N., Hidalgo, C.O., Morán, E., et al. 2008. Serum free embryo culture medium improves in vitro survival of bovine blastocysts to vitrification. Theriogenology 69: 1013–21.

30. Guerif, F., McKeegan, P., Leese, H.J. & Sturmey, R.G. 2013. A Simple Approach for COnsumption and RElease (CORE) Analysis of Metabolic Activity in Single Mammalian Embryos. PLoS One 8.

31. Gwatkin, R.B. & Haidri, a a. 1974. Oxygen requirements for the maturation of hamster oocytes. J. Reprod. Fertil. 37: 127–129.

32. Hardy, K., Hooper, M.A., Handyside, A.H., Rutherford, A.J., Winston, R.M. & Leese, H.J. 1989. Non-invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos. Hum. Reprod. 4: 188–191.

33. Harrison, M. & Langley-Evans, S.C. 2009. Intergenerational programming of impaired nephrogenesis and hypertension in rats following maternal protein restriction during pregnancy. Br. J. Nutr. 101: 1020–1030.

34. Hinrichs, K., Choi, Y.H., Love, C.C. & Spacek, S. 2014. Use of in vitro maturation of oocytes, intracytoplasmic sperm injection and in vitro culture to the blastocyst stage in a commercial equine assisted reproduction program. J. Equine Vet. Sci. 34: 176. Elsevier Inc.

35. Houghton, F.D. 2002. non invasive amino acid turn over predicts human embryo developmental capacity. Hum. Reprod.

36. Houghton, F.D., Thompson, J.G., Kennedy, C.J. & Leese, H.J. 1996. Oxygen consumption and energy metabolism of the early mouse embryo. Mol. Reprod. Dev. 44: 476–85. Wiley Subscription Services, Inc., A Wiley Company.

37. Jacobson, C.C., Choi, Y.-H., Hayden, S.S. & Hinrichs, K. 2010. Recovery of mare oocytes on a fixed biweekly schedule, and resulting blastocyst formation after intracytoplasmic sperm injection. Theriogenology 73: 1116–26. Elsevier Inc.

38. Kane. 1979. Fatty Acids as Energy Rabbit for Culture Morulae of One-Cell Ova to Viable essential acting. 323–332.

39. Kane, M.T. 1987. Minimal nutrient requirements for culture of one-cell rabbit embryos. Biol. Reprod. 37: 775–778.

40. Katz-Jaffe, M.G., Linck, D.W., Schoolcraft, W.B. & Gardner, D.K. 2005. A proteomic analysis of mammalian preimplantation embryonic development. Reproduction 130: 899–905.

41. Krisher, R.L., Heuberger, A.L., Paczkowski, M., Stevens, J., Pospisil, C., Prather, R.S., et al. 2015. Applying metabolomic analyses to the practice of embryology : physiology , development and assisted reproductive technology. 602–620.

42. Lane, M. & Gardner, D.K. 1998. Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. Hum. Reprod. 13: 991–997.

43. Lane, M. & Gardner, D.K. 1996. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. Hum. Reprod. 11: 1975–1978.

44. Leary, C., Leese, H.J. & Sturmey, R.G. 2014. Human embryos from overweight and obese women display phenotypic and metabolic abnormalities. Hum. Reprod. 30: 122–132.

45. Leese, H.J. 2012. Metabolism of the preimplantation embryo; 40 years on. 1–43.

46. Leese, H.J. 2002. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. Bioessays 24: 845–9.

47. Leese, H.J. & Barton, A.M. 1984. Pyruvate and preimplantation embryos and. 2: 1–5.

48. Leese, H.J., Hugentobler, S. a, Gray, S.M., Morris, D.G., Sturmey, R.G., Whitear, S.-L., et al. 2008. Female reproductive tract fluids: composition, mechanism of formation and potential role in the developmental origins of health and disease. Reprod. Fertil. Dev. 20: 1–8.

49. Leese, H.J. 2014. Effective nutrition from conception to adulthood. Human Fertility. 17: 252-256

50. Leroy, J.L.M.R., Rizos, D., Sturmey, R., Bossaert, P., Gutierrez-Adan, a., Van Hoeck, V., et al. 2012. Intrafollicular conditions as a major link between maternal metabolism and oocyte quality: A focus on dairy cow fertility. Reprod. Fertil. Dev. 24: 1–12.

51. Leroy, J.L.M.R., Van Hoeck, V., Clemente, M., Rizos, D., Gutierrez-Adan, a., Van Soom, a., et al. 2009. The effect of nutritionally induced hyperlipidaemia on in vitro bovine embryo quality. Hum. Reprod. 25: 768–778.

52. Lopes, A.S., Lane, M. & Thompson, J.G. 2010. Oxygen consumption and ROS production are increased at the time of fertilization and cell cleavage in bovine zygotes. Hum. Reprod. 25: 2762–2773.

53. Lopes, A.S., Larsen, L.H., Ramsing, N., Løvendahl, P., Räty, M., Peippo, J., et al. 2005. Respiration rates of individual bovine in vitro-produced embryos measured with a novel, non-invasive and highly sensitive microsensor system. Reproduction 130: 669–679.

54. Macháty, Z., Thompson, J.G., Abeydeera, L.R., Day, B.N. & Prather, R.S. 2001. Inhibitors of mitochondrial ATP production at the time of compaction improve development of in vitro produced porcine embryos. Mol. Reprod. Dev. 58: 39–44.

55. Manser, R.C. & Houghton, F.D. 2006. Ca2+ -linked upregulation and mitochondrial production of nitric oxide in the mouse preimplantation embryo. J. Cell Sci. 119: 2048–2055.

56. McKeegan P.J. & Sturmey, R.G. 2012. The role of fatty acids in oocyte and early embryo development.

57. Moley, K.H., Chi, M.M., Knudson, C.M., Korsmeyer, S.J. & Mueckler, M.M. 1998. Hyperglycemia induces apoptosis in pre-implantation embryos through cell death effector pathways. Nat. Med. 4: 1421–1424.

58. Orsi, N.M. & Leese, H.J. 2001. Protection against reactive oxygen species during mouse preimplantation embryo development: role of EDTA, oxygen tension, catalase, superoxide dismutase and pyruvate. Mol. Reprod. Dev. 59: 44–53.

59. Pantasri, T., Wu, L.L., Hull, M.L., Sullivan, T.R., Barry, M., Norman, R.J., et al. 2015. Distinct localisation of lipids in the ovarian follicular environment. 593–601.

60. Picton, H.M., Elder, K., Houghton, F.D., Hawkhead, J. a., Rutherford, A.J., Hogg, J.E., et al. 2010. Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development in vitro. Mol. Hum. Reprod. 16: 557–569.

61. Reis, a., Rooke, J. a., McCallum, G.J., Staines, M.E., Ewen, M., Lomax, M. a., et al. 2003. Consequences of exposure to serum, with or without vitamin E supplementation, in terms of the fatty acid content and viability of bovine blastocysts produced in vitro. Reprod. Fertil. Dev. 15: 275–284.

62. Reynolds, K.A., Boudoures, A.L., Chi, M.M. & Qiang Wang. 2015. Adverse effects of obesity and / or high-fat diet on oocyte quality and metabolism are not reversible with resumption of regular diet in mice. 716–724.

63. Rieger. 1992. Developmentally related changes in the metabolism of glucose and glutamine by cattle embryos produced and co-cultured in vitro. J. Reprod. Fertil.

64. Rieger, D., Loskutoff, N.M. & Betteridge, K.J. 1992. Developmentally related changes in the uptake and metabolism of glucose, glutamine and pyruvate by cattle embryos produced in vitro. Reprod. Fertil. Dev. 4: 547–557.

65. Rizos, D., Gutiérrez-Adán, a, Pérez-Garnelo, S., De La Fuente, J., Boland, M.P. & Lonergan, P. 2003. Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. Biol. Reprod. 68: 236–243.

66. Smeets, H.J.M. 2013. Preventing the transmission of mitochondrial DNA disorders: Selecting the good guys or kicking out the bad guys. Reprod. Biomed. Online 27: 599–610. Reproductive Healthcare Ltd.

67. Smith, D.G. & Sturmey, R.G. 2013. Parallels between embryo and cancer cell metabolism. Biochem. Soc. Trans. 41: 664–9.

68. Steptoe, P.C. & Edwards, R.G. 1978. Birth after reimplantation of a human embryo. Lancet ii 366.

69. Sturmey, R.G., Bermejo-Alvarez, P., Gutierrez-Adan, a., Rizos, D., Leese, H.J. & Lonergan, P. 2010. Amino acid metabolism of bovine blastocysts: A biomarker of sex and viability. Mol. Reprod. Dev. 77: 285–296.

70. Sturmey, R.G., Hawkhead, J. a, Barker, E.A. & Leese, H.J. 2009a. DNA damage and metabolic activity in the preimplantation embryo. Hum. Reprod. 24: 81–91.

71. Sturmey, R.G. & Leese, H.J. 2003. Energy metabolism in pig oocytes and early embryos. Reproduction 126: 197–204.

72. Sturmey, R.G., Reis, A., Leese, H.J. & McEvoy, T.G. 2009b. Role of fatty acids in energy provision during oocyte maturation and early embryo development. Reprod. Domest. Anim. 44 Suppl 3: 50–8.

73. Sturmey, Roger G. 2009. DNA damage and metabolic activity in the preimplantation embryo. Hum. Reprod.

74. Sutton-McDowall, M.L., Gilchrist, R.B. & Thompson, J.G. 2010. The pivotal role of glucose metabolism in determining oocyte developmental competence. Reproduction 139: 685–95.

75. Thompson, J.G., Partridge, R.J., Houghton, F.D., Cox, C.I. & Leese, H.J. 1996. Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. Reproduction 106: 299–306.

76. Thompson, J.G., Simpson, A.C., Pugh, P. a & Tervit, H.R. 1992. Requirement for glucose during in vitro culture of sheep preimplantation embryos. Mol. Reprod. Dev. 31: 253–257.

77. Thomson, J.G.., Simpson, A.C., Pugh, P. a, Donelly, P.E. & Tervit, H.R. 1990. Effect of oxygen concentration preimplantation sheep and cattle embryos. J. Reprod. Fertil. 89: 573–578.

78. Trimarchi, J.R., Liu, L., Porterfield, M., Smith, P.J., & Keefe, D.L. 2000. Oxidative phosphorylation-dependent and -independent oxygen consumption by individual preimplantation mouse emrbyos. Biol Reprod, 62: 1866-1874.

79. Van Hoeck, V., Leroy, J.L.M.R., Arias Alvarez, M., Rizos, D., Gutierrez-Adan, a, Schnorbusch, K., et al. 2013. Oocyte developmental failure in response to elevated nonesterified fatty acid concentrations: mechanistic insights. Reproduction 145: 33–44.

80. Van Hoeck, V., Sturmey, R.G., Bermejo-Alvarez, P., Rizos, D., Gutierrez-Adan, A., Leese, H.J., et al. 2011. Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. PLoS One 6.

81. Wale, P.L. & Gardner, D.K. 2012. Oxygen Regulates Amino Acid Turnover and Carbohydrate Uptake During the Preimplantation Period of Mouse Embryo Development. Biol. Reprod. 87: 24–24.

82. Wale, P.L. & Gardner, D.K. 2010. Time-lapse analysis of mouse embryo development in oxygen gradients. Reprod. Biomed. Online 21: 402–410. Reproductive Healthcare Ltd.

83. Watkins, A.J., Ursell, E., Panton, R., Papenbrock, T., Hollis, L., Cunningham, C., et al. 2008. Adaptive responses by mouse early embryos to maternal diet protect fetal growth but predispose to adult onset disease. Biol. Reprod. 78: 299–306.

84. Wilmut, I., Beaujean, N., de Sousa, P. a, Dinnyes, a, King, T.J., Paterson, L. a, et al. 2002. Somatic cell nuclear transfer. Nature 419: 583–586.

85. Xu, J. & Sinclair, K.D. 2015. One-carbon metabolism and epigenetic regulation of embryo development. 667–676.

86. Young, L.E., Sinclair, K.D. & Wilmut, I. 1998. Large offspring syndrome in cattle and sheep. Rev. Reprod. 3: 155–163.

SHORT COMMUNICATIONS

FERTILITY EFFECTS OF PERFORMING OVUM PICK UP AT YOUNG AGE

AARDEMA A, DOTINGA F, FLAPPER H, VD BRINK A, PIETERSMA N, KNIJN H, SCHOUTEN J AND MULLAART E

CRV BV, PO Box 454, 6800 AL Arnhem, The Netherlands

To shorten the generation interval, and to increase the genetic progress, CRV started to perform Ovum Pick Up (OPU) at 9 months instead of 12 months of age (Reproduction Fertility and Development 12/2014; 27;:209). We demonstrated that animals that had their (first) estrus before the first OPU produced significantly more embryos than animals that did not show estrus before OPU. It is however not known however what the effect is of performing OPU on such young animals on their fertility (i.e. flushing results and AI index).

The aim of this study is to check the fertility of animals that have been used for OPU at young age. To investigate this we compared the flushing results and AI index of these animals.

Embryos were produced by OPU-IVP (once every week during a period of 4-9 weeks), followed by flushing (two times) and insemination (AI) to make the animals pregnant. We used 3 groups of animals, (1) 12 young animals (9-10 months) that had their first estrus before the OPU, (2) 24 young animals that did not had their first estrus before the OPU and a (3) control group of 16 older (12-14 month) animals.

The flushing results from young animals that had their first estrus before OPU (Group 1) were comparable with those of the control group (both 6.5 embryos per flush). However, flushing results from young animals that did not had their first estrus before OPU (group 2) were clearly lower and had only 4.1 embryo per flush.

Interestingly, the insemination results (AI index) showed the same tendency, e.g. animals that had their first estrus before needed 2.1 semen straws to get pregnant, while animals that did not had their estrus before OPU needed 2.6 straws.

It is therefore concluded that in young animals that showed estrus before the first OPU no difference in flushing results and AI index fertility results later in life were observed as compared to older animals. However, when no estrus was observed before the fist OPU, fertility results were lower. It is not known if this is due to the OPU at young age or that these are less fertile animals having estrus at a later stage.

Notes

CUMULUS CELLS PROTECT THE OOCYTE AGAINST FREE FATTY ACIDS

AARDEMA H, VAN TOL HTA, ROELEN BAJ

Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University

Cumulus cells have an intimate contact with, and provide metabolites to, the oocyte. The importance of cumulus cells for the oocyte extends into potential protection of the oocyte against free fatty acids (FFA)[1]. Exposure of cumulus-oocyte-complexes (COCs) to elevated FFA levels results in massive lipid accumulation in cumulus cells and normal developmental competence of oocytes[1]. Two potential mechanistic routes by which cells are protected against saturated FFA are lipid storage and β-oxidation[2]. To further unravel the presumed protection against FFA by cumulus cells, oocytes with and without cumulus cells were exposed to FFA. To investigate the potential mechanism by which cumulus cells may protect the oocyte, gene expression of cumulus cells from COCs matured in the presence or absence of FFA was analysed for DiGlyceride-AcylTransferase (*DGAT*; lipid storage), Carnitine-PalmitoylTransferase-1A (*CPT-1A*; β-oxidation) and Stearoyl-CoA-Desaturase *(SCD)*, the enzyme that converts saturated FFA into unsaturated.

COCs were collected from bovine slaughterhouse ovaries and during 23h matured with or without 250μM saturated stearic acid followed by standard fertilization and culture. After 8h of maturation, cumulus cells were removed from part of the COCs and oocytes were placed back in maturation medium. Gene expression of cumulus cells from COCs was analysed by QPCR for *DGAT, CPT-1A* and *SCD* before and after 23h culture with or without FFA, and from cumulus cells without an oocyte for *CPT-1A* and *SCD*. Statistical analysis was performed by a paired sample t-test (gene expression) and general linear model (culture data). Materials and methods according to Aardema et al.[1]

Removal of cumulus cells after 8h maturation resulted in oocytes with normal developmental competence (27±2.8%; 24±1.1% for COCs). Exposure to stearic acid resulted in strongly reduced developmental competence of oocytes cultured without cumulus during the last 15h (1±1.0%; $P<0.01$) compared to oocytes matured as COC (18±4.2%). Expression of *CPT-1A* ($P<0.01$) and *SCD* ($P<0.01$) in cumulus cells increased during maturation of COCs, independent of the presence of FFA. *DGAT* expression was not different among groups. The presence of an oocyte during culture resulted in higher *SCD* expression levels in cumulus cells after 23h of culture ($P<0.05$).

These data indicate that cumulus cells are essential to protect the oocyte against saturated stearic acid. The increase in *CPT-1A* expression was independent of the condition and is in line with the necessity of β-oxidation during COC maturation. *SCD* expression has to our knowledge, not been investigated before and showed a marked, oocyte dependent, increase during maturation. We suggest that conversion of saturated FFA into harmless unsaturated FFA by cumulus cells protects the developmental competence of the oocyte.

1. Aardema et al., BoR, 2013; 88, 164
2. Henique et al., JBC, 2010; 285, 36818-827

Notes

LIVE CONFOCAL MICROSCOPY TIME-LAPSE IMAGING CHOLESTEROL INCLUSION TO PLASMA MEMBRANE OF MATURE BOVINE OOCYTES PRIOR TO VITRIFICATION

ARCARONS N [1]; MOGAS T [1]; MORATÓ R [2]

[1]Unit of Animal Reproduction, Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, Spain. [2]Biotechnology of Animal and Human Reproduction (TechnoSperm), Department of Biology, Institute of Food and Agricultural Technology, University of Girona, Spain.

The present study aimed to evaluate the effect of 2 mg/mL of methyl-β-cyclodextrin (MβCD) as a cholesterol loader to change mature oocyte plasma membrane and increase its tolerance to vitrification procedure. In a first set of experiments, a time-lapse imaging by confocal microscopy was conducted to determine at which time the MβCD loaded the cholesterol in the plasma membrane. Once the timing of cholesterol integration to plasma membrane was defined, we evaluated the effects of a pre-treatment with 2 mg/mL of MβCD for 35 min and 1h prior to vitrification on survival and embryo development rates. Analysis in all groups were performed through an ANOVA, followed by the Sidak's post-hoc test. In all cases, significant level was set at $P < 0.05$. *In vitro* matured oocytes exposed to 2 mg/mL of MβCD showed a clear immunofluorescence in the plasma membrane after a minimum of a 35 min and up to 1h pre-treatment. Higher survival rates were observed when oocytes exposed to 2 mg/mL of MβCD pre-treatment for 35 min (60.6%) were vitrified/warmed compared to 1h pre-treatment (47%). Similar results were observed when blastocyst yield was determined. Blastocyst rate on D7 was higher after 35 min MβCD treatment compared to the 1h MβCD treatment (3.1% vs 0%). However, vitrified oocytes showed lower embryo development rates than fresh non-vitrified oocytes (21.1%). Hence, our results warrant further research to be conclusive.

Notes

THE USE OF NEUTRAL RED AS A VIABILITY INDICATOR HAMPERS *IN VITRO* DEVELOPMENT OF SEMI-NUDE BOVINE OOCYTES TO THE BLASTOCYST STAGE

BAETENS E, ANDRIES S, BUS A, DE PORTE HFM, LEROY JLMR, BOLS PEJ

Gamete Research Centre, Veterinary Physiology and Biochemistry, Department of Veterinary Sciences, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium

Women suffering from premature ovarian failure due to cancer treatment can appeal to oocyte vitrification to preserve their fertility. An important factor to increase the effectiveness of the procedure is viability assessment of the cryopreserved oocytes after warming. To date, survival is predominantly assessed on the basis of morphological criteria by conventional light microscopy, a subjective assessment method that depends largely on the expertise of the observer. Therefore, there is a great need for an objective method to assess viability in a fast and non-invasive way. Oocytes can be cryopreserved at the immature or mature stage. After choosing to use mature oocytes, based on literature, this experiment examined whether the relative non-toxic stain Neutral Red (NR) can be used as an oocyte viability marker without affecting subsequent development to blastocysts. NR is taken up by lysosomes of metabolically active cells. Briefly, immature cumulus-oocyte-complexes (COCs) were subjected to routine *in vitro* maturation (IVM) for 21 or 24 hours, whereupon the 270 mature COCs were divided into 3 groups (2 replicates). A control group with an intact cumulus oophorus (24h IVM; LAB CTRL) and 2 groups of COCs with only the corona radiata (21h IVM), the semi-nude (SN) and Neutral Red group (NR) respectively. In view of future vitrification and IVF, cumulus cells were partially removed (semi-nude) by pipetting to facilitate oocyte handling and future cryoprotectant penetration. Following 30 minutes incubation with 15µg NR/ml maturation medium and a subsequent 1h washout period (NR group), all 3 groups were subjected to routine IVP (cultured under oil for 8 days). Cleavage and blastocyst rate were observed at respectively 2 and 8 days post-insemination. Developmental competence data were analyzed using a binary logistic regression including treatment as fixed factor and replicate as random factor (IBM SPSS version 22). Although there is a significant difference in cleavage (75 vs 55,8%) and blastocyst (36 vs 20,9%) ratio between the LAB CTRL and SN group, our results demonstrate that semi-nude oocytes still have an acceptable fertilization rate that can definitely be improved. However, oocytes from the NR-group significantly failed to cleave (42,9%) and develop to the blastocyst stage (2,4%) as compared to the CTRL and SN group. In conclusion, Neutral Red clearly affects cleavage and blastocyst formation of semi-nude oocytes in the above used conditions and therefore is not suitable for semi-nude oocyte viability assessment.

Notes

IMMUNORADIOMETRIC ASSAY (IRMA) OF PREGNANCY-ASSOCIATED GLYCOPROTEINS (PAG) IN BOVINE MILK: DETERMINATION OF PROFILES IN ONGOING AND FAILED PREGNANCIES

BECKERS JF[1]*, TCHIMBOU A[1], BARBATO O[2], SOUSA NM[1]

[1]*Laboratory of Animal Endocrinology and Reproduction, Faculty of Veterinary Medicine, University of Liège, Bd de Colonster n. 20, B41, 4000, Liège, Belgium.*
**Corresponding author: jfbeckers@ulg.ac.be*
[2]*Department of Veterinary Medicine, University of Perugia, via S. Costanzo 4, 06126, Perugia, Italy.*

Pregnancy-Associated Glycoproteins (PAGs) are used since early eighties as pregnancy markers in cattle and other ruminant species. Until now, they are mainly assayed in plasma or serum samples by using radioimmunoassay or ELISA systems. In cattle, concentrations of PAG are detectable in maternal blood from Day 28 to Day 30 after fertilization. Milk concentrations are 20-30 times lower than in blood samples and cannot be quantified by existing immunoassay systems.

Recently, a new sensitive and robust immunoradiometric assay (IRMA) was developed allowing PAG quantification in bovine milk. Purified bovine PAG 67kDa was used as standard at concentrations ranging from 100 to 50,000 pg/mL. Highly purified immunoglobulins (hp-Ig) were obtained from two distinct rabbit polyclonal antisera by using a specific affinity chromatography (anti-PAG 4B-Sepharose gel). The hp-Ig708 (purified from polyclonal antiserum raised against caprine PAG 55kDa+59kDa) was used as capture antibody (0.01 µg/tube). The hp-Ig727 (purified from polyclonal antiserum raised against purified boPAG67kDa) was used as detection antibody (1:8,000). Radiolabeled streptavidin (125I-Strep; 50,000 cpm/100 µL) was used to reveal the Ab-Ag-Ab-Biot complexes.

The aim of this study was to quantify PAG concentrations in bovine milk for pregnancy follow-up in cattle. Milk was collected from pregnant cows (n=20) during the whole duration of lactation until dry-off. Samples were frozen until assay. Before analysis, milk samples were thawed at 37 °C, centrifuged (2,500 x g) and fat was removed. Samples giving high PAG concentrations were serially diluted in order to fit with standard curve range. In non-pregnant cows, concentrations remain lower than 40-50 pg/mL at all time points. In pregnant cows, milk PAG concentrations increased from Week 10 (56.9 ± 13.1 pg/mL) to Week 11 (93.5 ± 20.4 pg/mL) and Week 12 (135.2 ± 27.7 pg/mL). Thereafter, PAG concentrations increased regularly until Week 32 (2,177.6 ± 496.2 pg/mL) and slightly decreased until dry-off at Week 35 (1,615.9 ± 663.9 pg/mL). Immediately after parturition, PAG concentrations reached 5,615.3 ± 615.7 pg/mL and decreased continuously until Week 11 postpartum (36.6 ± 2.1 pg/mL). In this experiment, we could also follow three cows with pregnancy failure (2330, 7722 and 7725). Two of these cows (7722 and 7725) showed very low levels of PAG before pregnancy failure. In Cow 2330, PAG concentrations clearly decreased around the time of pregnancy failure.

In conclusion, in the present study we describe the use of a sensitive and quantitative IRMA allowing pregnancy follow-up in dairy cows. This approach offers the possibility (in time or in retrospective studies) of an individual follow-up without any additional manipulation of female neither any stress induced by the investigator.

Notes

INFLUENCE OF CAFFEINE SUPPLEMENTATION PRIOR TO *IN VITRO* MATURATION ON BOVINE OOCYTE DEVELOPMENTAL CAPACITY

BERNAL SM[1,2], HEINZMANN J[1], HERRMANN D[1], BAULAIN U[1], HADELER KG[1], ALDAG P[1], LUCAS-HAHN A[1], NIEMANN H[1]

[1]*Institute of Farm Animal Genetics, Biotechnology, Friedrich-Loeffler-Institut (FLI), Mariensee, Germany*
[2]*Facultad de Ciencias Agropecuarias, Universidad de Ciencias Aplicadas y Ambientales -U.D.C.A-, Bogotá, Colombia*

Although, *in vitro* oocyte maturation (IVM) is common practice in the cattle industry, it is known that the mechanisms involved in meiotic resumption begin in a non-physiological way. The cyclic AMP pathway plays an important role in resumption of meiosis. When cumulus-oocyte-complexes (COC) are mechanically released from the follicle to perform IVM, cAMP levels in immature COC rapidly decrease, which in turn triggers meiosis continuation. It has been proposed that modulation of cyclic AMP prior to IVM can increase bovine blastocyst rates *in vitro.* Caffeine is a non-specific competitive phosphodiesterases (PDE) inhibitor and can inhibit meiotic resumption of oocytes due to maintenance of cAMP levels. It has been reported that gamete treatment with caffeine can increase developmental potential. The present study evaluated the influence of pre-IVM culture in the presence of different concentrations of caffeine on meiotic progress, developmental rates and blastocyst cell numbers. Bovine ovaries were collected from a local abattoir. A total of 4378 cumulus-oocyte-complexes were obtained by slicing. Four different concentrations of caffeine (Merk, Darmstadt, Germany) were used during slicing, searching and 2h pre-IVM culture: 1, 5, 10, 20 mM. A control group, using 2h pre-IVM without caffeine (0mM) and a standard control were also included. After pre-IVM, oocytes were washed and cultured for 24h *in vitro* without caffeine. Following maturation, oocytes were fertilized *in vitro* for 19h and zygotes were cultured *in vitro* for eight days to assess embryo development. Some oocytes were fixed in 2% glutaraldehyde at 9, 20 and 24 h after IVM. Hoechst staining was performed to evaluate nuclear status. Cleavage and blastocyst formation rates were evaluated. Expanded blastocysts from all treatments were submitted to differential staining. One-way ANOVA from R software was implemented to evaluate differences in progression through meiosis, cleavage and blastocysts rates and blastocyst cell numbers. Caffeine maintained the meiotic arrest after 9h IVM in a concentration dependent manner (GV: 100± 0.0%, 61.3±21.3%, 40.7±5.4 %, 36.2±11.4% 11.9± 6.3%, 28.5±10.0% for 20, 10, 5, 1, 0 mM and standard, $p<0.05$, mean±SEM). Cleavage (57.7±4.9%, 56.5±3.8%, 62.7±3.2%, 52.5±5.1%, 54.4±6.0%, 60.3±2.3% for 0, 1, 5, 10, 20 mM and standard, $p>0.05$, mean±SEM) and blastocyst rates (26.2±3.0%, 14.9±2.8%, 22.4±3.8%, 23.7±2.1%, 21.4±4.1%, 26.6±2.4% for 0, 1, 5, 10, 20 mM and standard, $p>0.05$, mean±SEM) and number of cells (ICM: 46.0±4.1, 43.2±3.7, 61.4±7.8, 53.0±6.5, 49.4±5.6, 50.0±4.4; TE:111.6±13.6, 115.4±7.8, 106.4±3.5, 102.6±8.3, 118.4±14.6, 119.6±11.7 for 0, 1, 5, 10, 20 mM and standard, $p>0.05$, mean±SEM) did not differ significantly among *in vitro* treatments. Although caffeine supplementation prior to IVM delayed resumption of meiosis, it did not affect subsequent embryo development and quality.

Notes

TEMPORAL PATTERN OF STEROID HORMONE CONCENTRATIONS DURING IN VITRO MATURATION OF BOVINE OOCYTES

BLASCHKA C, WRENZYCKI C

Clinic for Veterinary Obstetrics, Gynaecology and Andrology
Justus-Liebig-University Giessen, Germany

Present in vitro maturation (IVM) systems do not completely mimic the in vivo situation resulting in oocytes of reduced quality. Steroid hormones are regulators in the fine-tuned mechanism of follicular and oocyte maturation and development. During final maturation a switch from estradiol dominance to progesterone dominance within the follicle is well-described. This change is accompanied by the resumption of meiosis and results in the maturation of the oocyte. It also suggests the important role of these hormones in this process. Aim of the study was to determine the temporal pattern of steroid hormone concentrations in the IVM medium of bovine cumulus-oocyte-complexes (COC) supplemented with different gonadotropin concentrations.

COC were obtained from abattoir-derived ovaries and were matured in medium TCM 199 (Tissue Culture Medium 199) supplemented with three different compounds of gonadotropins employing a standard protocol. The three combinations of gonadotropins were: 1. equine (eCG) and human chorionic gonadotropin (hCG), 2./3. follicle-stimulating hormone (FSH) and luteinizing hormone (LH), each in two different concentrations 0.05 IU or 0.01 IU, and 4. without any supplementation of gonadotropins. Groups of 30 COC were matured for 24 hours at 39°C and 5% CO2 without oil overlay. 17β-estradiol (E2) und progesterone (P4) were measured in maturation medium before use (0h, control) and after specific time points of IVM via radioimmunoassay (RIA). So far, the following results could be obtained.

Treatment 1: TCM with eCG and hCG: P4 and E2 could not be detected in the control medium (0h). During IVM, P4 concentrations increased in the medium (4h: 3.3 ± 1.0 ng/ml; 8h: 6.2 ± 3.3 ng/ml; 12h: 6.5 ± 2.0 ng/ml; 16h: 6.8 ± 1.1 ng/ml; 20h: 7.3 ± 1.8 ng/ml; 24h: 10.4 ± 1.6 ng/ml), whereas the E2 concentrations stayed similar (4h: 52.8 ± 12.1 pg/ml; 8h: 54.6 ± 7.9 pg/ml; 12h: 63.8 ± 15.2 pg/ml; 16h: 54.2 ± 16.3 pg/ml; 20h: 77.1 ± 40.1 pg/ml; 24h: 74.7 ± 32.4 pg/ml).

Treatment 2/3: *TCM with FSH and LH:* Supplementation of 0.05 IU each, E2 concentrations stayed at the same level as with eCG and hCG (E2 0h: 6.2 ± 5.7 pg/ml, washing medium: 26.0 ± 10.8 pg/ml, after 24h: 59.7 ± 20.1 pg/ml). With the supplementation of 0.01 IU each, P4 and E2 concentration also stayed at the same level as with eCG and hCG (E2 0h: 3.3 ± 3.2 pg/ml, washing medium: 19.6 ± 4.2 pg/ml, after 24h: 58.4 ± 24.1 pg/ml, P4 0h: ≤0.25 ng/ml, washing medium: 0.3 ± 0.1 ng/ml, after 24h: 17.3 ± 3.5 ng/ml).

Treatment 4: After 24 h of IVM *without gonadotropins* the following hormone concentration could be detected: E2: 129.4 ± 88.8 pg/ml and P4: 6.7 ± 0.8 ng/ml.

During IVM, the temporal pattern of E2 and P4 did not correspond with the pattern during final maturation in vivo. This underlines that present conditions of IVM do not reflect the in vivo situation and require further optimisation.

We gratefully acknowledge the financial support of the German Research Foundation (DFG; FOR 1369, WR 154/3-1).

Notes

EFFECT OF EPIDERMAL GROWTH FACTOR ON NUCLEAR AND CYTOPLASMIC *IN VITRO* MATURATION OF GUINEA PIG OOCYTES

CAÑÓN-BELTRÁN K[1,2], CAJAS YN[1,2], GARCIA-GARCIA RM[1], LORENZO PL[1], CARRERA R[2], REBOLLAR PG[3], ARIAS-ÁLVAREZ M[4]

[1]Dpto. Fisiología (Fisiología Animal), Facultad de Veterinaria, Universidad Complutense de Madrid, Spain.
[2]Dpto. de Ciencias Agropecuarias y de Alimentos, Universidad Técnica Particular de Loja, Ecuador.
[3]Dpto. Producción Animal, ETSI Agrónomos, Universidad Politécnica de Madrid, Spain.
[4]Dpto. Producción Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Spain.

The guinea pig may represent an animal model for research on ovarian infertility and improvement of the in vitro maturation (IVM) conditions is needed in this species. The aim of the present work was to immunolocalize the Epidermal Growth Factor (EGF)-Receptor in the guinea pig ovaries and to study the effect of EGF on meiotic and cytoplasmic maturation, and apoptotic rate in cumulus-oocyte-complexes (COCs). Immunohistochemistry was performed in paraffined ovaries using a rabbit polyclonal antibody EGF-R (1:100; Santa Cruz Biotechnology) and the ABC Vector Elite kit (Vector Laboratories). For the IVM, COCs were collected by aspiration of follicles >700µm under a stereoscopic microscope. They were cultured at 37°C in 5% CO2 during 17 h with TCM-199 supplemented with glutamine, pyruvate, BSA, and different concentrations of EGF (Sigma) [0 (control), 10, 50 or 100 ng/mL] or 10% Fetal Calf Serum (FCS). After IVM, 564 oocytes were fixed and stained with 10 µg/mL Hoechst to assess nuclear configuration in terms of Metaphase II (MII) rate. A total of 143 oocytes were treated progressively with 0.5% pronase, 4% paraformaldehyde, 0.02% Triton X-100, 7.5% BSA and 100 µg/mL FITC-LCA for cortical granule (CG) staining. Also, 78 oocytes were stained with 180 nm MitoTracker RedCMXRos (Molecular Probes Inc) for active mitochondria visualization. CG and mitochondria patterns were analyzed with laser scanning confocal microscopy (Leica TCS SP2). Apoptosis rate in cumulus cells (n=58 COCs) were visualized with TUNEL (In Situ Cell Death Detection Kit, Roche) and analyzed with Image J software. Chi-square test was used to compare nuclear maturation, CG and mitochondria migration rates. The apoptotic index was analyzed by a one-way ANOVA using Duncan post-hoc test. Positive immunostaining for EGF-R was found in granulosa and theca cells and oocytes in all follicular stages. MII were significantly higher in oocytes supplemented with 50 ng/mL EGF group (75.9%) compared to other experimental groups (43.5, 51.8, 53.7 and 59.5% for 0, 10, 100 ng/mL EGF and 10% FCS, respectively, $P<0.05$). Group matured with 50 ng/mL EGF showed higher rate of oocytes with peripheral migration pattern of CG (compatible with cytoplasmic maturation) compared to control group (71.9 vs. 32.4%; $P<0.05$) and migrated mitochondrial pattern compared to the control group and the group supplemented with 100 ng/mL EGF (80.0% vs. 27.8% and 31.3%, respectively; $P<0.05$). Apoptotic rate was lower in 50 ng/mL EGF (17. 2±0.9%) and 10% FCS (16.0±1.2%) groups related to the control one (28.7±1.4%) ($P<0.05$). In conclusion, the presence of EGF-R in guinea pig ovaries, suggests that EGF may exert a direct effect on ovarian function. A dose of 50 ng/mL EGF seems to be the most appropriate concentration for IVM of guinea pig oocytes, since it improves nuclear and cytoplasmic oocyte maturation and reduces apoptosis in the cumulus cells. We acknowledge Cooperation Project UCM 2013.

Notes

TIME-LAPSE ANALYSIS OF EARLY CLEAVAGE IN BOVINE EMBRYOS PRODUCED IN SERUM-FREE MEDIUM

CATTEEUW M[1], WYDOOGHE E[1], PIEPERS S[1], SMITS K[1], DESTOUNI A[2], TSUIKO O[3], VERMEESCH JR[2], VAN SOOM A[1]

[1]Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Belgium; [2]Laboratory of Cytogenetics and Genome Research, Center of Human Genetics, KU Leuven, Belgium; [3]Department of Biotechnology, Institute of Molecular and Cell Biology, University of Tartu, Estonia

Two decades ago, early cleaving embryos were considered as developmentally more competent than slow cleaving embryos. But this theory has been challenged, since moderately developing embryos have decreased chromosomal abnormalities, normal *H19* and *Snrpn* imprint maintenance and potentially higher pregnancy rates. We want to analyze the incidence of chromosomal instability (CIN) in bovine cleavage stage embryos and relate this with developmental kinetics. Hence, we need a culture system allowing individual identification and selection of cleavage stage embryos for single cell analysis (SCA). In this preliminary study, we used time-lapse cinematography (TLC) as a non-invasive tool to describe kinetics and to use timing of early cleavages as a parameter predictive of blastocyst development. Bovine embryos were produced from immature oocytes derived from slaughtered cattle. Oocytes were matured in 500 µL TCM199 supplemented with 20 ng/mL epidermal growth factor (EGF). After in vitro fertilization with frozen-thawed bull semen, 9 presumed zygotes (7 replicates) were cultured in a WOW dish in 30 µL Synthetic Oviduct Fluid (SOF) supplemented with 0.4% BSA, 5 µg/mL insulin, 5 µg/mL transferrin and 5 ng/mL selenium (ITS), covered with mineral oil. In total, 63 zygotes were observed with TLC (Primo Vision®, VitroLife, Göteborg, Sweden), and images were taken every 15 min for up to 90 hours post insemination (hpi). At 192 hpi, blastocyst formation was set as endpoint. Timing of the first (t_1; cleavage into 2-cell stage) and second mitosis (t_2; cleavage into 4-cell stage) and the interval time between those two parameters were analyzed ($t_{\Delta 1\text{-}2}$). The median observation of each parameter was set as a threshold value (t_1 29.00h; t_2 38.83h; $t_{\Delta 12}$ 10.87h). All data were analyzed using a binary logistic regression model. Significantly more embryos reached the blastocyst stage when they cleaved before 29.00h into 2-cell stage or before 38.83h into 4-cell stage (48.3% and 51.2%, respectively), compared to embryos with a later first or second mitosis (16.1% and 18.9%, respectively) ($P<0.05$). Furthermore, when the interval between the first and second mitosis ($t_{\Delta 12}$) was shorter than 10.87h more embryos reached the blastocyst stage (42.3%), compared to a longer interval $t_{\Delta 12}$ (21.1%) ($P<0.01$). This indicates that timing of early cleavage is predictive for further developmental potential, which is confirming earlier studies (Van Soom *et al*., Theriogenology, 38:905-919, 1992; Grisart *et al*., J Reprod Fertil, 101:257-264, 1994). It is however the first time embryos have been cultured in WOW-dishes in serum-free medium and monitored using TLC. WOW dishes offer the advantage of small group culture with individual embryo follow-up, which allows specific embryo selection at any time of the development. Next, we want to identify CIN in embryos with particular cleavage patterns using TLC with SCA and eventually transfer embryos with high and low predicted viability.

Notes

SPERM STORAGE TUBULES CULTURE: A NEW APPROACH FOR REPRODUCTIVE RESEARCH IN AVIAN SPECIES

CORDEIRO LAV[1], RIOU C[1], GERARD N[2]

[1] Postdoctoral researchers and [2] Senior Researcher, Cellular Interactions and Fertility Team, Physiology of Reproduction and Behaviors*, French National Institute for Agricultural Research (INRA), F- 37380 Nouzilly, France. Nadine.Gerard@tours.inra.fr*

Sperm storage tubules (SST) are epithelial structures found in the mucosa of distal half of the oviduct of all avian species studied. These tubules maintain and store sperm up to 70 days and this allows fertilization without insemination. The aim of this work was to set up epithelial SST cells culture for future use as an *in vitro* model for oviduct cells-sperm interaction. Hens (*Gallus gallus domesticus,* Unité de Recherches Avicoles [URA], INRA, Nouzilly.) were euthanized with sodium pentobarbital injection. Oviducts were isolated and removed and the uterovaginal villi was manually dissected under stereomicroscopy. The SST area on the top of isolated mucosal villi was dissected, scalped in small fragments, and enzymatically digested in 1µg/ml Collagenase for 10 min at 41ºC. The digested tissue was flushed for 30 times by pipetting. The enzymatic activity was blocked by washing the tissue twice with culture medium. A second enzymatic digestion was performed by incubating the tissue overnight at 4ºC in 1µg/ml Pronase. The tissue was flushed again and the enzymatic activity was blocked. SST were isolated in 2 / 4% Percoll density gradient centrifugation at 2000g for 30 min at 4ºC. An intermediate phase of Percoll column containing SST was harvested before being maintained in Medium 199 containing 10% BFS and Gentamicin, during 30 min at 41ºC for fibroblast attachment. The medium containing SST was distributed in Lab-Tek Chamber Slide System (Nunc). SST were cultured at 37ºC, 5% CO_2 atmosphere, for 6 days. Immunocytochemistry for epithelial cell type confirmation, was performed with overnight incubation with monoclonal primary antibodies anti-Pan-cytokeratin (1:300, Sigma), Tubulin (1:300, Sigma) and Vimentin (1:500, Sigma) and anti-species secondary antibodies. We observed that, at the end of the enzymatic process, 90% dissected SST was isolated. In phase contrast microscopy we observed integral SST as well as individual cells. After 2 days of culture we observed cell migration from SST borders to form a monolayer. Eighty % cells presented epithelial characteristics as demonstrated with Cytokeratin and Tubulin positivity and Vimentin negativity, in Confocal microscopy. The digestion and isolation processes need to be controlled to differentiate the epithelial surface mucosal cells from SST cells. This method is very effective to isolate the SST specific population of cells that can be used in different reproductive and physiological studies for epithelial cell-sperm interaction.

Notes

HORMONAL AND BLOOD GAS LEVELS OF HOLSTEIN COWS UNDER HEAT STRESS DURING SUPEROVULATION TREATMENT

CORTEZ LC, COSTA LLM, BERTEVELLO PS, FONTENELE IP, CORDEIRO LAV

Animal Science Department, Semi Arid Federal University (UFERSA), Av. Francisco Mota, 572, Mossoró / RN, 59.625-900, Brazil, lavcordeiro@ufersa.edu.br

Most of the Brazilian cattle production is located in the south tropical belt of the planet, dominated by high temperatures and high radiation. The Brazilian semi-arid climate is characterized by low humidity, typical of desert climates around 30-50%, presenting an average of 51.90 ± 9.65%, (from 28.5 to 69.8%) with no rainfall volume, aggravates the condition of sweating and water needs for animals. This work aimed to evaluate the effect of heat stress on maternal physiology, quantity and quality of embryos of Holstein cows submitted to superovulation protocol. Ten cows were kept in individual stalls of shading (SDW) and other 10 cows remained enclosure outside paddock to receive incident sunlight (SUN) throughout the day. Both groups were submitted to the same hormonal superovulation treatment. Statistical analyzes were performed by ANOVA and Tukey test at 5% significance. The values founded during the trial period characterized abiotic stress, with high temperatures on the Sun around 39°C, while the animals in shadow have an average of 6% less. However, the radiation difference between environments during the day can be near 1000 Wm^{-2}. The temperature on animal surface was 34.5 ± 0.25 and 38.3 °C ± 0.20, on SWD and SUN. Rectal temperature had a significant difference ($p < 0.01$) between groups. Respiratory frequency of the SUN animals were 10 movements more per minute when compared to 51 ± 1 mov/min measured in shade. The sweating significant difference ($p < 0.01$), averaging 74.26 ± 3.97 and 149.66 ± 3.10 Wm^{-2}, respectively, for SUN and SDW, respectively. FSH and LH serum levels had no significant difference but Cortisol, T3 and T4 hormones increase at the time of AI. Blood gas analysis showed compensation of animals through the respiratory route: increased respiratory rate reduces O_2 uptake ($\downarrow pO_2$); decreases the release of CO_2 ($\downarrow$pCO2); raising the voltage plasma CO_2 ($\uparrow TCO_2$, Total Carbon Dioxide) with pH elevation ($\uparrow$pH), which is offset by compensation mechanisms for the formation of plasma HCO_3 ($\uparrow HCO_3$), corroborated by BE elevation, supporting the metabolic alkalosis which there are animals in the SUN. The number of embryos classified as morula and compact morula did not differ between groups, however only the SUN group had unfertilized oocytes collected. In contrast, blastocysts were collected in a greater number in the group SDW. These results show that animals under direct Sun were forced to use thermoregulatory mechanisms, more than on shade, causing metabolic alkalosis on animals. The superovulation protocol was effective even under stress conditions, however, the negative impact on embryos can determine post-transfer embryonic losses caused by low embryo quality than obtained on shade.

Notes

INTEGREATED ANDROLOGICAL EVALUATION IN ANGORA GOAT

DASKIN A[1], STELLETTA C[3], PASCALE L[3], OZTUTAR F[3], ALEMDAR H[1], TURRI F[2], TIRPAN B[1], VENCATO J[3], TEKIN K[1], PIZZI F[2]

[1]Department of Reproduction and Artificial Insemination, University of Ankara, Turkey
[2]National Research Council – Institute of Agricultural Biology and Biotechnology, Lodi, Italy
[3]Department of Animal Medicine Production and Health University of Padova, Italy

Integrated andrological evaluation (IAE) is a practice to identify Satisfactory (S), Questionable (Q) and Unsatisfactory (US) males. IAE procedure mainly involves classical and modern methods. The routine evaluation system contains physical and reproductive examination, while the innovative approach is more based on ultrasound detection of testicular and accessory glands, scrotal surface thermography, GnRH challenge test, CASA semen analysis (Computer Assisted Semen Analyzer), fluorescent staining, seminal plasma biochemistry, testicular fine needle aspiration cytology (TFNAC).

The aim of this trial was to conduct a pilot study with an IAE based evaluation system (except of the TFNAC) in four healthy adult (4/6 years) Angora goat in Kazan-Turkey. Physical traits such as age, BSC, hereditable defects on: eyes, mouth, legs/feet, prepuce, penis, scrotum and its components were recorded and scrotal circumference, testicular ultrasound (ESAOTE MyLab5, Genoa, Italy with convex probe 2.2 – 6.6 MHz) and scrotal thermography (Flir, E60 during GnRH challenge test with 8.4 µg Busereline IV) were performed. Semen parameters such as color, volume, concentration, sperm motility (SCA Microptics), viability and morphology (Eosin-Nigrosin stain), acrosome integrity (FITC-PNA) were measured in fresh and frozen-thawed semen samples. Correlation indices and mathematical tendencies were calculated using Sigma Stat Software 2.05 and Microsoft Excel version 14.4.9. Three males were evaluated as Q, and one as US because of the presence of feet and mouth defects. One buck has not been evaluated by reasons of higher delta Testosteronemia during GnRH Challenge Test and echotexture testicular classification (Lower Mineralization Index). All mature bucks showed similar scrotal thermal pattern. Seminal plasma mean values of cholesterol, glucose, LDH, triglycerides, total protein, GGT and magnesium were 30.5 mg/dl, 77.8 mg/dl, 470.1 u/L, 8.8 mg/dl, 82.5 g/l, 46.8 u/L and 2.03 mg/dl, respectively. Bucks with higher testicular functionality, according to the physical examination, had the best freezability (Delta Viability and Intact Acrosome) and higher levels of cholesterol (34.5 mg/dl) glucose (87.4 mg/dl), LDH (585.4 u/L), triglycerides (11.15 mg/dl), total protein (89.0 g/l), GGT (53.4 u/L) as well as the lower levels of magnesium (1.88 mg/dl) in the seminal plasma.

A correlation between testicular functionality and frozen-thawed semen parameters was also confirmed by sperm kinetic parameters, viability and morphology results. Application of IAE in Angora goat may indicate the buck selection for specific purposes such as breeding, cryopreservation or exclusion from any application.

Acknowledgements: The research was supported by Bilateral Agreement CNR/TUBITAK

Notes

THE EFFECTS OF HYPO- AND HYPERGLYCEMIA DURING LIPOLYSIS-LIKE CONDITIONS ON BOVINE OOCYTE MATURATION, SUBSEQUENT EMBRYO DEVELOPMENT AND GLUCOSE METABOLISM

DE BIE J[1], MAREI WF[1,2], DESMET KLJ [1], ANDRIES S[1], STURMEY RG[3], BOLS PEJ[1], LEROY JLMR[1]

[1]Gamete Research Centre, Veterinary Physiology and Biochemistry, Departement of Veterinary Sciences, University of Antwerp, Universiteitsplein 1, B2610 Wilrijk, Belgium, [2]Department of Theriogenology, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt,.[3]Centre for Cardiovascular and Metabolic Research, Hull York Medical School, University of Hull, Hull, HU6 7RX, UK.

Elevated follicular NEFA concentrations, commonly present in cattle in NEB or women suffering obesity or type 2 diabetes, are known to disrupt oocyte and embryo development and alter subsequent embryo metabolism. However, NEB cows exhibit systemic hypoglycemia whereas humans suffering metabolic disorders have hyperglycemic insults. Both metabolic features may affect oocyte development. Little is known about whether elevated NEFA concentrations in combination with hyper- or hypoglycemic conditions influences oocyte viability. In this study, we hypothesized that glucose interacts with high NEFA levels during *in vitro* oocyte maturation to affect developmental capacity and metabolism of the resulting blastocysts. Thus, 647 bovine grade I COCs were matured (3 repeats) under 4 conditions: 1) physiological NEFA (72µM; palmitic, stearic and oleic acid) and routine IVM glucose (GLUC) concentrations (5.50mM) (CNTRL), 2) pathophysiological NEFA (420µM) and routine GLUC (HI NEFA), 3) HI NEFA and high GLUC (10mM) (HI NEFA+HI GLUC) and 4) HI NEFA and low GLUC (2.75mM) (HI NEFA+LO GLUC). Subsequently, matured oocytes were routinely fertilized and cultured for 7 days. At day (D) 7 post insemination (pi) all blastocysts were individually cultured for 24 hours in 4µl drops of modified SOF medium under oil after which droplets were analyzed on GLUC concentrations as described by Guerif *et al.* (PLOSone, 8, e67834, 2013). Cleavage (48h pi), blastocyst rates (D8 pi) and the rates of D8 blastocysts from cleaved zygotes were recorded. Developmental competence and GLUC consumption data were compared between 4 treatments using a binary logistic regression model and mixed model ANOVA, respectively. Replicate, treatment and the interaction of both factors were taken into account (IBM SPSS Statistics 20). Significant lower cleavage rates were observed for HI NEFA+LO GLUC (56%) compared with CNTRL (73%; P=0.006) and HI NEFA+HI GLUC conditions (70%; P=0.048). At D8 pi, blastocyst rates of HI NEFA+LO GLUC exposed oocytes (18%) were significantly lower compared with CNTRL (38%, P<0.001), whereas development of HI NEFA+HI GLUC D8 blastocysts (25%) tended to be reduced compared with CNTRL (P=0.066). The capacity of cleaved zygotes to develop to blastocyst stage by D8 showed a similar profile: HI NEFA+LO GLUC (32%) significantly reduced and HI NEFA+HI GLUC (35%) tended to reduce development compared with CNTRL (53%; P=0.024 and P=0.066, respectively). Interestingly, with no significant difference in developmental stage at D7, these HI NEFA+LO GLUC blastocysts consumed significantly less GLUC from D7 to D8 (12.14 ± 4.10 pmol/embryo/h) compared with CNTRL (25.53 ± 2.96 pmol/embryo/h; P=0.020). In conclusion, low GLUC concentrations seem to be more deleterious than high GLUC concentrations in the presence of elevated NEFAs in terms of embryo development and the lower ability of the surviving D7 embryo to consume GLUC as an energy source for its further development.

Notes

3D VISUALIZATION OF BOVINE OOCYTE *IN VITRO* MATURATION BY CONFOCAL LASER SCANNING MICROSCOPY

DE MONTE E[1,2], REICHENBACH M[3], REICHENBACH HD[4], WOLF E[2,5], HABERMANN FA[1]

[1]Chair of Veterinary Anatomy, Histology and Embryology, LMU, Munich, Germany; [2]Chair of Molecular Animal Breeding and Biotechnology, LMU, Oberschleissheim, Germany; [3]Bayern-Genetik GmbH, Grub, Germany; [4]Institute for Animal Breeding, Bavarian State Research Center for Agriculture, Grub, Germany; [5]LAFUGA, Gene Center, LMU, Munich, Germany

Regular nuclear and cytoplasmic oocyte maturation is a prerequisite for normal fertilization and embryo development. Improvement of *in vitro* maturation systems is a central issue in veterinary and human reproductive medicine. Thereby, better microscopic visualization of cellular processes and structures is essential for further extending our rudimentary knowledge and understanding of mammalian oocyte maturation. We used three-dimensional multicolor fluorescence microscopy to investigate critical steps of meiotic maturation *in vitro*. The primary aims of this study were to simultaneously gain information on the meiotic spindle apparatus, on the kinetics of meiotic progression, on the dynamic changes of the cytoskeleton and on the meiotic failures and aberrations.

In cattle, the cumulus oophorus is considered to play an essential role for normal oocyte maturation. This makes direct microscopic live cell imaging of the oocyte rather difficult. Thus, cumulus-enclosed grade I and II oocytes were collected from slaughterhouse ovaries and allowed to mature for variable times from 0 to 28 hours *in vitro*. The oocytes were denuded and then fixed with formaldehyde in a microtubule-stabilizing buffer in such a way that the three-dimensional cell architecture was maintained, and were stained for DNA, microtubules and f-actin microfilaments. In addition, serine 10-phosphorylated histone H3 was used as a marker for chromosome condensation and the spindle midbody. For three-dimensional imaging of the oocytes *in toto*, confocal serial sections were captured at 1 µm distance using a 40x objective (NA = 1.3). For imaging details we used a high spatial sampling density (pixel size 50 x 50 nm, z-step size 200 nm) and image restoration by maximum likelihood estimation (MLE) deconvolution.

A collection of more than 500 confocal image stacks gives a clearer and more detailed view of completion of meiosis I and progression to metaphase II. Qualitative and quantitative data analyses provide a basis for studies on molecular mechanisms e. g. on the dynamic localization and function of potential key proteins. Important is the detection of anomalies of meiosis I that result in irregular genomic configurations in the zygote: Main findings were (i) the failure of first polar body extrusion as a consequence of incorrect positioning or orientation of the meiotic spindle and (ii) lagging chromosomes, chromatin bridges and incomplete polar body cytokinesis due to irregular spindle formation, chromosome congression and segregation.

3D fluorescence microscopy allows to exactly determine the stage of oocyte meiosis and to diagnose fatal aberrations of meiotic maturation. Thus, high speed imaging systems could be used to test and to improve oocyte isolation methods and in vitro maturation systems.

This work is supported by the Deutsche Forschungsgemeinschaft (DFG FOR 1041).

Notes

OSMOTIC CHALLENGE OF BOVINE EARLY PRE-ANTRAL FOLLICLES WITH DIFFERENT CRYOPROTECTANT AGENTS

DE PORTE HFM, ANDRIES S, BUS A, LANGBEEN A, BAETENS E, LEROY JLMR, BOLS PEJ

Gamete Research Centre, Veterinary Physiology and Biochemistry, Department of Veterinary Sciences, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium

Significant advances in cancer diagnosis and treatments have stimulated interest in fertility preservation strategies as chemical or ionizing radiotherapy often threatens future reproduction. Ovarian tissue cryopreservation currently is the only option for preserving the reproduction potential of pre-pubertal girls and women whom cannot undergo hormonal stimulation, ovarian tissue cryopreservation currently is the only option. However, there is a huge concern regarding the possible presence of malignant cells in the retrieved ovarian tissue, which could lead to cancer reintroduction after reimplantation of the frozen-thawed tissue strip. Cryopreservation of isolated early preantral follicles (PAFs) (and subsequent *in vitro* culture (IVC), maturation (IVM) and fertilisation (IVF)) might therefore represent an interesting alternative. Existing protocols are based on protocols for freezing embryos and oocytes . However, in order to improve follicular survival after cryopreservation, it is essential to develop a protocol for follicles specifically. Indeed, follicles are quite different from both embryos and oocytes, if only because they are composed of two different cell types (namely the oocyte and the surrounding (pre-)granulosa cells). In order to provide a biophysical base for choosing optimal cryoprotectant agents (CPAs) that avoid severe volume changes and formation of intracellular ice crystals, in this experiment, two-day-old isolated bovine PAFs were osmotically challenged by exposing them to different concentrations of cryoprotectant agents (CPAs). Briefly, isolated bovine early PAFs were exposed to either ethylene glycol (EG) or dimethyl sulfoxide (DMSO) in different final concentrations: 1 M, 2 M, 3 M, 4 M and 5 M at room temperature, and photographed at different time points (every half minute between 0 and 5 minutes) after the onset of exposure to calculate their volume over time (5 - 10 follicles per CPA and per concentration). Although there was a high variability in the individual response of the follicles to this CPA challenge, all follicles showed a typical 'shrink/swell' curve. Analysis with two-way ANOVA showed no interaction between the type of CPA and the respective concentrations. This means that volume differences in time between the minimum and maximum for both EG and DMSO were uniform across concentrations. Across all concentrations, time until the post stimulus maximum (i.e. the maximum volume to which follicles re-expand after shrinkage) appeared significantly longer in the EG group ($P = 0.04$), indicating that bovine early PAFs are less permeable to EG than DMSO. To our knowledge, this is the first time that isolated bovine early PAFs were osmotically challenged by exposing them to different concentrations of penetrating CPAs. This has provided us with some basic insights in follicular permeability to CPAs. These insights are a first step in the design of cryopreservation protocols for isolated early PAFs specifically.

Notes

ASSESSMENT OF THE QUALITY OF COMMERCIAL BELGIAN BLUE SEMEN STRAWS?

DE ROOVER R, DE ROOVER ET

Rondia 21, 1348 Louvain La Neuve, Belgium

Our practice carries out flushes for Belgian Blue farmers. In a retrospective analysis of our flushing results of 2 years, we observed that 25% of the flushes yielded no transferable embryos. The analysis further showed an important influence of the AI centre on flushing outcome.

The aim of this study was to determine (1) if semen straws commercialised by the AI centres can be considered a consistent product, and (2) whether the quality consistency of such straws should be taken into account in a future prospective study of flushing results.

Materials and methods: several AI centres are active on the Belgian Blue semen market in Belgium. Three centres (referred to here as N, H and J) account for over 90% of all flushes.

Semen straws were obtained from the liquid-N_2 container of the farmer and transported to the department of reproduction at the veterinary faculty of the University of Ghent. At our request and expense the straws were examined *secundem artem* with a Hamilton Thorn sperm analyser system for total sperm cell count, mobile and progressively mobile sperm cell counts (PMS).

The number of straws examined, per AI centre, was proportionate to the number of problems encountered with flushes for that centre. Results are shown in Table 1.

Table 1: Results of PMS analysis of Belgian Blue semen straws. *n* is the number of straws analysed.

		number of straws with PMS			PMS range	median PMS
Centre	n	$>10.10^6$	$5\text{-}10.10^6$	$< 5.10^6$		
N	33	6	15	12	1.6 – 28.5	5.7
H	13	7	4	2	2.1 – 26.9	11.2
J	5	5	0	0	10.2 – 24.3	15.6
Overall	51	18	19	14	1.6 – 28.5	7.4

The University of Ghent's recommended PMS per dose is 10 million.

Conclusions: 1) Commercial Belgian Blue semen straws are not a consistent product. The quality of a considerable portion is poor. 2) For future prospective studies of flushing results, quality of the straws is a factor that should be considered.

Notes

EFFECT OF ASYNCHRONOUS EMBRYO TRANSFER ON GLUCOSE TRANSPORTER EXPRESSION IN EQUINE ENDOMETRIUM

DE RUIJTER VILLANI M, GIBSON C, STOUT TA

Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, The Netherlands

Equine pregnancy is characterized by an unusually long pre-implantation period (40 days) during which the conceptus is entirely dependent on uterine secretions for nutrient provision. Moreover, horse embryos tolerate a wide range of uterine asynchrony following embryo transfer (ET); however negative asynchrony (recipient behind the donor) of more than 5 days markedly retards conceptus growth and development, and thereby offers a unique tool for studying the effect of the uterine environment on early development. Glucose is an important nutrient during pre-implantation development, however little is known about its transport from the endometrium into the uterine lumen. The aim of the current study was to evaluate the effect of uterine asynchrony on glucose transporter expression in the equine endometrium. Day 8 horse embryos were transferred to recipient mares that ovulated on the same day (synchronous; n=10), or 5 days after (asynchronous; n=10) the donor mare. The resulting conceptuses and matched endometrial biopsies were collected 6 or 11 days after ET (14 or 19 days of embryo development: n=5 per group). Endometrial expression of mRNA for glucose transporters was evaluated by qRT-PCR, and the effects of asynchronous ET and stage of pregnancy were analyzed by two-way ANOVA followed by independent-samples t-tests.

Gene expression for SLC2A3, 4, 5, 8, 10 and SLC5A1 was stable over time and treatment, whereas endometrial SLC2A1 mRNA expression was down-regulated in the asynchronous group at day 14 of embryonic development ($p<0.05$), but did not show differences between the two treatment groups at day 19. In summary, the expression of SLC2A1, one of the main glucose transporters in the endometrium, is negatively affected by asynchronous ET and, although its expression seems to be restored by day 19 of conceptus development, this might be a contributor to the delayed development observed in asynchronous pregnancies.

Notes

CONCENTRATION OF PROCAINE AND EXPOSURE TIME INFLUENCE *IN VITRO* FERTILIZATION RATE IN THE EQUINE

DOUET C[1], JEANNERAT E[2], GOUDET G[1]

[1] *INRA, UMR 85, Physiologie de la Reproduction et des Comportements, CNRS, Université François Rabelais, IFCE, 37380 Nouzilly, France*
[2] *Swiss Institute of Equine Medicine, Agroscope and University of Berne, Avenches, Switzerland*

Most wild equids are currently endangered or threatened, as mentioned in the Red List of the International Union for the Conservation of Nature and several domestic horse breeds are at risk of extinction. Genome resource banking requires cryoconservation of semen, oocytes and/or embryos. Embryo production in equids is limited *in vivo*, since routine induction of multiple ovulation is still ineffective. Embryo production *in vitro* allows the production of several embryos per cycle that could easily be frozen owing to their small size. Intracytoplasmic Sperm Injection (ICSI) has been widely adopted to generate horse embryos *in vitro*, however ICSI is time-consuming and requires expensive equipment and expertise in micromanipulation. We have established an efficient *in vitro* fertilization (IVF) technique in the equine (Ambruosi et al., 2013 Reproduction, 146: 119-133) but IVF zygotes have a low developmental competence. Incubation of gametes with procaine, necessary for induction of sperm hyperactivation, may have a deleterious effect on embryos quality. Our objective was to increase the developmental competence of the IVF zygotes by decreasing procaine concentration or exposure time. Immature cumulus-oocyte complexes were collected from slaughtered mares in a local slaughterhouse, cultured for 26 hours in an *in vitro* maturation medium and pre-incubated for 30 minutes in oviductal fluid collected from slaughtered females. Fresh sperm was collected, diluted to $10x10^6$ spermatozoa/ml, incubated for 5 hours in a capacitating medium and diluted to $1x10^6$ spermatozoa/ml. Spermatozoa were then added procaine (1mM or 5mM) and co-incubated with oocytes for 2, 4 or 18 hours. Zygotes were cultured in DMEM-F12 for 48 hours post-IVF, fixed and analyzed. In experiment 1, spermatozoa were added 5mM procaine and co-incubated with oocytes for 2 hours *vs* 18 hours. The percentage of zygotes 48 hours post IVF was higher for 18 hours co-incubation (62%, 13/21) than for 2 hours (0%, 0/22) (Chi2 test $p<0.05$). In experiment 2, spermatozoa were added 5mM procaine and co-incubated with oocytes for 4 hours *vs* 18 hours. The percentage of zygotes 48 hours post IVF was similar for 18 hours (44%, 7/16) and 4 hours co-incubation (32%, 6/19) (Chi2 test $p>0.05$). In experiment 3, spermatozoa were added 5mM *vs* 1mM procaine and co-incubated with oocytes for 18 hours. The percentage of zygotes 48 hours post IVF was higher for 5mM procaine (48%, 13/27) than for 1mM (19%, 5/26) (Chi2 test $p<0.05$). In the 3 experiments, zygotes contained at least 2 highly decondensed pronuclei, pronuclei decondensation being the first step of embryo development. We also observed 2 cleaved embryonic structures in the group 5mM during 18 hours, but the quality of these embryos was poor.

In conclusion, decreasing procaine concentration or exposure time influence IVF rate and doesn't improve equine embryo quality.

Notes

DETECTION OF PREGNANCY-ASSOCIATED GLYCOPROTEINS (PAGS) IN PROLIFIC AND NON PROLIFIC EWES FROM EARLY TO LATE GESTATION AND POSTPARTUM

EL AMIRI B[1], EL ABBADI N[2] , MELO DE SOUSA N[3] AND BECKERS JF[3]

[1]INRA, Centre Régional de la Recherche Agronomique de Settat, BP= 589, Morocco
[2]CNESTEN, Unité Radio-Immuno-Analyse, Département Sciences du Vivant, B.P 1382, R.P. 10001, Rabat, Morocco
[3]Faculté de Médecine Vétérinaire, Université de Liège, B-4000, Belgique

Pregnancy-associated glycoproteins (PAGs) are placental antigens that were initially characterized as pregnancy markers in the maternal circulation of bovine species (Zoli et al., 1992, Biol. Reprod. 46: 83-92). After that, the measurement of such molecules in maternal blood as a method for pregnancy diagnosis in other ruminants has been demonstrated in several species. It can give useful information to develop appropriate feeding strategies for pregnant females and to assure requirements of the mother and the growing of fetuses and to avoid metabolic disorder associated to pregnancy. The aim of the present study was to investigate the use of a PAG ELISA-Sandwich kit (Ref. code E.G.7. CER. Marloie, Belgium) *vs* two homologous radio-immunoassay described in El Amiri et al. (2007; Reprod. Domest. Anim. 42:257-62) to detect PAGs in blood samples collected from Boujaâd (non prolific, n=8) and Boujaâd x D'man (prolific, n=20) sheep from early to late gestation and postpartum. Ewes were assumed to be pregnant when PAG concentrations were higher than 0.8 ng/ml in ELISA and 0.3 in RIAs. In addition the samples were also explored by the double immunodiffusion radial (El Amiri et al., 2003, Theriogenology. 59:1291-301) after PAG extractions. The results show that in both systems (ELISA *vs* RIAs), the PAG concentrations were significantly lower in Boujaâd a non prolific sheep than in Boujaâd x D'man a prolific sheep. Furtemore, the concentrations in RIAs were 3 folds higher than those in ELISA. In all systems, the concentrations decreased rapidly after lambing (21 weeks) reaching basal values at fourth week postpartum in ELISA *vs* RIAs. In ELISA all pregnant females showed PAGs level above 1.4 ng/ml from day 24. The double radial immunodiffusion showed positive reactions in ewes carrying more dead fetus. In conclusion, the plasma PAG investigated in the present study showed that the ELISA technique is proved to be a convenient and reliable means for early pregnancy diagnosis as well as for pregnancy fellow up in sheep. From 24 days of gestation, its reliability achieved 100% and, therefore, matches conventional approaches of pregnancy detection. The PAGs could also be detected after extraction from plasma of pregnant ewes using the double radial immunodiffusion. However, for using this technique in routine, further studies are necessary.

Acknowledgement
The authors owe to thank Dr. Delahaut P and Colment Y from the Centre d'Economie Rurale (CER), Marloie, Belgium for supporting the ELISA-Kits.

Notes

RECIPIENT PREGNANCY RATES AFTER TRANSFER OF VITRIFIED IN VIVO PRODUCED OVINE EXPANDED OR HATCHED BLASTOCYSTS

EMSEN E[1], DEMIREZER H[3], KUTLUCA KORKMAZ M[2], DEMIRAG KM[3]

[1]*Ataturk University, Department of Animal Science,25240, Erzurum,Turkey*
[2]*Ataturk University, Ispir Hamza Polat Vocational School,25900,Ispir, Erzurum,Turkey*
[3]*Pan Small Ruminant Reproductive Technology Service, Gaziantep, Turkey*

The development of embryo transfer technology pushed freezing technology in domestic species. Controlled (traditional) slow freezing and vitrification (ultra-rapid freezing) have been the two major techniques used for embryo cryopreservation (Fahy and Rall, 2007). The ultra-rapid technique, such as vitrification, has reduced time and cost of the procedure since it does not require any special equipment and is, therefore, well adapted to routine field use (Baril, 2001). Sheep and goat embryos are able to survive vitrification procedures and with further research this method may provide an economical alternative to the current freezing methods. Shirazi et al., (2010) indicated that the survival of IVP ovine embryos following vitrification progressively increases as the developmental stage of the embryo proceeds.

We studied the effect of vitrification on the developmental stage of the embryo monitored after ET. Donor ewes (10 Romanov breed) were used to produce vitrified embryos kept under semi-extensive husbandry conditions and fed on a maintenance diet (ARC 1990). Superovulation was induced by treatment with ovine follicle stimulating hormone, FSH (Ovagen, Immuno-Chemical Products Ltd) that was administered in 8 equal doses at 12-hourly intervals (total dose equivalent to 9 mg NIADDKoFSH- 17) commencing 60 hours prior to the end of progestagen treatment (12 d). Embryos were recovered by the surgical procedure on day 6 following insemination and were assigned on their developmental stage and quality grade according to standards of the International Embryo Transfer Society (Savoy, IL). Within 2 to 4 h after collection, expanded (n=18) and hatched (n=18) blastocysts were first washed in phosphate-buffered saline (PBS) supplemented with 0.5 mM sodium pyruvate, 3.3 mM glucose and 10% FCS. The embryos were vitrified at room temperature (~23 °C) as follows: 10% EG+10% DMSO for 3 min, followed by 20% EG+20% DMSO+0.5 M Sucrose for 30 s, loaded into OPS and directly plunged into LN2. Before ET the embryos were warmed directly by plunging them into tissue culture medium-199 (TCM-199) + 20% foetal calf serum (FCS) at 37 °C for direct dilution. Following the direct dilution, the embryos were transferred as single into synchronised recipient ewes and allowed to go to term. Pregnancy rates were assessed by ultrasound scanning at 50 days after transfer. We used the *chi-square* test to compare *pregnancy rates.*

Late stage embryos produced in vivo to the expanded blastocyst stage before cryopreservation had a significant ($P<0.05$) higher (86%) of pregnancy rate than those recorded (60%) for the hatched stage blastocyst. Our results were higher than the results reported by Garcia-Garcia et al., (2005). They worked with in vivo produced ovine embryos in early stages (2-12 cells) and cultured to the blastocyst stage and frozen thereafter. In that study it was reported that blastocyst stage of embryos had a significantly higher viability than their counterparts frozen at earlier cleavage stages, (66.1% versus 23.1%). The results indicate that in vivo produced embryos up to expanded blastocyst stage can be successfully cryopreserved by vitrification while vitrification success decreases when the stage of development reaches hatched blastocyst.

Notes

REPRODUCTIVE SUCCESS IN INTERBRED EWES AFTER FRESH EMBRYO TRANSFER

EMSEN E[1], KUTLUCA KORKMAZ M[2], DEMIREZER H[3]

[1]*Ataturk University, Department of Animal Science,25240, Erzurum,Turkey*
[2]*Ataturk University, Ispir Hamza Polat Vocational School,25900,Ispir, Erzurum,Turkey*
[3]*Pan Small Ruminant Reproductive Technology Service, Gaziantep, Turkey*

Estrous response and ovulation rate of Turkish Native Sheep (N; n=27) and the prolific Romanov crossbred breed (NR; n=22) for the use as recipients in embryo transfer programs were evaluated in the anoestrus season (April). Estrus of recipient ewes was synchronized with vaginal sponges containing 30 mg FGA for 12 days and ewes received an i.m. injection of 400 I.U. PMSG at sponge removal. Estrus and ovulation rates, time to onset of estrus and site of the ovulation were determined. A total number of 3 Romanov breed donors ewes were superovulated using FSH-p with 200 mg NIH-FSH-P1 (total of 20 ml) (Folltropin-V; Vetrepharm, Canada) applied in 8 decreasing doses of 1.5, 1.5, 1.5, 1.25, 1.25, 1, 1, 1 ml i.m. at 12 h intervals, starting 60 h before sponge withdrawal. Donors received 1ml Estrumate and 100 I.U. PSMG 36h prior to sponge removal and finally an additional 200 I.U. PMSG was injected at sponge removal. Donors were mated with their own breed of rams. Ewes were observed for estrus (d 0) and were surgically flushed by laparotomy 6 or 7 d later to recover embryos. The number of ovulations and transferable embryos were 18.7 and 14.3, respectively. Embryos with Grade I, II and III with the stage of morula to expanded blastocysts were used in twin fresh transfer. The success rate of the synthetic progestagen treatment to establish an estrus out of season (April) was found to be 59.3% and 52.4% for N and NR, respectively. Time between the removal of the sponges and the onset of estrus was similar between the two breeds of recipient ewes (N: 53.06±0.95h and NR: 52.27±1.07h). For the recipient ewes at ET the ovulation rates were found significantly higher ($P<0.05$) in N ewes (1.0±0.00) than NR ewes (0.72±0.14); the ovulation site was mainly located on the right ovary in NR ewes (87.5%) compared to N ewes (42.9%). We transferred embryos as pairs to save number of recipients as advised by Gimenez-Diaz (2012) who indicated that pregnancy success for number of embryos transferred (single versus twin) was similar. Recipient ewes with fertile estrus (estrus accompanied with ovulation) received similar stages of embryos fallowing the laparoscopic measurements (location, number and quality score) of CL in recipients. Pregnancy and embryo survival rates were similar in N (64.3% and 77.8%) and NR (75.0% and 75.0%) ewes. The sex ratio of twin transferred embryos was higher in N (75% male) than those observed in NR (22.7% male) ewes. These preliminary results show that, Romanov crossbreed recipients with a lower ovulation rate and ovulation occurred mostly in right ovary had more overall MOET success (66% vs 57%; no of lambs born/no of embryo transferred) and were found more favourable with more female lambs from fresh embryo transfer compared to Turkish Native Sheep.

Notes

PRESERVATION OF RAM SEMEN WITH EXTENDER SUPPLEMENTED WITH SPERMATHECAL FLUID OF QUEEN BEE

EMSEN STEINMAN B, EMSEN E

Ataturk University, Department of Animal Science,25240, Erzurum,Turkey

Reproductive biotechnologies have been developed for routine, microbiologically safe propagation of genetic material among breeding populations. Semen still is the "cheapest" component of artificial breeding. Therefore, AI is the most prevalent reproductive biotechnology used on a global scale and, depending on the species, requires the preparation of liquid, chilled, or frozen thawed insemination doses. Honey bee (Apis mellifera) sperm remains viable in the spermatheca of mated female honey bees for several years. During this time, the sperm retains respiratory activity, placing it at risk of the damaging effects of reactive oxygen species common to many biological processes. Antioxidative enzymes might help reduce this damage. Understanding the extreme measures needed to maintain viable sperm in social insects should clarify both general mechanisms of sperm preservation in insects and mechanisms unique to the evolution of insect colonies. The study conducted by Kelnk et al., (2004) showed for the first time that a high concentration of protein is found in the spermathecal fluid in Apis mellifera (from 8.5 mg/ mL to 15.3 mg/mL). In honey bees, female derived proteins seem to play a major role in sperm storage. The researcher hypothesized that in Apis mellifera, in addition to the high pH, many proteins are produced by the queen which have a function in long sperm storage. Impaired sperm function is a general cause of male infertility. An unbalanced, excessive production of reactive oxygen species (ROS) and decreased level of antioxidant enzymes cause decreased sperm motility and viability, and increased sperm defects by initiating an oxidation chain reaction damaging proteins, lipids and DNA in rams. Due to the high percentage of polyunsaturated fatty acids (PUFA) in the plasma membrane, sperm are highly susceptible to ROS-induced damage (Asadpour et al., 2012). Lipid peroxidation of these PUFA can lead to diminished sperm membrane fluidity and, therefore, compromise the fertilizing ability of human and bovine, rabbit, boar and ram sperm. Seminal plasma has an antioxidant system that seems to be very relevant to the protection of sperm. The sperm oxidative defence enzymes predominantly include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase in mammalian and a reduction in the protective enzyme capacity by semen dilution has been shown (Maxwell and Stojanov, 1996). The spermatheca of queen bees provides a specialized physiological environment, in which the spermatozoa are densely packed and show decreased metabolism. For the prolonged survival of these non-dividing and non-regenerating cells, protection from oxidative stress should be of paramount importance. Therefore, the spermatheca or spermathecal gland appear to be the more plausible sources of increased or sustained enzyme activities. As an organ not involved in any major metabolic activity, the spermatheca of the mated queen showed remarkably high activities for these enzymes in the postmitochondrial fraction (spermathecal fluid, microsomes and cytosol) of the homogenized tissue (Weirich et al., 2002).

Development of better cryopreservation methods are on top of the research agenda and therefore it was suggested to develop a new protocol with the natural additives from spermathecal fluid in Apis mellifera to increase fertility of ram semen which is documented widely that is one of the species with poor semen freezing capacity.

Notes

β-DEFENSIN 126 AND SPERM FUNCTION IN CATTLE

FERNANDEZ-FUERTES B[1], NARCIANDI F[2], MEADE KG[3], O'FARRELLY C[2], FAIR S[4] AND LONERGAN P[1]

[1]School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland. [2]Trinity Biomedical Sciences Institute, Trinity College, Dublin 2, Ireland. [3]Animal & Grassland Research and Innovation Centre, Teagasc, Grange, Co. Meath, Ireland. [4]Department of Life Sciences, University of Limerick, Ireland.

β-defensins are antimicrobial peptides also thought to have a role in sperm function. In cattle, β-defensin 126 (BD126) has been only detected in the male reproductive tract, with preferentially in the epididymis (Narciandi et al., Immunogenetics 63, 641–651, 2011). The macaque ortholog has been shown to enhance the ability of sperm to migrate through cervical mucus (Tollner et al., Hum. Reprod. 23, 2523–2534, 2008). A mutation in the BD126 gene has been linked to subfertility in men, only explained by reduced ability to penetrate through mucus *in vitro* (Tollner et al., Sci. Transl. Med. 3, 92ra6, 2011). The aim of this study was to examine the role of bovine BD126 in sperm function. Western blot (WB) analysis with a BD126 specific monoclonal antibody demonstrated significant BD126 on bovine sperm which previously published methods for macaque sperm failed to remove. WB analysis also revealed that while BD126 is present on sperm and in seminal plasma from intact bulls, it is undetectable in the ejaculate of vasectomised animals, indicating that it does not originate in the accessory glands. Further analysis demonstrated that the peptide is uniquely present in the cauda epididymis and is absent from sperm recovered from other epididymal regions, thus providing a model to study its function. Confocal analysis revealed immunofluorescent labelling of BD126 specific to the tail and acrosomal region in cauda sperm only, suggesting a role in sperm motility. We therefore hypothesized that addition of cauda fluid to corpus sperm would improve motility and ability to penetrate cervical mucus *in vitro*, and that this may be due to the activity of BD126. Testes were collected from adult bulls at an abattoir and sperm from the corpus and cauda epididymis, as well as cauda epididymal fluid (CEF), were recovered. Corpus sperm were incubated for 1 h with CEF in the absence or presence of BD126 antibody (Ab); untreated corpus and cauda sperm were used as controls. A higher number of cauda than corpus sperm migrated through cervical mucus ($P<0.001$) and addition of CEF increased the number of corpus sperm migrating through this matrix ($P<0.05$). The presence of the BD126 Ab failed to abrogate this effect. Analysis of motility using a computer assisted sperm analysis system indicated higher total and progressive motility in caudal sperm when compared with sperm from the cauda ($P<0.001$); again, addition of CEF increased progressive motility ($P<0.05$). In conclusion, we have characterised the expression of bovine BD126 as a protein in the cauda epididymis. Incubation of sperm from the corpus epididymis (which lack BD126) with CEF from the cauda (which contains BD126) resulted in enhanced sperm migration through cervical mucus, and higher motility. Further work will clarify the role of BBD126 and related β-defensins in mediating bovine sperm function.

Supported by Department of Agriculture, Food and The Marine under the Research Stimulus Programme (Grant No. 11S 104).

Notes

A NOVEL 3-D CULTURE SYSTEM TO STUDY BOVINE OVIDUCT PHYSIOLOGY, GAMETE INTERACTION AND EARLY EMBRYO DEVELOPMENT

FERRAZ MAMM[1], HENNING HHW[2], VOS PLAM[1], STOUT TAE[2], GADELLA BM[2,3]

Departments of [1]Farm Animal Health, [2]Equine Sciences, [3]Biochemistry and Cell Biology, Faculty of Veterinary Sciences, Utrecht University, The Netherlands

Successful fertilization depends on processes that take place in the oviduct. Due to its intra-abdominal location, it is difficult to study intra-oviductal processes *in vivo* in mammals. Instead, *in vitro* models that retain essential cell morphological and functional characteristics are being developed. In culture, bovine oviduct epithelial cells (BOECs) rapidly lose differentiated cell properties (e.g. secretory activity and cilia), while suspended cells have a limited lifespan. Progress with insert culture models and 3-D printing technologies prompted us to develop two independent BOEC culture systems, in which *in vivo*-like differentiation and function is re-established, to study oviduct physiology: (i) 3-D printed U-shaped inserts mounted with PET membranes with 0.4 µm pores (3D U-shaped culture) and (ii) hanging inserts (polycarbonate with 0.4 µm pores) containing 150 µL of Matrigel (3D culture). BOECs were harvested by scraping, and cultured for 24h to agglomerate into floating vesicles with outwardly oriented cilia. The vesicles were plated and, 7 days later, the resulting monolayers were scraped, washed and seeded onto the 2 systems described above and cultured at an air-liquid interface. For comparison, BOECs were also seeded onto coverslips as monolayers (2D culture). After 28 days, the apical side of all BOEC monolayers was washed to harvest secreted proteins, and the inserts were fixed for immunocytochemistry. Proteins (20 µg) were separated by SDS-PAGE and visualized by silver staining, or blotted onto nitrocellulose and immunostained for oviduct specific glycoprotein (OVGP1).

Epithelial cell differentiation was indicated by immunodetection of laminin and the presence of primary cilia. Ciliated cell presence (acetylated α-tubulin) and secretory activity (OVGP1) characteristics of BOECs in 3D cultures were comparable to freshly harvested BOECs. The 3D culture yielded 46 silver-stainable protein bands versus 30 in 2D cultures (n=3 per system). In 3D U-shaped cultures, the polarized state (laminin and primary cilia) and their amenability to direct fluorescence microscopy (allowing live cell imaging) are currently determined.

In conclusion, 3D culture methods promote polarization and differentiation of BOECs. The extent to which physiological function is maintained is under investigation. Studies in progress to assess the BOEC differentiation using the 3D U-shaped cultures include basolateral co-culture of stromal cells. Ultimately, we aim to develop an oviduct-like environment to study gamete activation, fertilization and early embryo development *in situ*.

Notes

ESTRADIOL ROUTE AND NON-SURGICAL EMBRYO RECOVERY IN SYNCHRONIZED SANTA INÊS EWES

FONSECA JF[1*], ZAMBRINI FN[2], PRATES JF[3], MARTINS TLT[3], CASTRO ACR[1], ESTEVES LV[4], SOUZA-FABJAN JMG[4], BRANDÃO FZ[4], GUIMARÃES JD[2]

[1]Embrapa Goats and Sheep, Sobral, Ceará, Brazil; [2]Viçosa Federal University, Viçosa, Minas Gerais, Brazil, [3]Embrapa Dairy Cattle, Juiz de Fora, Minas Gerais, Brazil; [4]Fluminense Federal University, Niterói, Rio de Janeiro, Brazil.
**jeferson.fonseca@embrapa.br*

Animal surgery procedures are being progressively restricted worldwide in the context of embryo transfer. In small ruminants, the needs for development of alternative and efficient non-surgical techniques for embryo transfer have been emphasized. Non-surgical embryo recovery is well consolidated in Brazil in cattle and goats, while in sheep it remains a challenge. The aim of this study was to check the efficiency of different ways of estradiol benzoate administration on cervix dilation and embryo recovery in synchronized Santa Inês ewes. A total of 23 pluriparous ewes were subjected to two doses of 37.5 μg d-cloprostenol by intravulvo-submucosal way seven days apart. After the second cloprostenol administration, ewes were checked for estrus at 12 h interval and mated with fertile rams during estrus. After mating, ewes were allocated according to estrous response into two treatment groups for embryo recovery seven days after estrous onset. In T1 (n=11), ewes received 37.5 μg d-cloprostenol and 1 mg estradiol benzoate 16 h before embryo recovery, plus 50 IU oxytocin i.v. 20 min before embryo recovery. In T2 (n=10), ewes received the same protocol as T1, but the way of estradiol administration was intravaginal. All ewes received 2 ml of lidocaine 2% without vasoconstrictor for epidural and 2 ml of lidocaine for contact cervical anesthesia plus acepromazine 1% (1 ml/kg live weight) before cervical passage as previously described in goats (Fonseca et. al.; Small Rumin. Res., 111:96-99, 2013). Qualitative and quantitative data were analyzed by chi-square test and ANOVA respectively with 5% significance. Estrous response after the second cloprostenol administration was 91.3% (21/23). There were no differences (P>0.05) in any parameter evaluated for T1 and T2: successful uterine flushing (90.9% and 80.0%), duration time of embryo recovery (20.3±8.0 and 26.2±5.3min), flushing recovery rate (PBS injected/PBS recovered; 90.1 and 90.5%), average structures recovered (1.0±0.4, 20% viable and 1.4±0.6, 33% viable). Considering that Santa Inês sheep have up to 1.3 lambs we can conclude that it is possible to perform efficient non-surgical embryo recovery in non-superovulated synchronized Santa Inês ewes, regardless the way of estradiol administration.

Financial support: Embrapa (Project 03.12.01.031.00) and Fapemig (CVZ-PPM 00042-14).

Notes

EFFECT OF THYMOSIN ON *IN VITRO* FERTILIZATION AND DEVELOPMENTAL COMPETENCE AND QUALITY OF PIG EMBRYOS

GAJDA B, PONIEDZIALEK-KEMPNY K, RAJSKA I, SMORAG Z

Department of Biotechnology of Animal Reproduction
National Research Institute of Animal Production
Krakowska 1, 32-083 Balice/Kraków, Poland

Thymosin (TH) is biological active polypeptide released by thym. It plays a vital role in the repair and regeneration of injured cells and tissue. It protects cells from damage and blocks apoptosis (Goldstein et al. 2012, Expert Opin Biol Ther, 12(1), 37-51). In our recent study we demonstrated that supplementation of maturation medium for pig oocytes with synthetic TH increased the number of matured oocytes with lower morphological quality. The aim of the present study was to investigate the effect of supplementation of maturation medium for oocytes with TH on *in vitro* fertilization and developmental competence and quality of pig embryos. Cumulus-oocyte complexes (COCs) were obtained by aspiration from antral follicles of ovaries collected from slaughtered gilts. COCs were selected based on their cytoplasm morphology and cumulus cells appearance and cultured in modified TCM-199 medium supplemented with 0.5 mg/ml of synthetic TH (LipoPharm.pl) (experimental group) or without TH (control group) for 42 h at 39^{o}C and in a humidified atmosphere containing 5% CO_2 in air. After maturation oocytes were assessed and *in vitro* fertilized (IVF). Semen for IVF was incubated in modified capacitation medium-M-199 for 1 h. Sperm fraction was introduced to the droplets containing oocytes and next gametes were coincubated for 4 h in modified TCM-199 medium. Presumptive zygotes were cultured *in vitro* for 144 h in NCSU-23 medium under the conditions stated above. Embryo quality criteria were cleavage, morula and blastocyst rates, total cell number per blastocyst and degree of apoptosis assessed by TUNEL. The results were analyzed statistically with Chi-square test. Treatment of oocytes with TH during culture slightly increased the ratio of matured oocytes (95/103, 92.3%) compared to the control group (134/150, 89.3%; no significant differences) cultured without TH. After IVF cleavage, and development to the morula and blastocyst stage, based on number of cleaved embryos, were not different between experimental (29.5, 71.4 and 32.1%, respectively) and control (25.4, 50.0 and 29.4%, respectively) group. The mean number of cells per blastocyst in experimental and control group was comparable (40.4 and 39.9; respectively). The mean number of apoptotic nuclei and apoptotic index was 0.67 and 1.66 in the experimental group and was significantly lower ($P<0.05$) than in the control group i.e. 1.66 and 4.35. In conclusion, the culture oocytes in a medium with TH supplementation had a positive effect on quality pig IVF blastocyst since they had a significant lower incidence of apoptosis. However, further studies are required to determine the competence of porcine blastocyst recovered from oocytes matured with TH for *in vivo* development.

Supported by Fund of Own Research IZ PIB, project no. 07-2.02.7 (2014)

Notes

A RETROSPECTIVE STUDY OF IN VITRO EMBRYO PRODUCTION FROM HIGH GENETIC MERIT COWS USING UNSORTED OR X-SORTED SPERM IN A COMMERCIAL PROGRAM

GAMARRA G[1], LACAZE S[1], MOUNEYRES M[1], MARQUANT LE GUIENNE B[2]

[1] *MIDATEST, Domaine de Sensacq, 64230 Denguin, France*
[2] *ALLICE, R & D Department, 78352 Jouy en Josas, France*

X-sorted sperm can be used in embryo transfer programs to produce female progeny (Kaimio et al., Theriogenology 80, 950-954, 2013). However, X-sorted sperm is generally used in heifers as cow insemination results in lower numbers of transferable embryos (Hayakawa et al., Theriogenology 71, 68-73, 2009). We hypothesized that breeding programs based on IVP with X-sorted sperm may be a promising alternative. The aim of this study was to compare *in vitro* embryo production in cows using X-sorted or unsorted semen under commercial conditions performed at the Biotechnology MIDATEST Station located in Denguin, South West, France. Three to fifteen years old Holstein cows (n=26) and 16-22 months old heifers (controls: n=17) were used in an OPU-IVP program. Donor cows were stimulated with decreasing pFSH doses (Stimufol; Reprobiol, Liège, Belgium) twice daily during 3 days, (total dose: 350 µg for cows and 250 µg for heifers). Cumulus oocyte complexes (COCs) were collected by OPU 12 to 24 h after the last FSH injection and *in vitro* matured using a standard IVM protocol. Oocytes were fertilized with frozen–thawed unsorted (cows and heifers) or X-sorted (cows) sperm in modified Tyrode's bicarbonate buffered solution medium (fert-TALP) using different non pre-tested bulls (n=55). Presumptive zygotes were cultured in SOF medium (Minitub, Tiefenbach, Germany) plus 1 % cow serum up to Day 7 at 38.5 °C in 5% CO2, 5% O2 and 90% N2 atmosphere with maximum humidity. OPU/IVP was repeated one to 13 times (2.5 ± 2.6) for each donor cow or heifer. Grade 1 blastocysts and expanded blastocysts according to IETS classification were recorded on days 6.5 and 7. Viable embryos were frozen or transferred fresh. Embryo production was analyzed with ANOVA and blastocyst yield by Chi-Square. From 18 OPU sessions in heifers, a total of 168 COCs (9.3 ± 4.7 per session) were processed for *in vitro* maturation, and 5.4 ± 3.9 Grade 1 (G1) embryos were produced per session. In cows 42 sessions were performed with unsorted semen and 44 with X-sorted semen, 13.1 ± 9.6 and 8.9 ± 4.9 oocytes ($p<0.05$) were collected; 7.7 ± 5.5 and 4.1 ± 2.9 G1 embryos were produced, respectively ($p<0.05$). The mean embryo development rate (total number of G1 embryos / number of oocytes entering maturation process) was 59.1% (unsorted semen) and 46.3% (X-sorted semen; $p<0.05$). Although the number of collected oocytes was different, there were no differences in presumptive female embryos produced per session assuming a sex ratio of 90% (3.7 embryos per session) with sorted semen and a sex ratio of 50% (3.9 embryos per session) when using unsorted semen. In conclusion, our work confirmed the efficacy of OPU-IVP techniques to produce grade 1 embryos using X-sorted in high genetic merit cows. Furthermore this technique allows to get female calves based upon a lower number of recipients.

Notes

EXPRESSION OF B- NERVE GROWTH FACTOR IN RABBIT MALE TRACT AND SEMINAL PLASMA

GARCIA- GARCIA RM[1], MASDEU M[1], SÁNCHEZ-RODRÍGUEZ A[1], BAUTISTA JM[2], ARIAS-ALVAREZ M[3], LORENZO PL[1], REBOLLAR PG[4]

[1]Dpto. Fisiología (Fisiología Animal). [2]Dpto. Bioquímica y Biol. Mol. IV. [3]Dpto. Producción Animal. Facultad de Veterinaria. Universidad Complutense de Madrid. Madrid, Spain.
[4]Dpto. Producción Animal. ETSI Agrónomos. Universidad Politécnica de Madrid. Madrid, Spain.

Nerve growth factor (NGF) has been recently identified as an ovulation inductor factor (OIF) in the seminal plasma (SP) (Ratto et al. PNAS 2012; 109:15042-7). The presence of OIF in rabbit has been suggested but this protein has not yet been identified. Our aim was to study the mRNA expression in the rabbit male reproductive tract and to identify the protein β-NGF in the SP. Total RNA was extracted from prostate, testicles and seminal glands of 3 male rabbits (TRIzol® Plus RNA Purification Kit, Life Technologies) to subsequently isolate mRNA (FastTrack® MAG mRNA Isolation Kit, Ambion, Life Technologies,) for retrotranscription to generate cDNA. Specific primers were designed on the mRNA sequence deposited in GenBank (XM_008264614.1) to target a highly conserved region of NGF among species (5'-AGCCCACTGGACTAAACTGCA-3';5'-TCGCACACCGAGAACTCTCC-3'; product size: 305 nucleotides). PCR was performed on cDNA to obtain the expected 300 pb fragment that was sequenced confirming the presence of NGF-mRNA in seminal plasma, testicle and prostate. To determine the expression of mature NGF protein in SP, an aliquot was prepared from collected semen, centrifuged at 3000xg for 30 min at 4ºC and stored at -20°C. For Western blot (WB) analysis, samples were loaded in 12% SDS–PAGE and electrotransferred onto nitrocellulose membranes. The membranes were probed with mouse β-NGF antibody (Promega) using donkey anti-mouse as secondary antibody (Li.Cor Biotechnology). Blots were scanned in an Odissey Infrared imaging system. In addition, NGF was purified by offgel technique with the 3100 OFFGEL Kit pH 3–10 (Agilent Technologies Inc) and the recovered fraction recognized with the mouse β-NGF antibody was used for mass spectrometry analysis (MS) (4800 Plus Proteomics Analyzer Applied Biosystems,). MS was operated in positive reflector mode with an accelerating voltage of 20,000 V. For protein identification NCBInr was used. Database without taxonomy restriction and a home-made database with the sequence of NGF (gi|655847230) downloaded from NCBInr was searched using MASCOT v 2.3. The probability scores of NGF sequences from several species were greater than the score fixed by MASCOT as significant with a p-value < 0.05. Our results show that expression of NGF-mRNA were clearly identified in the rabbit male tract organs above described and the corresponding mature protein band with a mass of ~60 kDa was also identified by WB whereas a ~13 kDa band was detected in the basic fraction (pH=8.24-8.83) obtained when offgel electrophoresis was performed. Furthermore, protein identification by mass spectrometry revealed the existence of NGF in the SP. In conclusion, mRNA and protein NGF are present in rabbit male reproductive tissue and SP respectively, providing the basis to undertake further functional analysis for its potential role in rabbit reproduction. Acknowledgements: Funds from AGL2011-23822. L. Gutierrez (Genomics and Proteomics Center, UCM).

Notes

MOBILIZATION OF INTRACELLULAR LIPIDS BY SUPPLEMENTATION OF IVM AND IVC MEDIA WITH L-CARNITINE IMPROVES BOVINE EMBRYO QUALITY

GHANEM N, RÄTY M, LIDAUER P, PEIPPO J

Natural Resources Institute Finland, Green Technology, Finland, 31600 Jokioinen, Finland.

Mobilization of embryo lipid by supplementing culture media with metabolic activator is one of the promising tools to improve quality of in vitro produced bovine embryos. Therefore, the present study investigated the effect of L-carnitine supplementation during in vitro maturation (2.5 mM) and embryo culture (1.5 mM) on embryo developmental rates, quality and gene expression profiles. Cumulus-oocyte complexes recovered from slaughter house ovaries were morphologically evaluated and only grades 1and 2 were used in this study. Treatment groups were: T1=IVM+LC, T2=IVC+LC, T3=(IVM and IVC)+LC and control. In vivo produced embryos were included in all analyses. Development rate was calculated based on the number of embryos reached blastocyst stage at day 8 of culture. Total cell count as well as number of apoptotic cells was evaluated using Tunnel-Hoechst assay. The activity of mitochondria and intensity of lipid was measured using fluorescent probes. Expression of embryo selected candidate genes was profiled using quantitative real-time PCR. Our results showed no differences ($P < 0.05$) in cleavage rate between L-carnitine treated groups and control. Although there was an increase in blastocyst rate in T2 (44.4%) and T3 (42.1%) groups compared to T1 (39.2%) and control (38.2%), it was not statistically significant. Embryos cultured with L-carnitine and in vivo group had greater total cell number (T1: n=140.2, T2: n=164.8, T3: n=155.9 and in vivo: n=160) than the control (n=129.4). On the other hand, the percentage of apoptotic cells from total number of cells was greater ($P < 0.05$) in control (11.2%) than L-carnitine treated (T1: 4.2, T2: 3.8 and T3: 2.9%) and in vivo derived blastocysts (0.3%). Cytoplasmic lipid content was reduced by 1.8, 2.7, 2.4 and 5.1 times in T1, T2, T3 and in vivo produced blastocysts compared to their control counterparts. Whereas, intracellular mitochondria density was increased by 2.0, 4.8, 4.5 and 6.3 folds in embryos cultured with L-carnitine and in vivo group. Genes regulating lipid oxidation (CPT2 and CPT1B), fatty acid transport (SLC27A1) and mitochondria transcription (TFAM) were up-regulated while a lipid storage marker transcript (PLIN2) was down-regulated in embryos cultured in presence of L-carnitine and in vivo ones compared to control. Collectively, the lipolytic effect of L-carnitine was linked with increased mitochondrial activity, reducing apoptotic cells and modulating gene expression of in vitro produced embryos which will most likely enhance their survival after cryopreservation and transfer to recipients.

Notes

INTRAUTERINE EXPRESSION OF INSULIN-LIKE-GROWTH FACTOR FAMILY MEMBERS DURING EARLY EQUINE PREGNANCY

GIBSON C, DE RUIJTER-VILLANI M, STOUT TAE

Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, The Netherlands

Insulin-like growth factor (IGF) family members are known to regulate fetal and placental growth and development. Insulin (INS), IGF1 and IGF2 stimulate cell proliferation and differentiation via their receptors INSR, IGF1R and IGF2R. The actions of IGF are further regulated by the IGF-binding proteins. The horse is unique with regard to an unusually long pre-implantation period (40 days) offering a unique tool to study the dialogue between conceptus and endometrium. We evaluated the expression of IGF system components in equine conceptus membranes, and endometrium during the cycle and early pregnancy. Endometrial biopsies were harvested on days 7, 14, 21 and 28 from pregnant mares, following conceptus collection, and days 7, 14 and 21 from cycling mares (n=4 per group). Bilaminar trophoblast was isolated from day 14 and 21 conceptuses, and the yolk-sac and allantochorion from 28 day conceptuses were separated. Expression of mRNA for IGF system components (INS, INSR, IGF1, IGF1R, IGF2, IGF2R) were investigated by qRT-PCR. The effect of conceptus developmental stage was analyzed by one-way ANOVA, and the effects of pregnancy and days after ovulation on endometrium by two-way ANOVA followed by independent-sample T-tests. INS mRNA was not detected in endometrium or conceptus membranes. IGF1 and IGF2R mRNA levels were uniform in cycling and pregnant mare endometrium. INSR gene expression increased in the endometrium of pregnant mares only from day 7 to 14 ($p<0.05$) and showed a higher expression than in cyclic mares on day 21 of pregnancy ($p<0.05$). IGF2 mRNA increased sequentially from day 7 to 14 to 21 of pregnancy ($p<0.05$). IGF1R expression was elevated on day 14 in both cyclic and pregnant mares ($p<0.05$). In the conceptus membranes, mRNA expression for INSR, IGF1, IGF1R, IGF2 and IGF2R was low on days 7 and 14 but showed up-regulation from day 21 ($p<0.05$). In summary, IGF family members are expressed uniformly in endometrium from cycling mares whereas endometrial expression increases during early pregnancy. Conceptus membrane expressions of IGF family genes increases from day 21, when the blastocyst capsule would start to disintegrate. We propose that the INS/IGF system plays an important role in early equine embryonic growth and the preparation for placentation.

This study was supported by the EpiHealthNet project (European FP7 Marie Curie ITN Project number 317146).

Notes

BOVINE OVIDUCT EPITHELIAL CELLS: AN *IN VITRO* MODEL TO STUDY EARLY EMBRYO-MATERNAL COMMUNICATION

HAMDI M[1], LOPERA R[1], MAILLO V[1], NÚÑEZ C[1], GUTIÉRREZ-ADÁN A[1], LONERGAN P[2], BERMEJO-ÁLVAREZ P[1], RIZOS D[1]

[1]Department of Animal Reproduction, INIA. Avda Puerta Hierro 12, local 10, 28040, Madrid, Spain. [2]School of Agriculture and Food Science, University College Dublin, Belfield, Dublin, Ireland

We aimed in this study to: (1) assess the expression of oviduct epithelial cells markers on bovine oviduct epithelial cells (BOECs) cultured *in vitro* under two different systems (suspension or monolayer) and (2) determine the BOECs response to the presence of early bovine embryos. BOECs were mechanically extracted by squeezing the isthmus regions of oviducts collected from slaughtered heifers during the early luteal phase, determined by the appearance of the corpus luteum. Part of the oviduct extract was frozen in liquid nitrogen and stored at -80^{o}C for gene expression analysis (fresh BOEC), while the rest was cultured in SOF+10% FCS for either 24 h (suspension) or 7 days (monolayer). Suspension or monolayer BOECs were co-incubated for 24 h with Day 2 (2- to 4-cell) or 3 (8- to 16-cell) bovine embryos produced *in vitro* to determine the embryonic effect on BOECs. A control group without embryos was included for each BOEC culture. RNA extraction from BOECs was carried out by Trisure™ (Bioline, Madrid, Spain) and Dynabeads (Dynal Biotech, Oslo, Norway) and gene expression was analyzed by qPCR, using *ACTG1* as housekeeping gene. Statistical differences were assessed by ANOVA. *OVGP1*, *GPX4* and *FOXJ1* were chosen as markers for oviductal epithelial cells and based on their function to support early embryo development, protect gametes against oxidative stress, and cilia formation, respectively. *KERA* and *PRELP* are genes implicated in extracellular matrix and *ROCK2* and *SOCS3* are genes involved in cytokinesis, all of which were found to display a response to the early embryo *in vivo* (Maillo et al., Biol Reprod 2015, DOI:10.1095/biolreprod.115.127969). Among BOECs markers, *OVGP1* and *FOXJ1* were significantly downregulated in suspension cells compared with fresh BOECs, losing their expression in a monolayer; however, *GPX4* was significantly higher in monolayer than fresh and suspension BOECs, suggesting that although monolayer BOECs lost some of their functional characteristics, they still conserved others like protection against oxidative stress. Regarding the effect of the embryos on *in vitro* cultured BOECs, only suspension BOECs showed an embryonic effect on gene expression: *ROCK2* and *SOCS3* were significantly upregulated in cells co-cultured with Day 2 compared with Day 3 embryos.

In conclusion, based on the markers studied, BOECs cultured *in vitro* lost some of their functional characteristics, with suspension cells being closer to *in vivo* controls than monolayer. In addition, under our experimental conditions, suspension cells were more adequate to detect possible embryo signals than monolayer.

Notes

PRESENCE OF L-CARNITINE DURING MATURATION IMPROVES EFFICIENCY OF FERTILIZATION IN PORCINE OOCYTES

HULINSKA P, HANZALOVA K, MILAKOVIC I, KNITLOVA D, HANULAKOVA S, JESETA M, MACHATKOVA M

Veterinary Research Institute, Department of Genetics and Reproduction
Brno, Czech Republic

The lipid-rich pig oocytes might be an excellent model to understand the role of fatty acid metabolism during oocyte maturation, their subsequent monospermic fertilization and preimplantation embryo development. Recently, it was described that L-carnitine stimulates mitochondrial oxidation of fatty acids and increases energy supply of mammalian oocytes. The aim of the study was to characterize the effect of L-carnitine during maturation on the efficiency of fertilization of porcine oocytes with different meiotic competence. Cyclic sows, checked for the ovarian cycle status, were used as oocyte donors. Meiotically more competent (MMC) and meiotically less competent (MLC) oocytes were isolated either from medium (6–9 mm) or small follicles (<5 mm). They were matured separately in IVM medium supplemented with 0, 4 and 10 mM L-carnitine (Sigma-Aldrich Co., Prague, Czech Republic) and fertilized by frozen-thawed spermatozoa of a boar proven in the IVF system using standard protocols (Hulinska et al. 2011, Anim Reprod Sci, 124: 112–117). The presumptive zygotes were incubated in PZM-3 medium (Yoshioka et al. 2002, Biol Reprod, 66: 112–211) for 15 h, fixed in 2.5% aqueous glutaraldehyde solution (v/v), stained with bisbenzimide-33258 Hoechst (Sigma-Aldrich Co., Prague, Czech Republic) and examined by epifluorescence at a magnification of 400 ×. The proportion of penetrated oocytes from the inseminated ones and proportions of monospermic and polyspermic oocytes from the penetrated ones were assessed. Total efficiency of fertilization (%) of oocytes was calculated according to the formula (ratio of monospermic oocytes (n) to inseminated oocytes (n) × 100). The results were statistically analysed by the ANOVA procedure using the Chi-square test. In MMC-oocytes total efficiency of fertilization increased (51.1, 54.3 and 57.6%) when the oocytes were matured with 0, 4 and 10 mM L-carnitine. Similarly in MLC-oocytes, total efficiency of fertilization increased (42.1 vs 48.8%) in oocytes matured with 4 mM L-carnitine compared to those matured without L-carnitine. On the other hand, total efficiency of fertilization decreased when MLC-oocytes were matured with 10 mM L-carnitine (37.9%). It can be concluded that supplementation of medium with L-carnitine during maturation positively influenced fertilization efficiency of porcine oocytes independently of their meiotic competence. However meiotically more competent oocytes were more capable of utilizing L-carnitine in comparison with meiotically less competent porcine oocytes in which the abundance of L-carnitine had a negative effect on fertilization.

The study was supported by Grant LD14104 of the Ministry of Education, Youth and Sport and Grant QJ 1510138 of the Ministry of Agriculture of the Czech Republic.

Notes

EFFECTS OF RESVERATROL SUPPLEMENTATION DURING *IN VITRO* MATURATION AND *IN VITRO* FERTILIZATION ON DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES

KORDOWITZKI P[1], BERNAL SM[1], HERRMANN D[1], ALDAG P[1], LUCAS-HAHN A[1], NIEMANN H[1]

[1]*Institute of Farm Animal Genetics, Department for Biotechnology, Friedrich-Loeffler-Institut (FLI), Mariensee, Neustadt, Germany*

Resveratrol (3,4',5-trihydroxystilbene) is a phytoalexin - isolated from various plant species, particularly grapevine peel. Recently, resveratrol gained scientific interest because of its strong antioxidant effects it may have health benefits, including protection against cardiovascular diseases. In addition, it has been shown to increase lifespan in several species and activates the SIRT1 gene. The aim of this study was to investigate its effects in bovine early embryo development. We employed three different resveratrol concentrations during *in vitro* maturation (IVM) and *in vitro* fertilization (IVF). Bovine oocytes (n=1648) were collected from slaughterhouse ovaries and subjected to IVM medium supplemented with 0.2µM, 1µM, and 20µM Resveratrol® (Sigma-Aldrich, Buchs, Switzerland) for 24 h followed by IVF with the same concentrations of resveratrol for 19 h. IVM and IVF medium without resveratrol (control) and DMSO supplementation as vehicle control were included in this experiment. Presumptive zygotes were cultured *in vitro* until day 8 to assess embryo development. Maturation rates, cleavage and blastocyst formation were determined. Maturation rates did not differ significantly (0.2µM: 64.2±7%; 1µM: 82.3±4%; 20µM: 68.8±2%; control: 74.6±5% and vehicle control: 70.2±6%, respectively, $p \leq 0.05$) did not differ dramatically. Oocytes cultured in 1µM resveratrol supplemented maturation medium showed distinct detachment of cumulus cells. Cleavage was reduced in the 0.2µM and 20µM group (0.2µM: 44.21±2%; 1µM: 58.4±3%; 20µM: 40.9±5%; control: 56.6±2% and vehicle control: 55.2±6%, respectively, $p \leq 0.05$). Blastocyst development was impaired in the low and high resveratrol concentration group compared to the other groups (0.2µM:11.3±1%;1µM: 28.4±6%; 20µM: 8.2±4%; control: 22.7 ±4% and vehicle control: 20.8 ±2%, respectively, $p \leq 0.05$). These preliminary results indicate that very low and high concentrations of resveratrol impair the development to the blastocyst stage. In conclusion, a 1µM resveratrol supplementation during IVM and IVF seems to improve the developmental competence of oocytes, which is reflected not only in elevated blastocyst rates but also in the higher degree of expansion of cumulus cells after IVM and the maturation rates.

Notes

EFFECT OF HIGH HYDROSTATIC PRESSURE (HHP) STRESS ON INTERCELLULAR ATP CONTENT IN PIG EMBRYO

KUCIA M[1], GAJDA B[2], GOGOL P[2], SMORĄG Z[2], ROMEK M[1]

[1]*Department of Cell Biology and Imaging, Institute of Zoology, Jagiellonian University, Krakow, Poland*
[2]*Department of Biotechnology of Animal Reproduction, National Research Institute of Animal Production, Krakow/Balice, Poland*

Embryos exposed to high hydrostatic pressure (HHP) have a greater resistance to further stress and a higher survival rate in cryopreservation or nuclear transfer processes (Pribenszky C., Biology of Reproduction 83; 690-697, 2010). It is known that efficient metabolism is one of the main factors response for a proper development of pig embryos (Romek M., Reproduction in Domestic Animals 46; 471-80, 2011). In addition, preliminary measurements of the inner mitochondrial membrane potential ($\Delta\Psi m$) has shown lower $\Delta\Psi m$ in HHP embryos compared to untreated embryos. If the HHP directly affects on metabolism of embryos reducing the $\Delta\Psi m$, perhaps the total amount of adenosine triphosphate (ATP) content is changing. The aim of this study was to examine the effect of HHP treatment of porcine zygote, on ATP level in embryos at various stages of development.

Pig zygotes (number of 217 embryos) used in the experiment were collected surgically from superovulated gilts breed polskiej zwisłouchej. Gilts were superovulated by an intramuscular injection of 1500 IU of PMSG (pregnant mare serum gonadotrophin, Serogonadotropin, Biowet) followed 72 h later by 1000 IU of hCG (human chorionic gonadotropin, Chorulon, Biowet). Embryos were treated by HHP in HHP device (Cryo-Innovation Ltd, Hungary) for 1 h in 39°C at a pressure of 20 MPa. Afterwards cultured in vitro in medium NCSU-23 in 39°C and 5% CO2. Before ATP analysis, embryos from experimental and control groups were frozen in 5µl Gibco® HEPES buffer (Thermo Fisher Scientific Inc., MA USA) in 1.5ml eppendorf (4-8 embryos in each tube). Analysis of ATP content was performed using Adenosine 5'-triphosphate (ATP) bioluminescent somatic cell assay kit (Sigma Chemical Company, USA) and the luminometer Lumat^3 LB 9508 (Berthold Technologies, USA). In order to examine the statistical differences a one-way ANOVA were used.

The intracellular ATP content in HHP treated group (A) and control group (B) at zygote stage (a), 8-16 cells (b), morula (c) and blastocyst (d), looks like this: Aa 1,63 ± 0,26 pmol/embryo, Ab 1,55 ± 0,25 pmol/embryo, Ac 0,97 ± 0,09 pmol/embryo, Ad 0,88 ± 0,23 pmol/embryo, Ba 1,51 ± 0,40 pmol/embryo, Bb 1,40 ± 0,12 pmol/embryo, Bc 1,01 ± 0,35 pmol/embryo, Bd 0,62 ± 0,15 pmol/embryo.

Pig embryos treated by HHP at zygote stage show not significant differences in intercellular ATP content compared to control group. Significant differences in ATP content between zygote, 8-16 cells and morula, blastocyst stages in both groups of HHP treated and untreated embryos were observed. It means that ATP content in pig embryo is changing during development.

The project was funded by Jagiellonian University based on decision number K/DSC/002336.

Notes

DEVELOPMENTAL COMPETENCE OF PORCINE OOCYTES THAT HAVE FINISHED GROWTH PHASE FROM FOLLICLES OF DIFFERENT DIAMETER

KUZMINA T[1], KRAVTSOV V[1], ALM H[2], TORNER H[2], BRÜSSOW KP[3]

[1]*Russian Research Institute for Farm Animal Genetics & Breeding, St.Petersburg-Pushkin, Russia*
[2]*Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany*
[3]*University of Life Sciences, Poznan, Poland*

Numerous factors determined developmental competence of the oocytes. Brilliant cresyl blue (BCB) staining has been used for selection of oocytes from several mammalian species, including pigs (Ericsson S. et al, 1993 Theriogenology, 39(1): p.214). The aim of the present study was to evaluate the developmental competence of porcine oocytes that have finished growth phase (BCB^+) depending on diameter (d) of follicles (d < 3 mm, 3 - 6 mm, >6 mm) and to detect expression of estrogen receptor (ER) in cumulus cells of BCB^+ and BCB^- oocytes. Before IVM compact cumulus oocyte complexes (COC) were incubated in BCB solution for 60 minutes. Treated oocytes were divided into BCB^- (colorless cytoplasm) and BCB^+ (colored cytoplasm). Only BCB^+ oocytes were used in the experiments. The medium used for oocyte maturation was NCSU 23 supplemented with 10% follicle fluid (FF), 0.1 mg/ml cysteine,10 IU/ml eCG and 10 IU/ml hCG. FF was collected from follicles with 3 - 6 mm in diameter, COC cultured in maturation medium with pieces of wall (600-900 µm in length) from non atretic healthy follicles (d 3-6 mm). After 20-22 h of culture, COC and pieces of wall were washed and transferred into the same maturation medium but without hormonal supplements for another 20-22 h of culture. After IVM oocytes were fertilized in vitro and embryos were cultured by standard protocols (Stokes P. et al., Developmental Biology, 284, p.62 – 71, 2005). All chemicals used in this study were purchased from Sigma-Aldrich (Moscow, Russia). The question was: have BCB^+ oocytes from follicles of different diameters the same developmental competence? We did not find significant differences between the level of cleavage and blastocyst in all groups of experiments. Percentages of cleavage and blastocyst in groups were: follicles d < 3 mm - 43% (27/63) and 29% (18/63); follicles d 3 - 6 mm - 46% (45/98) and 35% (34/98); follicles d > 6 - 48% (28/58) and 28% (16/58) (χ^2 test).Immunocytochemical analysis was used for detection of ***estrogen receptor*** expression (ER) in cumulus cells of 53 BCB^+ and 33 BCB^- oocytes. Immunocytochemical staining was performed using the first rabbit polyclonal anti-human ER antibodies (NCL-ERp, Novocastra, OOO BMS, St.Petersburg, Russia). The visualization system (ABC-universal kit) consists of avidin–biotinilated peroxidase (DakoCytomation) was applied. 3,3'-diaminobenzidine was used as it was recommended from manufacture Novocastra (OOO BMS, St.Petersburg, Russia). Hematoxylin *(Abrisplus,* St.Petersburg, Russia) was used to stain cells. Antigen optical density was measured using morphometric VideoTest (Russia) computer program. Positive immunocytochemical reaction was mainly observed in the nuclei membrane and slightly on the cytoplasmic membrane of cumulus cells (probably as non-specific background). It was shown that cumulus cells of BCB^+ oocytes had a significantly more pronounced expression of the ER than the cumulus cells of BCB^- oocytes ($p<0,001$, Mann-Whitney test).

*This study was supported by grant No.**14-04-90038 Bel_a** from Russian Foundation of Basic Research*

Notes

METABOLOMIC ANALYSIS REVEALED DIFFERENCES BETWEEN BOVINE CLONED EMBRYOS WITH CONTRASTING DEVELOPMENT ABILITIES

LALOË D[1], LE BOURHIS D[2,3], BROCHARD V[3], FERNANDEZ-GONZALEZ A[5] , DUBE D[3], TRIGAL B[4], MUNOZ M[4], MARTIN D[4], GOMEZ E[4], DURANTHON V[3]

1. INRA, UMR 1313 Génétique Animale et Biologie Intégrative, F-78350 Jouy en Josas, France;
2. Allice, Station de Phénotypage, Lieu-Dit Le Perroi 37380 Nouzilly, France.
3. INRA, UMR1198 Biologie du Développement et Reproduction, F-78350 Jouy-en-Josas, France;
4. Centro de Biotecnologíca Animal-SERIDA, Camino de Rioseco 1225, Gijon, 33394 Asturias, Spain;
5. Servicios Cientifico Tecnicos (Oviedo), Universidad de Oviedo, Oviedo, 33006 Asturias, Spain.

Cloning by somatic cell nuclear transfer has been established in various species but its efficiency remains low. Efforts have been made to improve cloning efficiency mainly focusing on reprogramming, using donor cells sourced from different somatic tissues and using epigenetic modifiers. Differences in reprogramming efficiency linked to the genotype of donor nuclei have been observed in mouse and bovine, but remained largely unexplained, especially in the bovine. This study is part of a set of analysis to understand the early differences between two types of bovine cloned blastocysts with different term development abilities (2.5 versus 12.5% birth rates) (Bui, Reproduction, 138(2), 289-99, 2009). Blastocysts were obtained by nuclear transfer of fibroblasts derived from ear skin of two different Holstein heifers (OV 0029 and OV5538) as described (Khan, PlosOne, 7(3), e34110, 2012). After activation, embryos were cultured in groups in SOF medium with 1% oestrus cow serum (39°C, 5%CO2, 5%O2). On Day 6 embryos were cultured individually for 24 hours in 12µl droplets of SOF (Minitube) plus Bovine Serum Albumin. On Day 7, 10µl spent culture medium (CM) and blank samples were collected and frozen until Fourier Transform Infrared Spectroscopy (FTIR) analysis. The metabolic fingerprint of spent CM of the two types of cloned blastocysts were compared. Briefly, samples (n=36 and 65, respectively for OV0029 and OV5538) were analyzed using a Golden-Gate ATR device mounted on a Varian 620-IR FTIR spectrophotometer. After correction for experimental series and subtraction of values obtained for blank samples from experimental values, the FTIR spectra were analyzed by redundancy analysis (RDA), a method which combines multiple regression with Principal Component Analysis. The model included embryo stage at day7, embryo grade at day7 (according to IETS grading; only grade 1 and 2 embryos) and embryo genotype. Significance of the effect "embryo genotype" was addressed with permutations tests. Analysis on the whole spectra did not show significant effects. However, focusing on sub-regions of the spectra pointed to a significant "embryo genotype" effect ($p=0.035$) for wavelengths between 2850 and 3030cm^{-1}. The above identified region of the spectra covers the CH_3 and CH_2 asymmetric stretching from lipids (Socrates, Infrared and Raman Characteristic Group Frequencies, Tables and Charts, Ed. Wiley, 2001 ch 23). Therefore, our results suggest differences in lipid metabolism between these two types of clones. Further analysis is in progress to confirm this hypothesis.

Supported by AGL2012-37772 and FEDER

Notes

LIPID PROFILE ANALYSIS OF BOVINE *IN VITRO* BLASTOCYSTS DERIVING FROM INSULIN TREATED OOCYTES BY DESORPTION ELECTROSPRAY IONIZATION – MASS SPECTROMETRY (DESI-MS)

LASKOWSKI D[1], PIRRO V[3], FERREIRA CR[3], SJUNNESSON Y[1], ANDERSSON G[2], HUMBLOT P[1], GUSTAFSSON H[1], COOKS GR[3], BÅGE R[1]

[1] Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden; [2] Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences, Uppsala, Sweden; [3]Department of Chemistry, Purdue University, West Lafayette, Indiana, USA

The aim of this study was to characterize the lipid profile of bovine blastocysts produced from oocytes exposed to different insulin concentrations during maturation by DESI-MS. Insulin is a key metabolic hormone and its concentration in blood and follicular fluid changes in situations of metabolic imbalance as obesity, diabetes or negative energy balance (NEB). The impact of insulin on the lipid profile of blastocysts can provide important insights on the metabolic changes induced by this hormone on early development. Blastocysts were produced from abattoir derived oocytes according to standardized IVP-protocols in our laboratory. Insulin treatment was performed during 22 h of maturation using 0 (INS0); 0.1 (INS0.1) or 10 (INS10) µg/ml bovine insulin. After maturation, all treatment groups were submitted to equal conditions during fertilization and culture. On day 8, blastocysts were separately frozen at -80 ºC in PBS with 0.1% PVA and individually transferred to glass sides in randomized order. A total of 63 blastocysts were used for DESI-MS lipid profile analysis. Lipids such as diacylglycerols (DAG), triacylglycerols (TAG) cholesteryl esters (CE), squalene and ubiquinone were detected in positive ion mode as silver adducts. Average full scan mass spectra of the three different treatment groups indicated few changes in the lipid profiles. Multivariate statistics by PCA (Principal Component Analysis) was used to comprehensively explore the chemical information of the full mass spectral dataset and visualize the grouping of samples resulting from chemical similarity. PCA showed some extent of discrimination between INS0 and INS10 whereas the discrimination between INS0 and INS0.1 was less evident. Data suggests down-regulated mitochondrial metabolism (indicated by ubiquinone abundance) in INS10 as well as few changes in TAG- and cholesterol metabolism comparing the treated groups (INS10 and INS0.1) with the control (INS0). Overall, the low extension of changes observed in the DESI-MS lipid profiles indicates minimal impact of insulin exposure during oocyte maturation on lipid content during preimplantation embryo development. The results of the lipid profile analysis shows that the lipid profile was not significantly different in the day 8 blastocyst after exposure of insulin during maturation. Possible explanations could be that the insulin exposure during the IVM period is not sufficient to promote extensive end-point metabolism changes in the lipids detected during preimplantation development, or that the early embryo strongly compensates for the impact of a metabolic stressor as insulin during oocyte maturation by a subsequent change in gene expression, leading to compensating mechanisms to obtain balance in the chemical profile and permitting a viable phenotype.

Notes

LAPAROENDOSCOPIC SINGLE-SITE SURGERY (LESS) APPROACH TO THE PORCINE OVIDUCT FOR IN VIVO EVALUATION OF PHYSICO-CHEMICAL PARAMETERS

LATORRE R[1], SÁNCHEZ HURTADO MA[2], SÁNCHEZ MARGALLO F[2], SORIA F[2], MORCILLO E[2], GUTIÉRREZ THA[1,3], VILLAMONTE-CHEVALIER A[4], PÁRRAGA E[1], LÓPEZ ALBORS O[1]

[1]Dept. Anatomy & Comparative Pathology, Campus of Excellence Mare Nostrum, Veterinary Faculty, 30100 Murcia, Spain
[2]Minimally Invasive Surgery Center Jesús Usón, 10071 Cáceres, Spain
[3]Dept. Animal Health, Faculty Veterinary Medicine, University National of Colombia.
[4]Dept. Medicine & Surgery, Campus of Excellence Mare Nostrum, Veterinary Faculty, 30100 Murcia, Spain

To define a surgical approach to the porcine oviduct capable of combining minimally invasive techniques with a time effective and accurate insertion of biosensors of physico-chemical parameters.

Gilts (n= 14) and sows (n=6), of a range weight of 85 to 280 Kg were used. Animals were anaesthetized and placed in lateral right recumbent position. Then, a 5-6 cm incision in the skin followed by layer-by-layer surgical approach to the abdominal cavity was done so as to place the single-site monoport device GelPOINT Advanced (Applied Medical®, Rancho Santa Margarita, California, USA). Under laparoscopy conditions -CO2 pneumoperitoneum (8-10 mmHg)- the left uterine horn was grasped with non-traumatic forceps. Pneumoperitoneum and the single port cap were then removed, and the reproductive organs pulled up towards the incision so as to allow a direct manipulation of the oviduct. The rapid identification of the abdominal opening allowed a rapid and effective insertion of biosensors within the lumen, thus allowing the evaluation of the oviduct microambient, i.e. pH, O2 or temperature. After settling and stabilizing the probes within the oviduct lumen, the organs were put back into the abdominal cavity and in vivo recording of physiological parameters started.

The laparoendoscopic single-site surgery (less) approach was successful in all the animals, independently of weight and reproductive maturity. Manipulation of reproductive organs was always minimal, although in 3 cases (2 gilts and 1 sow), small and slight hyperaemic areas caused by the forceps were observed in the uterine horn. During the approach no damage to the ovary, oviduct or any other abdominal organ such as intestine was produced. The average duration of the whole procedure since the beginning of the incision in the skin till the insertion of the biosensor within the oviduct was approximately 19,5 min (12-27 range), with a current duration of pneumoperitoneum conditions of 5,5 min (4-7 range). Independently of the LESS approach occasional bleeding of the mesosalpingeal vessels was observed during the manipulation required for stabilizing the probes within the oviduct.

The laparoendoscopic single-site (LESS) approach described here proved very efficient in terms of allowing a rapid, minimally invasive and hardly manipulative approach to the reproductive organs, and particularly to the oviduct lumen. This approach benefits from the advantages of both laparoendoscopy (minimal trauma) and traditional laparotomy (by-hand manipulation of organs). The LESS approach is been successfully used to evaluate pH, CO2, O2 and temperature within the oviduct.

ACKNOWLEDGEMENTS

Authors are grateful to U. Besenfelder for support and technical advice. Work funded by project AGL2012-40180-C03-03 (Ministerio de Economía y Competitividad).

Notes

ASSESSMENT OF BULL SEMEN QUALITY LOADED IN NEW SENSITEMP STRAWS USING SEMEN AND IVP TECHNOLOGIES

LE BOURHIS D[1], CAMUGLI S[2], SALVETTI P[1], SCHIBLER L[1], SCHMITT E[2]

[1] *ALLICE, Station de Phénotypage, Lieu-Dit Le Perroi 37380 Nouzilly, France*
[2] *IMV-technologies, Department R&D, ZI 1-est, 61300 l'Aigle, France*

SensiTemp, a new IMV bull straw concept, presents the advantage of color changing while the straw is thawed. The color of frozen straws is blue and straws start to become white when the temperature reaches 33°C, with a complete change of color at 37°C. The objective of this study is to assess quality after thawing of semen frozen in SensiTemp, *in vitro*, using Computer Assisted Semen Analysis (CASA), Flow Cytometry (FC) and *In Vitro* Fertilization (IVF). The ejaculates of 2 bulls, selected during preliminary experiments on high *in vitro* fertility, were harvested at CIA L'Aigle, France and split ejaculates were frozen in experimental (SensiTemp) and conventional (Control) straws. In experiment 1 after thawing semen from the two type of straws (*5 pooled straws each; 2 replicates*), motility was assessed using the IVOS CASA system (Hamilton Thorne Inc., Beverly, MA, USA) and membrane integrity was evaluated through FC with Cytosoft software (Millipore-Guava Technologies Inc., Hayward, CA). In experiment 2, IVF was used to evaluate the non toxicity of SensiTemp and control straws. Cumulus-oocyte complexes (COC; *n=1178; 4 replicates*) collected from slaughterhouse ovaries were matured in IVM medium (TCM-199 with bicarbonate, Sigma-Aldrich, Saint Quentin Fallavier, France; 10µg/ml FSH-LH, Reprobiol, Liège, Belgium and 10% FCS, Thermo Fisher, Illkirch, France) for 22 h. After fertilization, presumptive zygotes of each group (SensiTemp and control for each bull) were cultured in synthetic oviduct fluid medium (SOF, Minitube, Tiefenbach, Germany) with 1% ECS and 0.6% BSA (Sigma-Aldrich, France) up to 8 days. All cultures were conducted at 38.5C in 5%CO2, 5%O2. The cleavage and blastocysts rates were evaluated on Day 3 and 7, respectively for each group. Embryo quality was recorded on day 7 according to the IETS evaluation. Data from each bull were analyzed separately using the Chi square test ($P<0.05$). In experiment 1, neither sperm motility from bull 1 (61.2 and 60.5%) and bull 2 (66.2 and 66.5%) nor membrane integrity from bull 1 (58.6 and 52.2%) and bull 2 (61.0 and 61.9%) were different between SensiTemp and Control, respectively. Results from experiment 2 showed no difference ($P>0.05$) in cleavage rate between SensiTemp and Control for the two bulls: 92.1 and 91.7% for bull 1 and 94.2 and 94.6% for bull 2 respectively. The blastocysts rate on day 7 did not differ ($P>0.05$) among groups (47.5, 47.1 and 51.3, 50.4% for SensiTemp and Control bull 1 and bull 2, respectively) nor the quality of embryos retrieved in the different groups : 25.4, 23.3 and 30.8, 29.6% in grade 1 embryo for SensiTemp and Control bull 1 and bull 2, respectively. Those results demonstrate, *in vitro*, that the new SensiTemp straws were non toxic and did not affect the semen quality after thawing nor did the SensiTemp straws affect the ability of sperm cells to fertilize oocytes and produce 8 days old embryos.

Notes

INTERPRETATION OF EQUINE *IN VITRO* PRODUCED EMBRYO MORPHOLOGY.

LEWIS N[1*], HINRICHS K[2], SCHNAUFFER K[3], ARGO CMCG[4]

[1] *Institute of Aging and Chronic Disease, University of Liverpool, UK.*
[2] *Department of Veterinary Physiology and Pharmacology and Large Animal Clinical Science, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, USA.*
[3] *Hewitt Fertility Centre, Liverpool Women's NHS Foundation Trust, UK.*
[4] *School of Veterinary Medicine, Faculty of Health and Medical Sciences, University of Surrey, UK.*

Objective: To better understand the correlation between equine *in vitro* produced embryo morphology and nuclear status.

Materials and Methods: Oocytes were recovered from abattoir-derived ovaries, matured *in vitro,* subjected to conventional ICSI and cultured *in vitro*. Assessment of nuclear status by staining with Hoechst 33258 and using fluorescent microscopy was performed at the following times after ICSI: Group A) 20 hours to evaluate pronuclear (PN) status; Group B) Day 2, 3, 4, or 7 to determine nucleus number and correlation of morphological cleavage; and Group C) Day 7 to 11 to determine blastocyst development. Only normal nuclei were included in the number of nuclei recorded; nuclei with signs of degeneration (vacuolization, condensation or fragmentation) were disregarded. Confirmed blastocysts contained > 64 normal nuclei and showed arrangement of an outer rim of nuclei in a presumptive trophoblast layer. Two Day-9 presumptive blastocysts were transferred to the uterus of a recipient mare to evaluate viability.

Results: A total of 109 oocytes were subjected to ICSI in groups A and B. In Group A, the rate of PN formation was 43%. In Group B, 64% demonstrated apparent morphological cleavage but only 17% had ≥ 2 normal nuclei on staining and only 6.5% had a number of nuclei that matched the number of visible blastomeres and were appropriate for age. The other stained embryos that appeared cleaved morphologically possessed only degenerated nuclei or were completely anuclear. In Group C (n = 138 injected oocytes), 17 embryos were presumed to have developed to the blastocyst stage based on morphological criteria. Of these, 7 were confirmed blastocysts by staining and 8 were degenerating embryos. One embryo, presumed to be degenerated, was also revealed to be a blastocyst. Notably, as uncleaved oocytes were placed in a separate droplet at Day 4 but were kept in culture, we could evaluate changes in these oocytes over time. Several known uncleaved oocytes increased in diameter on Day 9, which on simple morphological evaluation, could have led to mistaken classification as blastocysts. Transcervical transfer of two Day-9 presumptive blastocysts to the uterus of a recipient resulted in 2 embryonic vesicles detected on Day 14 after ICSI. The smaller vesicle was manually reduced and the remaining vesicle developed normally and is > 250 days gestation. Overall in Group C, including the two transferred embryos, the rate of confirmed blastocyst development per injected oocyte was 7.2%.

Conclusions: To the best of our knowledge, this is the first report documenting the morphology and DNA staining of equine *in vitro*-produced blastocysts vs. blastocyst-like structures. Our findings reinforced the importance of removing uncleaved oocytes to limit uncertainty in later assessments of blastocyst development, and of staining embryos for DNA to definitively establish blastocyst development.

Notes

PERIOVULATORY pH WITHIN THE PORCINE OVIDUCT AND UTERUS OBTAINED BY LAPAROENDOSCOPIC SINGLE-SITE SURGERY

LÓPEZ ALBORS O[1], SÁNCHEZ HURTADO M[2], SÁNCHEZ MARGALLO F[2], SORIA F[2], GUTIÉRREZ THA[1,3], COY P[4], BORONAT T[1], GIL F[1], LATORRE R[1]

[1]*Anatomía Veterinaria, Dep. Anatomía y Anatomía Patológica Comparadas, Facultad de Veterinaria, 30100 Murcia, España*
[2]*Minimally Invasive Surgery Center Jesús Usón, Ctra. N 501 SN, 10071 Cáceres, Spain*
[3]*Dept. Animal Health, Faculty Veterinary Medicine, University National of Colombia.*
[4]*Dept. Physiology, Campus of Excellence Mare Nostrum, Veterinary Faculty, 30100 Murcia, Spain*

To determine in vivo pH values within the oviduct (ampulla and isthmus) and uterus in the porcine species with a minimally invasive approach.

8 pre-pubertal gilts (G) and 7 sows (S) were used. G were treated with intramuscular 1500 IU of eCG and 750 UI of hCG to induce ovulation. 36-44 hours after hCG injection (G) or the onset of oestrus external signs (S) pigs were anaesthetized and placed in lateral right recumbent position. A left lateral paralumbar laparoendoscopy single-site surgical approach (GelPOINT Advanced, SingleMedical®) was carried out under CO2 pneumoperitoneum (8-10 mmHg). Laparoscopy non-traumatic forceps were used to pull up the ovary towards the incision and upon visual inspection pigs were assorted into preovulatory (PreO) or postovulatory (PostO) stages. A flexible 1.6 mm diameter pH probe (MI508, Microelectrodes®, New Hampshire, USA) was sequentially inserted into the ampulla (Amp), isthmus (Isth) and uterus (Ut) for a time period of 10-12 min after signal stabilization. A reference electrode (MI401, Microelectrodes®, New Hampshire, USA) was also required for measurements. To simulate the physiological ambient registers were obtained after replacing back the organs -with the pH probe inserted- into the abdominal cavity and the surgical port closed. Anova of repeated measures was carried out with SPSS 19 (IBM®) to evaluate for a significance level of $p<0.05$.

pH values (mean ± SD) within the Amp and Isth were significantly different (7.41 ± 0.17 and 7.10 ± 0,21 respectively, $p<0.001$). pH within the uterus (7.55 ± 0.16) was within the range of the Amp ($p>0.05$) and significantly higher than in the Isth ($p<0.001$). Regarding the PreO and PostO stages pH differences were found in the oviduct ($p=0.02$) for either the Amp (7.45 ± 0.15 vs 7.34 ± 0.12) or the Isth (7.15 ± 0.24 vs 7.04 ± 0.12), but not for the Ut (7.57 ± 0.15 vs 7.52 ± 0.07). While no differences between G and S were observed (7.35 ± 0.24 vs 7.33 ± 0.25) a significant interaction between sex maturity (G vs S) and the phase of the estrous cycle (PreO vs PostO) was found ($p=0.02$).

The recorded pH values in the oviduct were lower than those of Nichol (Can. J. Physiol. Pharmacol. 75:1069, 1997), which could be related with the use of a different pH probe and surgical approach. The pronounced pH contrast between the Amp and Isth, and between the Isth and Ut is a relevant result that should be considered to better understand the microambient experienced by the porcine gametes and early embryos.

ACKNOWLEDGEMENTS

Authors are grateful to U. Besenfelder for support and technical advice. Work supported by project AGL2012-40180-C03-03 (Ministerio de Economía y Competitividad).

Notes

EFFECT OF THE REWARMING TEMPERATURE ON SURVIVAL RATE OF IVP BOVINE EMBRYOS VITRIFIED IN TRIACETATE CELLULOSE HOLLOW FIBER INCORPORATED INTO A NEW VITRIFICATION DEVICE.

MALENKO GP, KORNIENKO EV, NESTEROV II,
POPOV DV, KOSOVSKY GYU

Centre of experimental embryology and reproductive biotechnology
ul. Kostyakova, 12, building 4, 127422 Moscow, Russia

Hollow fiber vitrification (HFV) was introduced by Matsunari et al. (J. Reprod. Dev. 58: 599–608, 2012) as a method for vitrifying groups of mammal embryos and was shown to be effective for cryopreservation of such cryosensitive objects as in vivo and in vitro produced porcine morulae. Due to the standard and simple vitrification procedure HFV method may be perspective for cryopreservation of bovine oocytes and IVP embryos in combination with embryo transfer methods. The objective of this work was to introduce a vitrification device that will allow effective storage of the triacetate cellulose hollow fibers (HF) that become fragile in liquid nitrogen and to show effectiveness of the HFV method for IVP bovine embryos rewarmed at room temperature (22–24 °C). IVP bovine embryos were cultured in modified SOF medium. Morphologically normal blastocysts and expanded blastocysts were collected at day 7 after IVF and used for vitrification. A vitrification device was constructed by connecting a piece of HF to a heat-pulled tip of a glass capillary. A protective sheath was fitted directly on the capillary. Embryos in groups of 5–10 were loaded into the vitrification devices in the equilibration solution containing 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO). Then HFs were exposed to vitrification solution containing 15% EG, 15% DMSO and 0.5M sucrose for 1 minute and immersed into liquid nitrogen. HFs were rewarmed in the solution with 1M sucrose at either 39 °C or 22–24 °C and were transferred into dilution and washing solutions stepwise. Rewarmed blastocysts were cultured for 72 hours in modified SOF medium. Reexpansion and hatching rates of embryos were assessed at 24 and 72 hours post rewarming, respectively. Results are presented as mean ± SD. Data was analyzed using Student's t-test. Significance was set at $p < 0.05$. After storage in liquid nitrogen for 2–12 months and transportation to the farm all HFs within vitrification devices remained intact. Volume of vitrification solution with loaded blastocysts within hollow fibers ranged between 0.024 and 0.030 mkl. Reexpansion rates of the blastocysts after rewarming at 22–24 °C and 39 °C were 82.65 ± 11.62% (159/191; 14 repeats) and 88.73 ± 5.99% (64/73; 7 repeats), respectively. Hatching rates were 53.77 ± 22.37% after rewarming at 22–24 °C and 64.74 ± 12.74 % at 39 °C. There were no statistically significant differences between two experimental groups. The introduced vitrification device is relatively simple in construction and protects HFs with loaded embryos from mechanical damage. Due to the very small volume of the samples within HFs, rewarming at room temperature did not significantly affect survival rate of the embryos. Rewarming at 22–24 °C can be advantageous for practical uses and may help to avoid temperature related effects of high concentrations of cryoprotectants, such as DMSO.

Notes

OMEGA-3 FATTY ACIDS ENHANCE DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES UNDER METABOLIC STRESS CONDITIONS *IN VITRO*

MAREI WFA[1,2], DE BIE J[1], DESMET KLJ[1], ANDRIES S[1], BOLS PEJ[1], AND LEROY JLMR[1]

[1]Gamete Research Centre, Veterinary Physiology and Biochemistry, Department of Veterinary Sciences, University of Antwerp, 2610 Wilrijk, Belgium. [2]Department of Theriogenology, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt.

Metabolic stress conditions such as negative energy balance in dairy cows are associated with fat mobilization and elevated saturated (stearic; SA, palmetic; PA) and monounsaturated (oleic; OA) fatty acids (FAs) in serum and follicular fluid. We have shown that these FAs have direct detrimental effects on oocyte quality (Van Hoeck et al., ARS, 149:19-29, 2014). In contrast, we demonstrated that polyunsaturated α-linolenic acid (*n-3* 18:3; ALA) can enhance oocyte competence (Marei et al., BOR, 81:1064-1072, 2009). Here, we examined the effects of ALA supplementation (at physiological follicular fluid concentration; 50 µM) during *in vitro* oocyte maturation on subsequent embryo development in the presence of high follicular fluid concentrations of SA, PA and OA (HNEFA, 425µM). Cumulus cell expansion was scored at the end of oocyte maturation (0-3: 0; not expanded, 3; fully expanded). The proportions of cleaved and fragmented embryos were recorded on day 2 post-fertilization. Blastocyst rates were recorded on day 7 and 8. Day 8 blastocysts were categorized as Normal (not expanded), Expanded, or Hatched, and were fixed and immunostained with anti-cleaved-caspase-3 antibody and Hoechst. Total cell counts and apoptotic cell indices were calculated. Data were obtained from 5 independent repeats using 1529 oocytes derived from slaughter house material. A total of 179 blastocysts were stained. Categorical data were analyzed by binary logistic regression using SPSS, and numerical data were analyzed using ANOVA. Pairwise comparisons were performed using Bonferroni correction. *P* values <0.05 were considered significant. Compared with FA-free solvent controls, supplementation with HNEFA resulted in: inhibition of cumulus cell expansion (score: 1.7±0.2 vs. 2.8±0.04, *P*<0.05); higher fragmentation rates (16.8% vs. 9.5%, *P*<0.05); and lower blastocyst rates on day 7 (*P*<0.05), either expressed as a proportion from the total number of fertilized oocytes (15.6% vs. 22.8%) or from the total number of cleaved embryos (20.4% vs. 30.6%). Hatched and expanded blastocysts produced from HNEFA-exposed oocytes had higher apoptotic cell indices. In contrast, these negative effects were alleviated by ALA supplementation. In the HNEFA+ALA group, cumulus expansion score (2.4±0.16), fragmentation (6.9%), blastocyst rate on day 7 (21.4% from total fertilized oocytes and 28.7% from cleaved embryos), and apoptotic cell index were similar to the controls. In addition, HNEFA+ALA group had significantly higher total cell numbers in expanded and normal blastocysts compared with those from HNEFA group. In conclusion, ALA supplementation enhanced oocyte developmental capacity during maturation under metabolic stress conditions. The underlying mechanisms of action are currently under investigation. These results may have clinical implications to improve fertility through dietary interventions in animals and humans suffering from metabolic disorders associated with lipolysis.

Notes

TRANSFER OF CATTLE EMBRYOS PRODUCED WITH SEX-SORTED SEMEN RESULTS IN IMPAIRED PREGNANCY RATE

MIKKOLA M AND TAPONEN J

University of Helsinki, Department of Production Animal Medicine, FIN-04920 Saarentaus, Finland

This study compared the pregnancy rates after transfer of day-7 *in vivo* embryos produced either with conventional or sex-sorted semen from numerous bulls commercially available and extensively used. In addition, mortality of calves born from sexed embryos and conventionally produced embryos was studied. The data consisted of 12,438 embryo transfers, of which 10,697 embryos were produced using conventional semen (CONV embryos) and 1,741 using sex-sorted semen from 97 bulls (SEX embryos), predominantly of Ayrshire and Holstein breeds. Quality codes of embryos were similar in both groups. Of the CONV embryos, 27.4% were transferred fresh, the proportion being 55.7% for SEX embryos. Recipient properties (breed, parity, number of previous breeding attempts and interval from calving to transfer) were similar for both embryo types, heifers representing 57.8% of recipients in the CONV group and 54.8% in the SEX group. Recipients that were not inseminated or did not have a new embryo transferred after the initial one, and had a registered calving in fewer than 290 days after the transfer, were considered pregnant. Data were analyzed with IBM SPSS Statistics, Version 21.The effects of sexing protocol, embryo type (fresh vs. frozen), developmental stage, quality and breed of embryo as well as parity (heifer vs. cow) and breed of a recipient on conception were analyzed using binary logistic regression. Pregnancy rate for recipients receiving CONV embryos was 44.1% and for those receiving SEX embryos 38.8%. The odds ratio for pregnancy in recipients receiving CONV embryos was 1.34 compared with SEX embryos ($P<0.001$). Other factors affecting the pregnancy rate were embryo quality ($P<0.001$), being highest for grade 1 (CONV 45.2%, SEX 42.8%) and lowest for grade 3 (CONV 29.2%, SEX 22.2%) embryos, and developmental stage of an embryo ($P=0.038$). Transfer of earlier developmental stages, i.e. compact morulas, resulted in lower pregnancy rates than transfer of later stages. Also recipient parity affected pregnancy rate ($P<0.001$), the odds ratio for pregnancy for heifers was 1.18 compared with that for cows. There was no effect of the breed on pregnancy rate, neither of an embryo nor of the recipient. The proportion of female calves was 49.6% and 92.3% in CONV and SEX groups, respectively. Calf mortality was 9.0% and 8.9% in CONV and SEX groups, respectively. Mortality of female calves was similar in CONV and SEX groups, 6.6% and 7.7%, respectively. For male calves, mortality was 9.2% in the CONV group but significantly higher, 16.0% ($P<0.05$), in the SEX group. This study showed that transfer of embryos produced with sex-sorted semen decreased the pregnancy rate by about 12% compared with embryos produced using conventional semen. Mortality of male calves born from SEX embryos was higher than for those born from CONV embryos.

Notes

SUPPLEMENTATION OF CULTURE MEDIUM WITH FOETAL CALF SERUM OR INSULIN – TRANSFERRIN – SELENIUM AFFECTS THE INTEGRITY OF EQUINE OVIDUCT EXPLANTS

NELIS H[1], VANDENBERGHE L[1], D'HERDE K[2], LEEMANS B[1], VAN SOOM A[1], SMITS K[1]

[1]Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine,
[2]Department of Basic Medical Sciences, Faculty of Medicine and Health Sciences,
Ghent University, Belgium

Equine oviduct explants provide an excellent tool to unravel embryo-maternal interactions. They can be cultured *in vitro* for several days in DMEM F12 and serum whilst remaining functionally intact and highly differentiated. However, dark cell degeneration (DCD) has been observed inside the explants (Nelis et al. 2014 RFD 26 954-966). Since serum has been reported to negatively affect cell and embryo culture (Fernandez-Gonzalez 2004 PNAS 101 5880-5885), we aimed to assess the effect of serum and the serum replacer insulin-transferrin-selenium on the prevalence of DCD, ciliary activity, membrane integrity and ultrastructure of equine oviduct explants. Oviducts ipsilateral to the ovulation side were gathered from mares in the early postovulatory stage. Oviduct explants were harvested by scraping and cultured for 6d in 50 µl drops under oil in 5% CO_2 in air in DMEM/F12 (control; Invitrogen, Merelbeke, Belgium), in DMEM/F12 with 10% foetal calf serum (FCS; Greiner Bio-one, Wemmel, Belgium) or in DMEM/F12 supplemented with 5 µg/ml insulin, 5 µg/ml transferring and 5 ng/ml selenium selenite (ITS; Sigma, Schnelldorf, Germany). Three replicates of 60 droplets per condition were performed. With an inverted microscope, every 24h, the percentage of explants with dark zones and the percentage of explants showing ciliary activity were determined. In addition, the percentage of membrane-damaged cells was determined using Trypan blue (Sigma-Aldrich, Diegem, Belgium). At d0, 3 and 6, ultrastructure was assessed by TEM. To compare DCD prevalence, ciliary activity and membrane integrity, binary logistic regression was implemented (SPSS 21 for windows; SPSS IBM, Brussels, Belgium). During the first two days, the prevalence of DCD was significantly lower in the FCS group (36%), when compared to ITS (68%, $P<0.0005$) and the control (67%, $P<0.0005$), indicating an initial protective effect of FCS. From d3 on, significantly more DCD was observed in the presence of ITS and FCS (87% resp. 92%, $P<0.0005$) compared to the control (81%). FCS and to a lesser extent ITS seem to sustain the percentage of explants showing ciliary activity (97%, $P<0.0005$ and 94%, $P<0.0005$) compared with the control (87%). In all groups, as shown by Trypan blue, the explants consisted of >98% membrane intact cells ($P=0.9$). No qualitative differences in the development of DCD was detected by TEM. The outer surface of explants in all groups was highly differentiated and intact. In conclusion, without affecting morphology, components of FCS, which may be depleted after 2 days of culture, turn out to partly protect while ITS enhances the development of DCD. Furthermore, FCS and ITS seem to preserve ciliary activity. Since the toxic margin of insulin and transferrin, but not of selenium, is far above the applied levels in our culture system, amongst others, selenium may play a role in the development of DCD. Further research is needed to unravel the exact cause in the development in DCD in oviduct explants.

Notes

REPRODUCTIVE RESPONSE OF PROLIFIC BREED AND ITS CROSSES IN INTRAUTERINE INSEMINATION PROGRAM

OCAK S[1], EMSEN E[2], DEMIREZER H[3]

Nigde University, Faculty of Agricultural Sciences & Technologies , Department of Animal Production and Technologies 51000, Nigde-TURKEY[1]
Ataturk University, Department of Animal Science, 25240, Erzurum, Turkey[2]
Pan Small Ruminant Reproductive Technology Service, Gaziantep, Turkey[3]

The success of laparoscopic AI (LAI) depends on events and factors that interrelate in a complex way. Once the selection and preparation of the ewe have been accomplished, one of the most important steps in the program is the successful synchronization of the ewe to deliver good quality ova to the site of fertilization at a specific time. However, a considerable variation in success rate exists when using this technology whereby conception rates range from 10 to 85%. A major and highly consistent finding from the studies reported showed to be a major difference among the different ewe breeds with respect to pregnancy rate after LAI regardless of the source of that semen. These results confirm the importance of the breed and therefore possible reasons for this effect needs to be elucidated.

In this study, we aimed to investigate the reproductive performance of yearling prolific Romanov breed and its half and quarter crosses with Turkish native breeds in a LAI program conducted during the breeding season. In addition to breed effect we also examined vaginal electrical resistance (VER) values which was reported by the previous researches (Bartlewski et al., 1999; Rezac, 2008) that ewes with lower VER, which means higher estrogen levels. A total number of 30 ewes, equally distributed for each genotype (Romanov: 10, F1 Romanov crosses (F1): 10 and quarter Romanov (Q breed): 10) were included in the experiments. All animals were treated with a vaginal sponge containing 30 mg fluorgestone acetate (FGA; Chrono-gest, Intervet, MSD, Turkey, for 12 d. Immediately following sponge removal, ewes received an injection of 500 IU, i.m. eCG. An experienced laparoscopic AI operator performed the inseminations using fresh diluted semen (100 x 10^6 motile spermatozoa/0,4ml) at 52-55h after sponge removal. The animals were screened for estrus beginning at 24 h after sponge removal and continuing up to 57 h. Animals that did not show any mating marks by 57 h were not inseminated. Electric resistances of vaginal secretions (VER) were measured with a vaginal probe (DRAMINSKI, Poland) that was gently inserted into the vagina prior to LAI. *Conception rate* was determined by *ultrasound* 40 days after AI. The Romanov breed showed the highest estrus response (83%; $P<0.05$)) and, the F1 (40%) and Quarter Romanov crosses (50%) were found similar estrus rates. Conception rates (CR) were 80%, 75% and 57% for Romanov, F1 and Quarter Romanov crosses, respectively ($P>0.05$). Correlation coefficient between vaginal mucous impedance and conception rates was computed as 0.025 and showed to be not significantly correlated with CR. However, compared to F1 and Q ewes Romanov ewes showed more tight VER values which is possibly related to the variation in the moment of estrus.

Notes

ADDITION OF OMEGA-3 DHA DURING IN VITRO MATURATION AFFECTED EMBRYO DEVELOPMENT

OSEIKRIA M[1234], ELIS S[1234#], UZBEKOVA S[1234]

[1] INRA, UMR85 Physiologie de la Reproduction et des Comportements, F-37380 Nouzilly, France; [2] CNRS, UMR 7247 Physiologie de la Reproduction et des Comportements, F-37380 Nouzilly, France; [3] Université François Rabelais de Tours, UMR Physiologie de la Reproduction et des Comportements, F-37380 Nouzilly, France; [4] IFCE, UMR Physiologie de la Reproduction et des Comportements, F-37380 Nouzilly, France

[#] Corresponding author

Several studies have suggested a positive effect of n-3 poly-unsaturated fatty acids (PUFA) on bovine reproduction. Indeed, n-3 PUFA reduced prostaglandin secretion in uterine environment, thus providing more favorable conditions for embryo development. Other studies suggested a direct effect of n-3 PUFA on the oocyte that could enhance fertility. In the present study, we aimed at investigating *in vitro* the effect of docosahexaenoic acid (DHA, C22:6 n-3, Sigma, Saint-Quentin Fallavier, France) on bovine oocyte maturation and developmental competence.

Oocyte cumulus complexes (OCC) were collected from slaughtered cows. In first experiment, in vitro maturation (IVM) with DHA 1, 10 and 100 µM was performed (n=3 replicates, 50-60 OCC per condition). After IVM, oocyte viability was assessed using Live/DEAD staining and then meiotic stages were determined by using Hoechst staining after oocyte fixation. Neither difference in viability nor in maturation rate was observed after IVM between control and treated oocytes whatever the DHA concentration. 83.1% of mature oocytes in control IVM and 78.9%; 84.0%; and 84.0% in presence of DHA at 1, 10, 100 µM, respectively, were observed.

In second experiment (n=5 replicates, 50-60 OCC per condition), after 26h IVM with or without DHA 1, 10 and 100 µM, oocytes were subjected to parthenogenetic activation (ionomycin 5 µM, 5 min and 6DMAP 2 mM, 4h). Oocytes were then *in vitro* developed in modified synthetic oviduct fluid supplemented with 1% estrus cow serum for 7 days. Cleavage rate and a number of blastomers were assessed in resulting embryos at day 2 post activation. Cleavage rate significantly increased after IVM with DHA 1µM (84.3%) but significantly decreased with 100µM DHA (66.2%) as compared to control (76.0%) embryos (Chi-square test p=0.02). Moreover, the percentage of embryos that progressed further than 4 cells at day 2 was significantly higher (p=0.02) in the presence of 1 and 10 µM DHA (40.8% and 40.4%, respectively) than in control (31.2%) and with DHA 100 µM (22.2%). At day 7, embryos from DHA 1 µM-treated oocytes encountered more cells than those from control and other DHA groups (10 and 100 µM).

Altogether these data suggest that a low dose of DHA (1µM) during IVM might improve oocyte developmental competence through possible effect on cytoplasm but not nuclear maturation. Also, we confirmed that a high dose of DHA (100µM) is deleterious for oocyte developmental potential.

Notes

THE APPLICATION OF BOVINE IN VITRO EMBRYO PRODUCTION TECHNOLOGY TO DEVELOP AN IN VITRO TEST BATTERY FOR THE SCREENING OF ESTROGENIC COMPOUNDS

QUADALTI C[1], CROTTI G[1], TURINI P[1], GALLI C[1,2,3] , LAZZARI G[1,3]

[1,2]Avantea, Laboratory of Reproductive Technologies, Cremona, Italy; [2]Dept. of Veterinary Medical Sciences, University of Bologna, Italy; [3]Fondazione Avantea, Cremona.

The objective of this study was to develop a battery of tests able to identify the two main mechanisms of action of estrogenic compounds: the receptor-mediated mechanism, naturally occurring in hormone-responsive tissues carrying specific receptors (Erα and Erβ) and the direct mechanism through which estrogen and estrogenic compounds bind spindle components and cause a depolymerizing effect on microtubules therefore inhibiting the correct formation of the meiotic spindle.

For this purpose two well-known compounds, diethylstilbestrol (DES) and 17β-estradiol (EST), were tested on four different in vitro assays: bovine oocyte in vitro maturation (bIVM) assay, bovine embryo in vitro culture (bIVC) assay and MCF-7 (human breast adenocarcinoma) and BALB/3T3 cell lines (mouse fibroblasts) proliferation and cytotoxicity assays, respectively.

For the bIVM assay immature oocytes were aspirated from abattoir ovaries, washed and transferred to oocyte maturation medium, which was supplemented with the test compounds. At the end of maturation the oocytes were denuded, fixed with acetic acid/ethanol (1:3) for 18-24h and stained with lacmoid solution. The completion of meiosis up to the metaphase II stage was considered as the toxicological endpoint. For the bIVC assay, bovine embryos were obtained by IVM and IVF, followed by in vitro culture. At day 7 after IVF, embryos were selected at the early blastocyst stage and exposed to test substances from this stage onwards. The toxicological endpoint was the development of embryos up to the expanded hatched blastocyst stage at day 11. For the other two assays MCF-7 cells were cultured in MEM without glutamine and phenol red supplemented with 10% Foetal Bovine Serum (FBS) charcoal stripped, 4 mM α-glutamine and 1 mM pyruvate and BALB/3T3 cells were cultured in DMEM:TCM199 (1:1) supplemented with 10% FBS charcoal stripped. Both cell lines were exposed to test compounds at increasing concentrations. The AlamarBlue® test was performed and data were analysed with a TECAN plate reader (Infinite F200 Pro).

Results indicate that only the MCF-7 proliferation assay can detect the receptor-mediated mechanism in the picomolar range of test compounds whereas a cytotoxic effect appeared in both cell lines in the micromolar range of test compounds. Moreover, the bIVM assay can detect the direct mechanism inducing spindle depolymerisation and abnormal nuclear configuration in the range of 1-20 microM. Finally the bIVC assay does not seem to be informative because only a cytotoxic effect is evident at the highest concentration tested, as for the BALB/3T3 assay.

In conclusion this battery of four tests can allow to discriminate between the two major mechanisms of action of estrogenic and estrogen-like compounds, the receptor-mediated pathway and the direct one.

Notes

USING PROGESTERONE ASSAY BEFORE SUPEROVULATORY TREATMENT IN BOVINE FARMS

QUINTON H*[1], MOREL A[1], EDE P[1]; CHARREAUX F[1]

[1]*Evolution, Rennes, France*

A French ET team had systematically realized progesterone assay for 15 years to help in the decision to start or not the superovulatory treatment for an embryo flushing in farm.

Since 2010, 2210 progesterone assays have been done from 1561 females (1 425 heifers; 1119 Holstein, 30 other dairy breeds, 266 dual-purpose breeds and 146 beef breeds) in 665 farms. The blood samples for progesterone assay were performed by the farmer from 4 to 13 days after a reference heat and sent to a hormonology lab (LNCR, Maisons-Alfort). The superovulation protocol (8 FSH injections) was : D-16 to D-8 = reference heat; D-10 to D-3 = blood sample for progesterone assay ; D-2 = input of an implant of norgestomet (Crestar®); D0, 8:00 = first FSH (Stimufol®) injection; D4 = 2 AI depending on heat observation ; D11 = embryo flushing. The ET team received the quantitative result from the lab 2 to 6 days after the blood sample. A qualitative result was determined: negative for quantitative result inferior to 1.2 ng/mL, positive for results superior to 1.8 ng/mL and dubious between 1.2 to 1.8 ng/mL.

The interval between the reference heat and the first FSH (from 8 to 16 days) didn't influence the number of collected embryos. No clear effect of parity (0, 1, 2, 3, 4 or 5 and more) or kind of breed could be shown, due to the great predominance of Holstein heifers (1 125).

Among the 2210 progesterone assays, 1961 (89 %) gave a positive result, 114 (5%) a dubious result and 135 (6%) a negative one. Among the planned embryo collections, 108 (5%) were not performed, 42 due to a negative progesterone result, 66 for other different reasons. The mean progesterone level increased significantly from 4 to 6 days after heat, but this increase was no more significant after 7 days. Actually, 70 % of the negative results were all the same followed by an embryo flushing (result received too late, recipient already prepared…). Of course negative progesterone levels, led to significantly ($p<0.0001$) less total and viable embryos collected than for positive ones : respectively 10.3 ± 8.6 and 5.7 ± 5.3 for positive versus 6.1 ± 5.2 and 3.0 ± 3.6 for negative. But, for the positive results, no effect of the level of progesteronemia on the number of collected embryos has been observed.

Because of the very low ratio of embryo flushing finally cancelled due to negative result of the progesteronemia (2%), it has been decided to stop the use of systematic progesterone assay.

Notes

BORN SIMMENTAL CALVES AFTER THE TRANSFER OF GENETIC EVALUATED DAY 7 BOVINE EMBRYOS

REICHENBACH M[1], JUNG S[2], WOLF E[3], PIMENTEL E[4], EMMERLING R[4], GÖTZ KU[4], DUDA J[5], GSCHOEDERER C[1], SCHERZER J[1],GRUPP T[1], FRIES R[2], REICHENBACH HD[4]

[1]Bayern-Genetik GmbH, Grub, Germany; [2]Chair of Animal Breeding, Technische Universität München, Freising, Germany; [3]Chair for Molecular Animal Breeding and Biotechnology, Ludwig-Maximilians-University, München, Germany; [4]Bavarian State Research Center for Agriculture, Institute for Animal Breeding, Grub, Germany; [5]LKV Bayern e.V., Germany.

Genomic selection can theoretically take place in any stage of an animal's life. The use of ET can increase the intensity of selection, but at the cost of keeping many recipients. This can be avoided by transferring only embryos with desired gender and best breeding values. Improvement of embryo micromanipulation and DNA amplification techniques allows for the direct genetic analysis of bovine embryos prior to implantation. The aim of our study was to set up and optimize a whole embryo production and evaluation line in Simmental cattle to determine gender, polled status, hereditary defects and reliable breeding values on blastomeres at the morula and blastocyst stages. For embryo recovery (n=45) German Simmental animals (n=17) were superovulated using a standard protocol. Embryos were biopsied immediately after recovery by a single operator under a mobile stereo microscope (Olympus) at 50x magnification with a single use special steel blade mounted on a blade holder (Bausch & Lomb, Germany) attached to a micromanipulator (Eppendorf, Germany). Two biopsy methods were compared, first embryos were splitted and one third of a half cut off (G1, n=161) or by cutting of the trophoblast (G2, n=146). Biopsied cells, approximately 10-15, were immediately used for whole genome amplification (Repli-g mini Kit, Qiagen) followed by PCR analysis of gender and polledness. Hereditary defects were analyzed using a 5'-exonuclease assay. Embryos were transferred to recipients after in vitro culture in SOF supplemented with 5% ECS, 40 µl/ml BME and 10 µl/ml MEM in four-well dishes, under mineral oil, at 39°C and gas mixture (5% CO2, 5% O2, 90% N2) for 24 h. DNA of the first 14 born calves was extracted from blood samples. These calves together with the corresponding embryos were genotyped with the Illumina Bovine 54k BeadChip. Call rates were recorded, correlations between embryo and calf genotypes calculated and breeding values estimated. The biopsy technique G1 resulted in the highest number of good quality transferable embryos G1 (1.37) vs. G2 (0.97) ($p<0.05$) in relation to the number of original embryos. However, better pregnancy rates were obtained by transferring 2 demi-embryos to one recipient (1 demi-embryo=28.6%; 2 demi-embryos=76.2%). Biopsy technique G2 resulted in 55.0% pregnancies. No discrepancies could be detected between gender, polled and hereditary defect status of born calves and corresponding embryos. The average call rate for the genotyped embryos was 0.922, ranging from 0.841 to 0.980. The call rate of the corresponding calves ranged from 0.998 to 0.999. The average concurrency of the obtained genotypes of embryos and calves was 98.7%, with an average correlation of 0.991. Gender, polledness and genotypes obtained from preimplanted embryos were consistent with genotypes obtained of the born calves. Therefore, our first results provide promising prospects for the optimized production line.

Funded by the Bavarian Research Foundation (AZ-1031-12).

Notes

EMBRYO COLLECTION IN CLONE CATTLE OFFSPRING

RICHARD C[1], GELIN V[2], NEVEUX A[2], HUE I[1], HEYMAN Y [1], CHAVATTE-PALMER P[1]

[1]INRA, UMR1198 Biologie du Développement et Reproduction, F-78350 Jouy-en-Josas, France;
[2]INRA, UCEA Bressonvilliers, 91630 Leudeville, France.

Our laboratory has been working on bovine clones for many years. These clones were studied and several cloned females were bred to obtain clone offsprings. All pregnancies were normal and calves developed as healthy individuals. The females were used for embryo collection after superovulation. The objective of this study was to compare the embryo recovery results between clone offspring and control animals. Altogether, 28 cows were used for this study (18 clone offspring and 10 controls). All the animals were born and raised in the same experimental farm, in the same time period and in the same rearing conditions. 90 flushes were performed to collect D9 to D21 embryos for research protocols. For early embryos on D9, a classical 3 way collection equipment (IMV, France) was used. To collect the late embryos D12-D21, the same equipment was modified so that larger embryos could be collected through the remaining larger hole (2 way collection) (Richard et al. 2015, Theriogenology 83,1101-9). All females were submitted to ovum pick-up to remove the dominant follicle and were subsequently superovulated with FSH (Stimufol®, Reprobiol, Belgium). Luteolysis was induced 48 hours prior to AI. Two AI were performed with frozen semen, 48 and 56 hours after PGF2 injection (Estrumate®, MSD Santé Animale, France). Before embryo collection, cows were treated with an epidural injection of 3-4 ml (Xylovet®, CEVA Santé Animale SA, France).The presence of Copora Lutea (CL) was checked and they were counted by rectal palpation. For all collections, the cervix was prepared with the initial introduction of a dilator. Then the catheter was introduced in one horn and the cuff was inflated as low as possible. For the collection of late stage embryos, 30 ml (Euroflush, IMV, France) was injected slowly twice to suspend the embryos prior to flushing the horn with 500 ml, and the same operation was performed on the second horn. Data were analyzed by unpaired t-test using Prim® software. There was no significant difference in the number of embryos collected per flush in clone offspring and controls (349 embryos collected, 5.05 ± 4.8 per flush vs 90 embryos collected, 4.28 ± 3.92 per flush, respectively). The number of CL was also not significantly different between groups (11.49 ± 7.32 and 8.43 ± 4.26 per flush, respectively). For late collections in all animals, the FSH dose (Stimufol®) was reduced to limit the number of embryos and preserve development (Richard et al. 2015). Retrospectively there was no significant difference for the necessary dose for superovulation (0.57 ± 0.08 for clone offspring and 0.54 ± 0.07 for controls). These data indicate that offspring of clones raised since birth in the same conditions as control heifers have the same ability to give embryos after superovulation treatment indicating equivalence of reproductive function.

Notes

COMPREHENSIVE PROTEOMIC ANALYSIS OF *GALLUS GALLUS* UTERINE FLUID

RIOU C[1, 2], BRIONNE A[3], GARGAROS A[4], CORDEIRO LA[1], HARICHAUX G[4], ALMINANA C[1], LABAS V[4], GAUTRON J[3], GERARD N[1]

[1] INRA, UMR INRA85, UMR CNRS 7247, université de Tours, IFCE, Physiologie de la Reproduction et des Comportements, Interactions Cellulaires et Fertilité, 37380 Nouzilly, France
[2] ALLICE, Station de Phénotypage, Lieu-Dit Le Perroi, 37380 Nouzilly, France
[3] INRA, UR83 Recherches Avicoles, Fonction et Régulation des Protéines de l'œuf, 37380 Nouzilly, France
[4] INRA, UMR INRA85, UMR CNRS 7247, université de Tours, IFCE, Physiologie de la Reproduction et des Comportements, Plate-forme d'Analyse Intégrative des Biomolécules, Laboratoire de Spectrométrie de Masse, 37380 Nouzilly, France

Domestic hens are able to keep spermatozoa in their genital tract for long periods, and can so produce fertilized eggs for up to 3 weeks after one insemination. An extensive description of the avian uterine fluid proteome will help to provide the basis for a better understanding of a number of diseases and processes, including sperm survival but also female infertility and cell storage. Uterine fluid was collected (n=10) into a plastic tube placed at the entrance of the everted vagina 10h after oviposition. Bottom up proteomic approach using SDS-PAGE and nano LC-MS/MS (ultimate 3000 RSLC system coupled to LTQ Velos Orbitrap mass spectrometer) was performed with a high-low resolution MS strategy. Data were matched against NCBInr database using Mascot 2.3 and identifications were validated by the peptide and protein Prophet algorithm using Scaffold 4.0 software. Bioinformatics treatments of data set was carried out to refine annotation of proteins using NCBInr database, and to describe uterine fluid proteins using SecretomeP 2.0 and SignalP 4.1 tools, InterproScan software, and, Exocarta, KEGG and UniprotKB databases. Among a total of 922 proteins that were identified, 836 (91%) were identified in *Gallus gallus* databases, whereas 86 (9%) were identified in others species, indicating unknown chicken isoforms. Deepens analysis of cellular component revealed three categories of proteins. The secreted proteins (165) known to be secreted with a peptide signal or by an unconventional pathway, the exosomal proteins (644) which match against exosomal databases (Exocarta, UniprotKB, KEGG) and the last category refers to proteins which are not annotated as exosomal or secreted (113). Secreted proteins are composed of protease inhibitors (11), cytoskeletal and extracellular matrix proteins (22), enzymes (metabolic, proteases etc.) (49) and others proteins implied in calcification of eggshell (OC-17, OC-116). Exosomal proteins mainly consist in enzymes (metabolic, oxidoreductase) (225), chaperon proteins (HSPA8, HSP90AA1,...) (26) and proteins implied in MVB biogenesis (Alix, TSG101, Clathrin,…) (25). We have isolated exosomes and confirmed the presence of exosomal markers (CD63, HSPA8) by western blot in the avian uterine fluid. The presence of exosomal proteins in avian uterine fluid may represent a novel and exiting mechanism of cell-cell interactions, that may explain at least in part, the long term sperm survival. We believe that the thorough catalogue of proteins presented here can serve as a valuable reference for the study of sperm interaction with the female genital tract. Moreover, it could be an interesting tool for biomarkers discovery involved in fertility.

Notes

PERICONCEPTIONAL BODY CONDITION INDUCES PLACENTAL ADAPTATIONS BUT DOES NOT AFFECT FOAL GROWTH AND METABOLISM IN HORSES

ROBLES M[1], WIMEL L[2], DUBOIS C[2], PEUGNET P[1], TARRADE A[1], CHAVATTE-PALMER P[1]

[1] *INRA, UMR1198 Biologie du Développement et Reproduction, Jouy en Josas, France*
[2] *IFCE, Station Expérimentale des Haras Nationaux, Domaine de la Valade, Chamberet, France*

Objectives: It has been shown in several species that the periconceptional environment can affect offspring long-term phenotype. This study aims to investigate the effects of periconceptional body condition on feto-placental biometry, post-natal foal growth and glucose metabolism.

Materials and methods: 32 saddlebred mares of similar size were allocated to one of two groups depending on their body condition score (BCS, 1-5 French scale) at the time of artificial insemination (AI). Group High (H, n=18) had a median BCS of 3.9 (range: 3–4.25) whereas group Low (L, n=14) had a significantly lower BCS (median: 2.5, range: 2–3.75, p=0.01). Both groups were kept in pasture until the 7th month of gestation when they were housed indoors and fed forage and concentrate (barley). Food intake was not different between groups. Mares were weighed every 2 weeks and their BCS was monitored monthly. Placentas and foals were weighed and measured at birth. Foals were measured and their fasting glucose assessed regularly until 12 months of age. A frequently sampled intravenous glucose tolerance test (FSIGT) was performed at 3 days and 4 months of age. Results were analyzed using a Mann-Whitney test.

Results: H mares maintained a significantly higher BCS (median ≥3.75) than L mares from AI until foaling (median at foaling: 3.75, p<0.0001). L mares reached a peak BCS of 3.75 at the 7th and 8th month and thereafter lost BCS until foaling (median BCS at foaling: 2.75). Mares' body weight was not different between groups at any time. Gestation length did not differ between groups. H placentas tended to be 15% lighter with a 10% reduced surface compared to L placentas (p=0.071). Foals' weight and measurements at birth were not different but the placental efficiency (foal/placental weight) tended to be 12% higher in H mares (p=0.078). There was no difference in foals' growth until 12 months. H foals' fasting glucose tended to be higher at 3 days (p=0.063) but there was no difference in the glucose response to the FSIGT. Plasma insulin concentrations are pending.

Conclusion: H mares tended to have a lighter placenta and with a reduced surface area that was more efficient than L mares. Their foals tended to have greater fasting plasma glucose than L foals at 3 days. The fact that the BCS of H and L mares throughout gestation matched their BCS at AI highlight the importance of periconceptional BCS. This study follows a previous one showing that feeding mares in the 2nd part of gestation with two different energy sources does not affect feto-placental biometry and foal development until the age of 6 months (Peugnet et al 2015). Nevertheless, periconceptional BCS appears to induce placental adaptations that are currently being characterized.

Reference: Peugnet et al. (2015) Plos One 10, e0122596.

Acknowledgements: This study was funded by the Institut du Cheval et de l'Equitation and by the Fonds Eperon (FOETALIM grant).

Notes

EFFECT OF LOW OXYGEN TENSION ON MITOCHONDRIAL ACTIVITY IN CULTURED PIG EMBRYOS

ROMEK M[1], GAJDA B[2], SMORAG Z[2]

[1]*Department of Cell Biology and Imaging, Institute of Zoology, Jagiellonian University, Kraków, Poland*
[2]*Department of Biotechnology of Animal Reproduction, National Research Institute of Animal Production, Krakow/Balice, Poland*

Mitochondrial membrane potential (ΔΨm) is a key factor of the normal pre-implantation embryonic development due to high correlation with the cellular energy production. Consequently, alteration of ΔΨm may improve performance of pig embryo produced *in vitro*. Therefore the goal of this study was to check whether decreasing of oxygen tension may influence ΔΨm during *in vitro* development of pig embryos. Pig zygotes were collected surgically from superovulated gilts after flushing the oviducts. Zygotes were cultured in NCSU-23 (North Carolina State University-23) medium at 39°C in an atmosphere containing 5% CO_2 and: (A) 21% O_2, (B) 5% O_2, (C) 2% O_2. Embryos at 2- to 4-cell, 8- to 16 cell and morula stages were selected on days 2, 3 and 4 of culture, respectively. To estimate ΔΨm embryos were labeled with 0.5 μM MitoTracker Orange CMTMRos (Molecular Probes Inc.) for 30 min. at 39°C and subsequently analyzed in LSM 510 META confocal microscope (Carl Zeiss GmbH). The amount of fluorescence emitted from the mitochondria in arbitrary unit which proportional to the ΔΨm were measured. Data were analyzed using one-way analysis of variance and post-hoc Tukey test. For zygotes ΔΨm (mean±standard error of the mean) equals 8.07±1.28 (N=19). In group (A) ΔΨm was: 7.74±1.65 (N=17), 14.28±2.45 (N=16) and 15.1±2.44 (N=17) for 2- to 4 cell, 8- to 16 cell and morula stage respectively. In group (B) ΔΨm was: 9.73±0.96 (N=11, 2- to 4 cell), 24.52±2.37 (N=20, 8- to 16 cell) and 28.3±1.33 (N=18 morula). For group (C), ΔΨm was: 10.15±1.19 (N=21, 2- to 4 cell), 26.45±1.88 (N=13, 8- to 16 cell) and 32.57±1.04 (N=21, morula). In all analyzed groups, at the 2- to 4 cell stage ΔΨm was very low with no differences between groups, while significantly increased later, at 8- to 16 cell and morula stage ($p<0.01$). In conclusion, significant differences between embryos at the same developmental stages cultured in different oxygen tension were detected. Mitochondrial membrane potential for 8- to 16 cell and morula cultured at ambient oxygen tension was lower than that of stage matched embryos cultured in hypoxia conditions. Further investigations regarding the oxygen-sensitive hypoxia-inducible factors expression during *in vitro* cultured of pig embryos under different oxygen tensions are required.

The project was funded by the National Science Centre based on decision number DEC-2012/07/B/NZ9/01326.

Notes

CRYOPRESERVATION OF SHEEP EMBRYOS BY SLOW FREEZING OR VITRIFICATION WITH OR WITHOUT CAFFEIC ACID

SAMARTZI F[1], THEODOSIADOU E[2], VAINAS E[1], MASTRANESTASIS I[1], BAKARAS C[1], REKKAS C[1]

[1]*Veterinary Research Institute, Hellenic Agricultural Organization, Thessaloniki, Greece;*
[2]*Faculty of Veterinary Medicine, University of Thessaly, Karditsa, Greece*

Reduced viability of embryos after cryopreservation has been associated with lipid peroxidation due to increased levels of free radicals. Thus the addition of antioxidants in the cryoprotectant solutions might be beneficial to embryo survival. Antioxidant caffeic acid, that has been shown to reduce the levels of lipid peroxidation markers in rat erythrocytes, was tested for its ability to improve the cryotolerence of sheep embryos under the two major methods used for embryo cryopreservation. Embryos were collected from 32 superovulated Lesvos ewes, on day 7 after the onset of oestrus, soon after slaughter, by uterine horns flushing and were evaluated under stereoscope. One hundred and thirteen freezable embryos (grades 1, 2), in the morula or in the blastocyst stage, were cryopreserved either by slow freezing (seeding at -6.5°C, 0.3°C/min to -35°C) or by vitrification. Unless differently specified, all chemicals were purchased from Sigma-Aldrich Co. (St Luis, MO, USA). Ethylene glycol, in a final concentration of 1.5M in ECM [Embryo Culture Medium = PBS+20%FCS (Biochrom AG, Berlin, Germany)], was used as cryoprotectant in slow freezing. A final concentration of 25% glycerol and 25% ethylene glycol in ECM was used for vitrification. In half of the cases in each method, 20μM of antioxidant caffeic acid was added in all the cryoprotectant solutions. After thawing / warming, the embryos were cultured in vitro, in SOF, for 72 hours and evaluated for development and hatching. Plasminogen activator activity (PAA), which has been linked to embryo development or degeneration, was determined spectrophotometrically in the media used during the removal of cryoprotectants and in vitro culture. Data was analysed using chi square test, t-test and regression analysis. Overall, 56.0% of the thawed / warmed embryos developed during in vitro culture. At the end of in vitro culture, 42.0% of all the incubated embryos were undergoing or had completed hatching; 42.3% after slow freezing and 41.7% after vitrification. Increased hatching ratio was observed in the embryos cryopreserved in the presence of caffeic acid (52.0% vs. 32.0%, $P<0.05$); this was apparent in both cryopreservation methods and the difference approached significance after slow freezing (53.8% vs. 30.8%, $P<0.10$) but not after vitrification (50.0% vs. 33.3%, $P<0.20$). At the end of in vitro culture, 47.0% of the embryos were degenerating; no statistically significant effect of cryopreservation method or the presence/absence of caffeic acid was observed. PAA in the culture medium at the end of in vitro culture was negatively associated with the ratio of degenerated embryos ($R^2=0.465$, $P<0.05$). In conclusion, addition of antioxidant caffeic acid seems to improve cryotolerence of sheep embryos and its effect seems to be more prominent when slow freezing is applied.

(Financed by SEE-ERA.NET Plus - Ref No ERA83).

Notes

ASSESSMENT OF FERTILIZING ABILITY OF IBERIAN IBEX (*CAPRA PYRENAICA*) VITRIFIED AND FROZEN EPIDIDYMAL SPERM BY *IN VITRO* HETEROLOGOUS FERTILIZATION OF BOVINE OOCYTES

SÁNCHEZ-CALABUIG MJ[1], PRADIEÉ J[1,2], BELTRÁN-BREÑA P[1], MAILLO V[1], O'BRIEN, E[1], CASTAÑO C[1], ESTESO MC[1], SANTIAGO-MORENO J[1], RIZOS D[1]

[1]*Department of Animal Reproduction, INIA, Avda.Puerta de Hierro 12, local 10, 28040 Madrid, Spain,*
[2]*Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), SHIS QI 1, Lago Sul - Brasilia DF, 71605-001 Brasil*

The aim of this study was to evaluate the fertilizing ability of vitrified and frozen Iberian ibex sperm by assessing heterologous IVF using bovine oocytes. Testes were obtained from mature ibexes that were legally hunted in the Tejeda and Almijara Game Reserve, in southern Spain. Epididymal spermatozoa were collected by the retrograde flushing method. Sperm from right epididymis was vitrified with TCG-6% egg yolk plus 100 mM sucrose while sperm from left epididymis was conventionally frozen with TCG-6% egg yolk and 5% glycerol. *In vitro* matured zona-intact bovine oocytes were subjected to heterologous IVF with vitrified-warmed (n=495) or frozen-thawed ibex sperm (n= 565) and homologous IVF (n=299). A non-fertilized group was included as control for parthenogenesis (n=81). For heterologous fertilization, sperm pool of three males was used for each treatment. Sperm-oocyte interactions were evaluated at 2.5 hours post-insemination (hpi) by the number of attached and bound spermatozoa whereas penetration and polyspermy were evaluated after 12 hpi. Presumptive zygotes were fixed and stained with Hoechst 33342 at 18, 20, 22, 24 and 26 hpi to assess pronuclear formation using a phase contrast and confocal microscopy. Besides, cleavage rate was evaluated in all groups at 24 hpi. Data obtained was analyzed using one way ANOVA (Sigma Stat, Jandel Scientific, San Rafael, CA) Results showed a higher number of bound and attached spermatozoa in both heterologous groups compared to homologous group (P<0.001). The homologous IVF group as expected, showed the highest percentage of pronuclear formation at 18 hpi (67.7±9.8%), significantly different to both heterologous groups (Frozen: 21.3±13.9%; Vitrified: 28.8±15.5%, P<0.05). Indeed, pronuclear formation was delayed in both heterologous groups with the highest percentage at 24 hpi (30.3 ± 15.1%) for frozen sperm and at 20 hpi (31.7 ± 21.5%) for vitrified sperm. In addition, cleavage rate was higher in homologous group compared with heterologous frozen and vitrified groups (76.1±15.9% *vs*. 31.3±27.2% and 45.1±24.4%, respectively, P<0.05). No differences were observed between heterologous vitrified and frozen sperm in all parameters evaluated. In conclusion, Iberian ibex epididymal sperm can be vitrified successfully, maintaining its fertilization ability in the same extend as frozen sperm. To our knowledge, this is the first report of successful epididymal sperm vitrification in a mammal species being capable of fertilization as a standard tool for genome conservation in threatened species.

Notes

VITRIFICATION OF INTACT AND SPLITTED IN VITRO PRODUCED DAY 7 BOVINE EMBRYOS

SAUCEDO MN[1], REICHENBACH M[2], KUROME M[1], WOLF E[1], REICHENBACH HD[3]

[1]Chair for Molecular Animal Breeding and Biotechnology, Ludwig-Maximilian University, Oberschleissheim, Germany;[2] Bayern-Genetik GmbH, Grub, Germany;[3] Bavarian State Research Center for Agriculture, Institute for Animal Breeding, Grub, Germany

In the present study, we compared the vitrification of intact and splitted *in vitro* produced bovine embryos with two vitrification methods: The CryoLogic Vitrification Method (CVM), CryoLogic® Australia and the Hollow Fiber Vitrification (HFV) Method (Matsunari et al. 2012). For IVP ovaries from slaughtered animals were used. Aspirated oocytes were *in vitro* matured (IVM) for 22 h, followed by *in vitro* fertilization (IVF) for 18 h. Presumptive zygotes were denuded and *in vitro* cultured (IVC) in SOF supplemented with 5% OCS. On D7, intact or splitted embryos were classified in grade I or II and vitrified, either by the CVM or by the HFV method. Embryos were loaded in 0.7-1.0 µl of vitrification solution. Vitrification and thawing procedures were performed as previously described (Saucedo et al., 30th Annual Meeting A.E.T.E., Dresden, Germany, 2014). After thawing, embryos were *in vitro* cultured until D12. Survival rate (judged by re-expansion) 24-48 h after thawing and hatching rate were recorded. Within the HFV method 273 (intact: HFV-) and 50 (splitted: HFV+), and within the CVM method 256 (intact: CVM-) and 312 (splitted: CVM+) embryos were cryopreserved. The percentage of lost embryos was lower in HFV- (0.7%) vs. HFV+ (9.0%) vs. CVM- (9.2%) vs. CVM+ (16.4%). The overall re-expansion rate was significantly higher with CVM than HFV (70.8 vs. 61.0%; $p>0.05$) and the highest results were obtained with blastocysts (73.6%) followed by early blastocysts (70.8%) and morulae (58.0%) ($p=0.004$, Kruskall-Wallis test). No significant differences were observed using intact or splitted embryos. Re-expansion rate of intact embryos resulted in 68.6% vs. 68.8% of splitted embryos ($p=0.835$; Mann-Whitney test). Survival of embryo regarding the time of culture between splitting and vitrification (3 or 20 h) showed a tendency to highest results after 20 h (63.0% vs. 72.1%; $p=0.807$, Mann-Whitney test). Demi-embryo survival and effect of embryo's stage on biopsy outcomes were evaluated. No significant difference was found among stages (59.0%, 67.2%, and 90.0%, for morulae, early blastocysts and blastocysts, respectively; $p=0.0568$ Kruskal-Wallis test) with regards to survival after splitting and biopsy. However, blastocysts leads to better survival after splitting and vitrification. In conclusion, both vitrification methods are suitable for intact or splitted bovine embryos, whereas the CVM seems to be more practical in handling.

Funded by the Bavarian Research Foundation (AZ-1031-12; DOK-153-12).

Notes

HYALURONIC ACID-BINDING ABILITY OF SPERMATOZOA AND ITS ROLE FOR SELECTION OF VACUOLE FREE HUMAN SPERMATOZOA IN HUMAN REPRODUCTION

SAYMÉ N [1], KREBS T[1], KLOCKE S[1], MAAS DHA[1], TÖPFER D[2]

[1] Team Kinderwunsch Hannover,
IVF-Labor, Brühlstraße 19, 30169 Hannover, Germany
[2] Tierärztliche Hochschule Hannover,
Institut für Reproduktionsbiologie, Bünteweg 2, 30559 Hannover, Germany

The type of spermatozoa selected in ART (assisted reproductive technology) influences the outcome in regard to embryo development, pregnancy, miscarriage and malformation. Sperm head nuclear abnormalities were identified earlier as vacuoles by motile-sperm organelle-morphology examination (MSOME). Blastocyst development and the pregnancy rates are negatively influenced if vacuoles containing sperm are used for ICSI. Thus, it is of importance to reliably select vacuole-free spermatozoa in assisted reproduction.

In a prospective, observer blinded study. Hyaluronic acid (HA) bound, standard morphological (SM) selected (200x) and unselected sperm were collected by different examiners. The evaluation of vacuoles by Nomarski differential interference contrast (DIC; 600x up to 7.200x) was performed observer blinded for all samples.

Eleven human semen samples were prepared by a 80% density gradient. From each sample a minimum of 20 sperm per method (HA and SM selection) were collected in separate PVP droplets. Additionally, 20 unselected spermatozoa were collected from each sample designated as control. The number of vacuoles in each sperm head was determined by means of DIC. One way analysis of variance was performed (Tukey-Test; Sigma Stat Version 3.5, DUNDAS Software LTD.).

Significantly more sperm without vacuoles were found in HA selected ($p<0.001$) and SM selected ($p<0.001$) than in unselected samples. The number of sperm with 1 or 2 vacuoles ($p<0.01$) and more than 2 vacuoles ($p<0.001$) was significantly higher in the unselected group. Furthermore, in HA selected sperm the appearance of 2 vacuoles was significant lower than in SM selected sperm ($p<0.05$).

Both selection methods provide spermatozoa containing less vacuoles than in the unselected samples, especially in the group with more than two vacuoles. This shows that HA selection is a good method to select spermatozoa in regard to the appearance of vacuoles. This is of significance since the HA selected spermatozoa are more mature, with less cytoplasmatic retention and higher DNA integrity than unselected sperm cells. Thus HA selection may be an effective method to identify spermatozoa with a higher potential in reproduction in order to improve safety and results in ART procedures.

Notes

DETERMINING INTRAFOLLICULAR CONCENTRATIONS OF CORTISOL AND PROGESTERONE IN HORSES AND THE EFFECTS OF CORTISOL ON IN VITRO MATURATION OF EQUINE OOCYTES

SCARLET D[1], ILLE N[1], ALVES BG[2], GASTAL GDA[2], GASTAL MO[2], GASTAL EL[2], AURICH C[1]

[1]*Centre for Artificial Insemination and Embryo Transfer, University of Veterinary Medicine, Vienna, Austria*
[2]*Department of Animal Science, Food and Nutrition, Southern Illinois University, Carbondale, IL, USA*

Increased glucocorticoid release and synthesis in response to acute or chronic stress has been shown to impair reproductive function in a variety of species and therefore may affect fertility. The aims of this study were 1) to determine cortisol and progesterone concentrations in equine follicular fluid and serum and 2) to assess the effects of cortisone supplementation to the maturation medium on IVM rates of equine oocytes. We hypothesized that challenging equine oocytes during IVM with higher doses of cortisone than physiological levels does not affect IVM rates. Light horse mares (n=9) used in this study were reproductively sound and cycling. Follicular fluid samples were collected by ultrasound-guided transvaginal follicle aspiration from the following follicle classes: G1: 5-9 mm, G2: 10-14 mm, G3: 15-19 mm, G4: 20-24 mm and G5≥25 mm. Blood samples were collected from each animal at the beginning and at the end of the aspiration period, respectively. Hormone determinations for cortisol (DE1887, Demeditec, Kiel-Wellsee, Germany) and for progesterone (ADI-901-011, Enzo Life Sciences, Farmingdale, NY, USA) were performed by ELISA. Cumulus oocyte complexes (COCs) were collected by OPU only from healthy, growing follicles, in the absence of a preovulatory follicle. Compact COCs (n=84) were randomly assessed either to control group, or to one of the treatment groups, in which hydrocortisone (H4001, Sigma Chemical, St. Louis, MO, USA) was added to the standard maturation medium in the following concentrations: 0.1 µg/ml, 1 µg/ml, 5 µg/ml and 10 µg/ml. After 30h, oocytes were denuded, stained with Hoechst (33342, Sigma) and IVM rates were assessed. Statistical analysis was done with the SPSS Statistics 22 software. As all data were normally distributed (Kolmogorov–Smirnov test, $p > 0.05$ for all parameters), one way ANOVA, Post-Hoc-Test and Pearson's correlation were applied for the hormones, whereas Chi-Square Test was used to analyse IVM rates. In follicular fluid from G5 follicles, concentrations of cortisol and progesterone were significantly higher ($p<0.05$) than in all other groups. Concentrations of cortisol and progesterone were positively correlated ($r=0.8$; $p<0.001$). In contrast, serum concentrations of progesterone and cortisol in mares did not differ at the beginning and the end of the aspiration period. There was no significant difference in the percentage of matured oocytes between groups, regardless of the concentration of cortisone added to the medium. Our results demonstrate a significant increase of cortisol in preovulatory follicles in vivo, suggesting its importance for oocyte maturation. Moreover, challenging equine oocytes in vitro with up to 100 times more cortisol than physiologically existent in follicles larger than 25 mm did not significantly affect IVM rates, suggesting that the equine oocyte is able to modulate cortisol levels and therefore to adapt to stress situations.

Notes

INVOLVEMENT OF PHOSPHODIESTERASE 5 (PDE5) ON LIPID ACCUMULATION IN BOVINE OOCYTES AND EMBRYOS PRODUCED IN VITRO

SCHWARZ KRL[1]; BOTIGELLI R[1,2]; DE CASTRO FC[1]; WATANABE O[3]; WATANABE YF[3]; LEAL CLV[1]

[1]Departamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, S.P – Brazil
[2]Instituto de Biociências, Universidade Estadual Paulista, Botucatu-S.\|P-Brazil
[3]Watanabe Tecnologia Aplicada, Cravinhos-S.P.-Brazil

The aim of this study was to investigate the involvement of PDE5 on lipid metabolism in bovine oocytes by assessing the effects of PDE5 inhibition during in vitro culture on lipid contents in oocytes and resulting in vitro produced (IVP) embryos, and their cryotolerance. In Experiment 1, cumulus-oocye complexes (COCs) from slaughterhouse ovaries were submitted to IVM in TCM199 supplemented with 0.4% BSA or 10% FCS associated or not with a PDE5 inhibitor (10^{-5}M sildenafil- Sigma-Aldrich) and after 22h oocytes were denuded and stained with Nile Red (1µg/ml, 30 min) to assess cytoplasmic lipid levels measured by fluorescence intensity. In Experiment 2, 10^{-5}M sildenafil (SDF) was included during IVM and/or IVC (SOFaa) during embryo development after IVF (TALP medium using frozen sperm from the same bull prepared by Percoll gradient). Controls were cultured without SDF and all groups were cultured with 10% FCS. After 22h IVM, 20h IVF and seven days IVC, embryos were assessed for cleavage (Day 4) and blastocyst development rates. Day 7 blastocysts (BL) were fixed and stained with Nile Red to evaluate lipids. In Experiment 3, the same groups were assessed plus two others including melatonin (10^{-7}M) as an antioxidant during IVC in SDF treated groups. Cleavage and BL rates were determined and embryos were vitrified. After thawing, BLs were cultured for 24h to assess reexpansion and 48-72 h for hatching. Cultures were at 38.5ºC under 5%CO_2 in air. Statistical analyses were performed by ANOVA followed by Tukey test using SAS and significance level was 5%. In Experiment 1, SDF reduced ($P<0.05$) lipid content in oocytes matured with BSA (13.1) or FCS (16.3) when compared to controls matured only with BSA (17.6). SDF groups were similar ($P>0.05$). Reduction in lipids was only observed in BLs produced with SDF in both IVM and IVC (30.2; $P<0.05$). Oocytes matured only with FCS had highest lipid content (20.1, $P<0.05$). In Experiment 2, there was no effect of SDF or melatonin on cleavage or BL rates (79 and 31%, respectively, $P>0.05$) or reexpansion and hatching (89 and 64%, respectively, $P>0.05$). In conclusion, PDE5 inhibition during IVM reduces lipid content in oocytes, but in embryos, inhibition is necessary during both IVM and IVC. Lipid reduction, however, did not translate into improved cryotolerance, neither did the addition of the antioxidant melatonin. PDE5 appears to be involved in lipolysis in bovine oocytes and embryos possibly related to cGMP levels and PKG activity and may be an interesting target for studies to understand lipid metabolism in oocytes and IVP embryos. To our knowledge, this is the first study to show the possible relationship between this pathway and lipid metabolism in bovine oocytes and embryos.

Acknowledgements: FAPESP, Brazil; WTA, Brazil.

Notes

IN VITRO PRODUCTION OF BOVINE EMBRYOS AS A TOXICOLOGICAL MODEL: IMPACT OF POLYCHLORINATED BIPHENYL (PCB) 126 DURING MATURATION

SJUNNESSON YCB[1], ÖRN S[2], PERSSON S[1]

[1] *Swedish University of Agricultural Sciences (SLU), Department of Clinical Sciences, Division of Reproduction;* [2] *Swedish University of Agricultural Sciences (SLU), Department of Biomedical Sciences and Veterinary Public Health, Section of Environmental Toxicology*

Many of the experimental animals used in toxicological studies are for assays involving reproductive toxicity and the vast majority use the small rodents as models for the human. There are many factors making the human and cow much more similar than humans and rodents. The aim of this study was to explore the bovine IVP system for the impact of PCB 126 during oocyte maturation. All PCB congeners are lipophilic persistent environmental pollutants, of which PCB 126 is the most dioxin-like (activates the aryl hydrocarbon receptor) and therefore considered to be the most toxic congener. For maturation, 254 abattoir derived oocytes were used (in three replicates). The oocytes were randomly divided into two groups for maturation and the treated group contained an addition of 100.6 pg/ml of PCB 126, a concentration previously found to affect cleavage and blastocyst development (Krogenæs et al., Reprod Toxicol 12:575-80 1998). Apart from the addition of PCB 126, the maturation, fertilization and culture were done according to standardized protocols (Abraham et al., Acta Vet Scand 54:36 2012). The embryo development was assessed through cleavage at 44 h after fertilization and blastocyst development (stage and grade) at day 7 and 8 after fertilization. At day 8 after fertilization the blastocysts were stained for number of nuclei (DraQ-5, Bionordica, Stockholm, Sweden) and neutral lipid (HCS LipidTOX, Invitrogen, Paisley, UK). The embryos (n = 63) were examined for number of nuclei and for neutral lipid staining intensity with fluorescent microscopy and ImageJ 1.48v (http://imagej.nih.gov/ij). Statistical analysis of the effect of PCB 126 on cleavage rate and blastocyst rate, stage and grade was done by logistic regression (logistic procedure of SAS, Milltown, USA). Continuous variables were analysed in the GLM procedure. Replicate was considered as an influencing factor and was included in all models. The mean cleavage rate for the control group was 76.3% ±0.12 (mean ±SD) and in the PCB 126 treated group 70.0% ±0.09. Blastocyst rate (calculated from number of oocytes to maturation) on day 7 was higher in the control group (19.5% ±0.1) than the PCB 126 group (10.4% ±0.04). On day 8 the corresponding figures were 28.5% ±0.06 (control) and 21.4% ±0.04 (PCB 126 group). The difference in blastocyst rate between the control and the PCB 126 group was significant (p=0.04) on day 7, but not on day 8. There was no effect of PCB 126 on blastocyst stages, grades or number of nuclei. The mean pixel intensity of the LipidTOX stain was lower in the control group (334 ±139) compared to the PCB 126 group (454 ±212) but this was not statistically significant (p=0.18). In conclusion, addition of PCB 126 during maturation seemed to affect early embryo development in this small study, and could possibly be related to lipid metabolism. Bovine IVP should be further explored as a model for toxicity on oocytes.

Notes

SERUM-FREE *IN VITRO* CULTURE OF EQUINE EMBRYOS

SMITS K, WYDOOGHE E, VAN SOOM A

Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium.

While bovine embryos are routinely cultured in serum-free conditions since serum culture is associated with the occurrence of the large offspring syndrome, equine embryo culture is still conducted in the presence of fetal calf serum (FCS). In the horse, a negative effect of in vitro culture on the foals has not been observed, but early embryonic loss and development of trophoblast-only pregnancies have been associated with in vitro production of equine embryos (Hinrichs et al, Theriogenology 68:521-529, 2007). Therefore, the aim of this study was to evaluate equine blastocyst development and quality in serum-free culture medium. Equine embryos were produced as reported previously (Smits et al. Reproduction 143:173-181, 2012). Briefly, oocytes were aspirated from abattoir ovaries, matured in DMEM/F12 based medium in 5% CO_2 in air and fertilized by piezo-assisted ICSI. Presumptive zygotes were further cultured in DMEM/F12 supplemented with either 1) 10% FCS, 2) 10% serum replacement (SR, Life technologies, Gent, Belgium) and 5 ng/ml selenium, or 3) 0.4% BSA (Sigma-Aldrich, Diegem, Belgium), 5µg/ml insulin, 5µg/ml transferrin and 5 ng/ml selenium (BSA-ITS) at 38.2°C in 5% O_2, 5% CO_2 and 90% N_2. At day 2.5 cleavage was assessed and at day 9 blastocyst rate was evaluated. Subsequently blastocysts were fixed in 2% paraformaldehyde during 20 minutes and stored in PBS with 0.5% BSA at 4°C until staining. Differential apoptotic staining was performed as described previously (Wydooghe et al, Anal Biochem 416:228-230, 2011) to determine total cell number (TCN), inner cell massa/trophectoderm (ICM/TE) ratio and apoptotic cell ratio (ACR). Cleavage and blastocyst rates were compared using binary logistic regression. Data concerning blastocyst quality (i.e. TCN, ACR and ICM/TE ratio) were analyzed using a mixed-model analysis of variance (SPSS statistics 22). Cleavage rates were similar in FCS (22/29, 75.8%), SR (20/28, 71.4%) and BSA-ITS (22/28, 78.6%). No blastocysts developed in the BSA-ITS. Blastocyst rates were not significantly different between FCS (7/29, 24%) and SR (4/28, 14%) and TCN and ICM/TE were not affected either. However, ACR was significantly higher in SR (4.16 % ± 0.49), when compared to FCS (0.88% ± 0.20 , $p<0.001$). In conclusion, serum-free IVC of equine embryos in the presence of SR does not impair embryonic development, but ACR in the resulting blastocysts is significantly increased, when compared with ACR in blastocysts cultured in the presence of FCS.

Notes

SEASONAL VARIATION OF TESTICULAR FUNCTIONALITY IN ALPACA (VICUGNA PACOS) RAISED IN ITALY

STELLETTA C[1], VENCATO J[1], OZTUTAR F[1, 2], MILANI C[1], DASKIN A[2], ROMAGNOLI S[1]

[1]*Department of Animal Medicine, Production and Health, University of Padova, Italy*
[2]*Departement of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, University of Ankara, Turkey*

Thermoregulatory functions of testicles are very important for sperm viability in terms of spermatogenesis and maturation phases. Unique characteristic are present in South American camelids related to the position, body mass/testicle volume ratio and anatomical features (epididymis orientation). Among different parameters to evaluate male there are testicular dimension measures (width, length and thickness). It has been observed different times the increase of testicular volume during heat season in animals with pendulous testicles because of circulatory impairment. Aim of this study was to monitoring physical and physics parameters besides the semen quality evaluation during two different seasons (summer, winter) in alpacas. Eight adult males are evaluated considering classical (testicular measures – caliper measurements) and innovative parameters as ultrasonography of the testicles. Semen collections were performed with a teaser and ejaculates obtained were destined to the quality assessment (volume, colour, viscosity, motility and concentration) and biochemical evaluation of the seminal plasma (energetic, protein and enzymatic profile – Hitachi 912 biochemical auto-analyzer). Data were analysed for ONE-WAY ANOVA considering the season as variable independent and the parameters evaluated as dependent variables using the statistical software SIGMASTAT 2.05. There was a significant difference among seasons with a general decrease of the semen quality during the hot season. The lower levels of volume, concentration, seminal plasma (SP) glucose, SP cholesterol, SP triglyceride, SP Phosphates and the higher levels of SP Gamma Glutamil Transferase, SP Alkaline Phosphatase, SP Magnesiumn clearly indicate a detrimental effect of high environmental temperature because the effect on testicular thermoregulatory capability. Negative correlation between Testicular Measures and semen quality parameters was significant (r: -0.64 - -0.45) . At the ultrasound evaluation was characterized the reason of increased testicular mass during the hot season considering the evidence of scrotal edema. The scrotal edema derived by a defect of local circulatory mechanism.

Testicular functionality may be influenced by the high environmental temperature and specifically in alpaca were the position of the gonads imposes a fine regulatory pattern. Hot season causes a testicular circulatory defect with a scrotal edema as results and a decrease of semen quality.

Acknowledgement: Thanks to Mr. Dr. Rene and Mrs. Esther Steiger, Poggio Piero Farm, to support this research.

Notes

CHARACTERIZATION OF ACCESSORY GLANDS ULTRASONOGRAPHY IN RAMS OF ENDANGERED VENETIAN SHEEP BREEDS

STELLETTA C[1], VENCATO J[1], OZTUTAR F[1,2], MILANI C[1], DASKIN A[2], ROMAGNOLI S[1]

[1]*Department of Animal Medicine, Production and Health, University of Padova, Italy*
[2]*Department of Reproduction and Artificial Insemination, Faculty of Veterinary medicine, University of Ankara, Turkey*

Evaluation of male needs standardized protocol for the male's classification. Breeding soundness evaluation (BSE) is a practice that is widely used, mainly in the bull, to evaluate the male starting from physical and reproductive parameters. BSE protocols for rams are already published around the world but complete evaluation is not yet raised for all the breeds with specific characteristics. The male can be classified as Satisfactory, Questionable and Unsatisfactory. When is not possible to evaluate it properly, the classification can be deferred. Among the innovative methods to perform the evaluation, there is the ultrasound exam of the reproductive organs. Testicles, epididymis, vascular cone and accessory glands ultrasound may increase the accuracy of the evaluation. In this study, an established sample of rams belonging to Veneto Agricoltura Center in Villiago (BL), Italy, has been evaluated with classical and innovative monitoring system. On these animals the entire BSE procedure was carried out. Moreover, the ultrasound evaluation (MyLabVet^{TM} One, ESAOTE S.p.A., Genova, 10 MhZ probe frequency) of testicles and vesicular glands has been performed for the first time in these breeds (18 adult rams: N=5 Brogna, N=5 Lamon, N=4 Foza, N=4 Alpagota). After the physical and physics exams all the males involved in the evaluation were collected using electro-ejaculator (Ruakura Ram Probe Plastic Products, Hamilton, New Zeland); the trans-rectal probe was inserted after a mucosal anesthesia (5 ml of Lidocaine 2 %) performed during the deferent ampullas massage. Procedure of semen quality evaluation considering general ejaculate parameters (color, volume, concentration) and specific microscopic observation about viability fresh-post thawed with differential staining (Eosin/Nigrosin, Spermac and Farrelly staining), kinetic CASA parameters (Ivos II, Hamilton Thorne, Germany). Data analysis (Pearson correlation indices) revealed important correlations among scrotal circumference, serum testosterone and semen kinetic parameters. Furthermore, increasing the testicular parenchyma echogenicity, the semen volume used to lower. Testicular and vesicular glands ultrasound exam give us important information about seminal plasma quantity. Particularly vesicular glands echogenicity has shown high relationship with quantity of seminal plasma and therefore low sperm concentration. Physics equipment as ultrasonography may optimize collection procedure performed with electro-ejaculator. Body mass and vesicular glands dimension can influence the induction success and the semen freezeability.

Acknowledgements: The research was supported by PSR 214H-BIONET Regione Veneto and Progetto di Ateneo "Development of an integrative model for assisted reproductive technology in farm animals" of the University of Padova, Italy

Notes

THE EFFECT OF DIMETHYLSULPHOXIDE ON BOVINE EMBRYONIC DEVELOPMENT IN VITRO

STÖHR J, STINSHOFF H, WRENZYCKI C

Justus-Liebig-University, Clinic for Veterinary Obstetrics, Gynecology and Andrology, Giessen, Germany

DMSO is routinely used as cryoprotectant or solvent for in vitro production (IVP) of embryos. Based on its high glassforming characteristics it is essential for vitrification but DMSO is also known for its toxicity at higher concentrations. Earlier studies deemed concentrations of up to 0.4% in in vitro maturation and 0.1% in in vitro culture (IVC) as safe with regards to morphological criteria.

In the present study, bovine IVP embryos employing standard protocols were exposed to the following DMSO concentrations during IVC: 0% (control group), 0.05%, 0.1%, 0.15%, 0.2% and 0.25%. At day 8 cleavage and developmental rates were recorded. The morphological quality of expanded day 8 blastocyst was assessed with differential cell stainings; live-dead-staining (live-dead ratio) and TUNEL staining (apoptotic index). Fat accumulation was determined by red-oil staining.

So far, the following results could be obtained: Cleavage and developmental rates did not differ ($p \geq 0.05$) between embryos of the various groups. Mean cleavage and development rates averaged at 58.3% ± 10.6% and 28.4% ± 9.2% (0 %), 59.5% ± 11.5% and 26.1% ± 7.4% (0.05%), 57.6% ± 6.6% and 21.7% ± 7.1% (0.1%), 58.1% ± 7.8% and 27.8% ± 5.6% (0.15%), 56.6% ± 7.3% and 24.5% ± 7.0% (0.2%), 56.3% ± 10.9% and 23.5% ± 9.9% (0.25%).

The live/dead cell ratio was significantly higher ($p \leq 0.05$) in those embryos derived from the 0.1% group [40.1% ± 23.1%] than that from embryos of the other groups [22.6% ± 13.5% (0%), 23.4% ± 10.4% (0.05%), 24.2% ± 14.6% (0.15%), 22.7% ± 14.0% (0.2%), and 20.3% ± 9.9% (0.25%)]. Apoptotic cells in embryos exposed with 0.1 % and 0.2% DMSO were significantly lower than in those of other groups and with 0.05% DMSO the apoptotic cells in this group are also slightly lower compared to those of control group ($p = 0.08$).

Apoptotic index was lower in embryos out of the groups supplemented with 0.1% and 0.2 % DMSO compared to those of the control group (0% DMSO: 3.8%±1.6%, 0.05% DMSO: 2.6%±1.6%, 0.1% DMSO: 2.3%±1.8%, 0.15% DMSO: 3.2%±1.5%, 0.2% DMSO: 2.2%±1.5%, 0.25% DMSO: 3.1%±1.7% [$p = 0.09$; $p = 0.06$]). Fat accumulation was significant higher [$p \leq 0.05$] in embryos stemming from the group supplemented with 0.15 % DMSO (0% DMSO: 6616.9 μm^2±2703 μm^2, 0.05% DMSO: 7346.3 μm^2±1981.3 μm^2, 0.1% DMSO: 6975.5 μm^2±1847.9 μm^2, 0.15% DMSO: 9301.1 μm^2±1703.3 μm^2, 0.2% DMSO: 8675.1 μm^2±2271.4 μm^2, 0.25% DMSO: 8300.7 μm^2±2711μm^2).

These findings show that DMSO concentrations of 0.1% and 0.2 % used during in vitro culture influences the quality of embryos at the morphological level. However, further analyses to verify these results at the molecular level via RT-qPCR are still needed.

The financial support of the Förderverein Bioökonomieforschung e.V. (FBF) is gratefully acknowledged.

Notes

THE JOINT TREATMENT OF SPERM BY PROLACTINE AND GTP HAVE DETERMINED THE INCREASE OF THE NUMBER ACROSOME-REACTED SPERMATOZOA IN BULLS

USSENBEKOV Y[1], KUZMINA T[2], BOYTSEVA E[2], DENISENKO V[2]

[1]*Kazakh National Agrarian University, 050010, Almaty, Kazakhstan*
[2]*Russian Research Institute for Farm Animal Genetics & Breeding, 189620 St.Petersburg-Pushkin, Russia*

There are contradictory opinions concerning involvement of prolactin (PRL) in the process of sperm capacitation and acrosome reaction (Vigil P. et al., 2011 Biol Res, 44:151-159). It was shown that PRL stimulates release of Ca^{2+} from IP3-sensitive stores, and GTP stimulates release of this ion from IP3-insensitive stores (Denisenko V. et al., 2015 Tsitologiya, 3:1-8). GTP forms a connection between IP3-sensitive and IP3-insensitive intracellular stores and promotes transition of Ca^{2+} between these stores (Mullaney J. et al., 1987 J. Biol. Chem. 262: 13865—13872). The aim of the present study was to examine the mobilization of Ca^{2+} from intracellular stores after the treatment spermatozoa by PRL and GTP and to evaluate the status of spermatozoa after these treatments. Intensity of fluorescence of membrane-bound Ca^{2+} was determined with a fluorescence spectrophotometer Hitachi MPF-4 (excitation: 380-400nm, emission: 530 nm) using 40 μM chlortetracycline (CTC) - (Denisenko V. et al., Tsitologiya 3:1-8, 2015). Intensity of fluorescence of membrane-bound Ca^{2+} was determined in Sp-TALP medium where the concentration of cells was adjusted to 1, 5 X 10^6 sperm/mL. The CTC assay was used to determine the functional status of spermatozoa (Ded L. et al., 2010 Reprod Biol Endocrinol, 8-87). Samples were examined with fluorescence microscope ***Zeiss Axo Imager*** M1. Ejaculates from three fertile bulls were used, and five replicates were performed for each experiment. In each sample, 200 cells were evaluated. Sperm were evaluated according to 1 of 3 CTC staining patterns: fluorescence over the entire head (precapacitated cells), fluorescence-free band in the postacrosomal region (capacitated cells) and low fluorescence over the entire head except for a thin bright fluorescent band along the equatorial segment (acrosome-reacted cells). All reagents that were used in this study were produced by Sigma-Aldrich (Moscow, Russia)**.** Data were analyzed by Student's t-test. Treatment spermatozoa by PRL (10 ng/ml) or GTP (10 μmol) resulted in release of Ca^{2+} from intracellular stores (0.70±0.019 and 0.69±0.017 vs 0.85±0.016; $P<0.001$). There was additional release of Ca^{2+} with the combined effect of PRL and GTP (0.62±0.011 *vs* 0.70±0.019 and 0.69±0.017; $P<0.001$). There was no additional release of Ca^{2+} after the joint action by the pair of these reagents in the presence of protein kinase C inhibitor (Ro 31-8220, 10ng/ml). The average percentages of capacitated spermatozoa did not change after treatment by PRL, GTP or both these reagents. The percentage of cells that underwent acrosome reaction have increased after treatment by PRL and GTP jointly (46% *vs* 62%, $P<0.01$); there was no such effect at preliminary treatment of sperm by Ro 31-8220 (10 ng/ml). Thus, Ca^{2+} transition between intracellular stores in bull spermatozoa after the treatment with PRL and GTP jointly is leading to increasing in the percentage of acrosome-reacted spermatozoa.

Notes

AUTHORS INDEX

AUTHORS INDEX

E

F

G

H

I

J

K

S

T

U

V

W

Z