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Association Européenne de Transfert Embryonnaire

European Embryo Transfer Association

30ème COLLOQUE SCIENTIFIQUE

30th SCIENTIFIC MEETING

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Dr. Klaus-Peter Brüssow

Special Celebration

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Dresden, Germany, 12th and 13th September 2014

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European Embryo Transfer Association

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Dr. K.P. Brüssow

A.E.T.E. Medalist 2014

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Professor Klaus-Peter Brüssow

Right at the beginning let me thank the Board of AETE for appointing me to make the laudation for Professor Klaus-Peter Brüssow. My first reaction was that it would be quite an easy task because I have known Klaus for 26 years already. And then very quickly I had to change my mind and came to the conclusion that introducing and praising such an old friend could and probably would be really complicated. What should I highlight, what is interesting for the people who do not know him and what for the colleagues who do know him? So I apologize for giving my personal view and forgive me for being moved even at writing these words. In explanation our very close working relationship and my friendship with Klaus began in 1988 – more than a quarter century ago.

He was born in 1949 but only on the 31 December, it means we can bargain almost one year from his age. His birthplace was the East German Wismar, a beautiful Hansa city at the Baltic Sea. German Democratic Republic – a country to the East of the iron curtain and not simple to understand for people who never saw it or to the younger generation born after 1990.
His father was an engineer and his mother a book-keeper. He had one older sister.
They moved to another Hansa city, Rostock when Klaus was 5 years old. And he is living in Rostock with his family still to this day.

He had a nice childhood with many friends, much sport and the obligatory shoe cleaning every evening as he once told me. He hated it. Apart from shoe cleaning he enjoyed his early year growing up. As many good East German high school students did he entered a Soviet university i.e. the University of Donetsk, Faculty of Biology. He completed it with "summa cum laude", and got his "Promotion A", in other words his PhD with "magna cum laude". And I have to tell you he completed all his scientific works, projects and manuscripts in the same manner. It was always great to learn from him his daily working method nevertheless it often made me nervous. I will come back to that later.

It was a good school in Donetsk. In subject of biology, let's focus on reproductive biology. The Russians had had long traditions and well based knowledge.
He brought his diploma back from Donetsk to Germany as well as his young wife Nadja. They have remained the same young couple for the last 25 years only with only some slight modifications in

hair colour. They had one little daughter and one little son who grew up very fast and have already produced three grandsons for Nadja and Klaus. Who can believe it? Frankly I cannot…

Klaus does not like to change anything very much. After graduation as a research assistant he entered the Department of Reproduction, Research Institute for Animal Husbandry in Dummerstorf located some 12 km from Rostock. It was a huge institute at that time with more than 1100 employees. Legendary in the Eastern block. Preciously scheduled basic and applied science adapted strictly to the need of centrally directed agriculture production. As mentioned above he got his "Promotion A" with "magna cum laude". He did it in 1979 at the Academy of Agricultural Sciences. At that time postgraduate education in Eastern countries was not given to universities. Only one "doctor school" existed in each countrys connected to the academy so it was rather complicated to get the degree at such a young age. Dummerstorf had excellent opportunities for dedicated researchers. Right after the PhD he became a research scientist and in 1985 was promoted to senior scientist. Head of the Experimental Research Station (Tiervesuchsstation = TVS), deputy head of Technology of Swine Reproduction and Reproduction Physiology unit. When East and West Germany merged the institute was renamed Research Institute for the Biology of Farm Animals (German abbreviation FBN) and actually Leibniz Institute for the Biology of Farm Animals. He was senior scientist at Department of Reproductive Biology, deputy head from 2004 to 2011, and head from 2011 to 2012.

No position interfered with his professional life style, he remained always the dedicated, enthusiastic researcher. And something else: nothing else but the pig. After so many years of cooperation and close friendship with Klaus I dare to tell you there is an emotional contact between him and the pigs. Not only as the subjects for research. But I think nearly all of us have similar emotions to different domestic species.

His scientific interest was centered around female pigs, mostly around gilts. During the young years he did a great many embryo transfers under the supervision of Dr. Bergfeld and an outstanding pictorial evaluation of pig embryos was published (Brüssow and Kauffold, 1982). It is well worthwhile seeing.

Also he worked on the ovarian function in gilts, of course. For many years it was the main stream of the porcine reproductive research in Dummerstorf. They described the different porcine ovarian features and classified the ovaries (Schnurrbusch et al, 1983). Descriptions in his research did not mean only a morphological characterization but a kind of dynamic observation of reproductive physiology, in collaboration with endocrinologists and nutrition physiologists etc. as the project demanded it. He wanted to understand the physiological events and how to influence them if the daily pig production needed i.e. estrous synchronization, stimulation of puberty, ovulation induction. He carried out very interesting studies in the '80's on bioelectric processes of the porcine uterus at different stages.

Moreover he investigated the fate of oocytes following follicle rupture. Many of us remember the IVF experiments that were very popular everywhere in the '90ies. In every species and every aspect. Frankly the in vitro studies were expanding at that time so quickly that

researchers forgot to look at the in vivo events. Experts in the labs wanted to make in vitro copies of in vivo processes without profound knowledge of how it occurred in life. Klaus began observing the porcine in vivo fertilization at the late '90ies and with well scheduled trials he greatly contributed to our knowledge of the role of follicular fluid and local endocrine parameters during the rendez-vous of ova and sperm cells in the oviduct. Later these achievements were instrumental in improving in vitro projects.

It was impossible for him to stop at the fertilization studies, he followed the early embryos in to the uterus and not only for transferring them to recipients, also Uterus-embryo interaction and uterine capacity in different breeds.

Now we are moving on to another wonderful topic of the past 15 years. It was not difficult to convince Klaus that indigenous European pigs breeds probably had different physiological characteristics compared to their modern day counterparts. We began to investigate the reproductive biology of the Hungarian Mangalica pigs and through many joint experiments we could concluded important results later used by farmers in reproductive management or artificial insemination. Some other native pig breeds attracted his interest, too and he assisted our projects in South East Asia.

As mentioned he always wanted to achieve an indepth and multilateral knowledge of porcine reproductive physiology and he liked to participate in the projects always including experts from a broad scientific area. Anyway he preferred to do clinical work himself like laparotomic and laparoscopic surgery, chronic venous catheterization. Among his achievements we can see his clinical development, amongst them a number of firsts e.g. worldwide first endoscopic ovum pick up and endoscopic embryo collection in pigs, oviduct manipulation etc. Also the projects for optimization of estrous synchronization at first with Suisychron, after that with Regumate and later with GnRH agonists and antagonists. So he divided himself between basic and applied science.

More than 10 years ago he established a good contact to leading Japanese researchers who utilize his expertise in the editorial board of Journal of Reproduction and Development. It is the scientific journal of the Japanese Society of Reproduction and Development.

He is also involved in the board of the Acta Veterinaria Hungarica, ISRN Veterinary Science, Electronic Journal of Polish Agricultural Universities, open Journal of Animal Science.

He never denied his roots and has strong professional and personal relations to East European countries. He is a habilitated professor at Faculty of Veterinary Science, Szent István University in Hungary. He has active cooperation in Poland with Poznan, Wroclaw Universities. He helped a lot for younger and elderly colleagues not only in Poland and Hungary but in Russia and Bulgaria, as well.

What else should I write here? No sense to analyze his publication list of 214 full publications and 146 conference papers. Anybody wants to do it there is an easy access to them either using internet or asking Klaus personally.

Allow me to close the laudation with some personal remarks. I have never met anyone else with such waste experience and such a well based knowledge in female porcine reproduction. I

have never met someone who plans his experiments so exactly, writing his papers with so much meticulous effort. As I mentioned sometimes he made me miserable when insisting on repeating the trials so many times to make sure of the scientific evidence. Many times I complained to his wife Nadja and she agreed with me. Anyway Klaus was always right.

And I declare I have never met such an honest, straightforward and good hearted man like Professor Klaus-Peter Brüssow. It was, is and will be good to work with him and it was and is great to be his friend.

Warm congratulations for the Pioneer Award of AETE in 2014.

Jozsef Rátky
former member of the AETE board
National Agricultural Research and Innovation Center
Research Institute for Animal Breeding, Nutrition and Meat Science
H-2053 Herceghalom
Hungary

FOOTPRINTS AROUND EMBRYO TRANSFER IN PIGS

KLAUS-PETER BRÜSSOW

Experimental Reproductive Biology Unit, Institute of Reproductive Biology, Leibniz Institute for Farm Animal Biology (FBN)
18196 Dummerstorf, Germany

Introduction

In 1991, i.e. 25 years ago and two years after the political change in the former socialist East-Germany (GDR), I had first the opportunity to attend an AETE Meeting. The 7th Scientific Meeting in Cambridge, UK, celebrates the 100th anniversary of the first embryo transfer (ET) in rabbits done by Walter Heape and the 50th anniversary of the first ET in swine by Alekseij Kvasnitski. So, this meeting was a double highlight for me and I could also present our results on the transfer of gene injected porcine embryos (Brüssow et al. 1991). However, at this moment I could never foresee to be awarded one day by our association.

Looking back to my scientific career, I feel that most of my research interests were close to embryo transfer in swine (ETS) and I hope to leave little "footprints". Some of them regarding (1) synchronization of estrus and ovulation, (2) research on oocytes, embryos and oviduct, (3) embryo transfer, (4) laparoscopy and oocyte-sperm-oviduct interaction, and (5) reproductive studies on Hungarian Mangalica pigs should be summarized.

Synchronization of estrus and ovulation – the impact of GnRH

Starting my profession 1975 in the Swine Reproductive Physiology Group of the Department of Reproduction, Research Center of Animal Production Dummerstorf, I had to focus on synchronization of estrus and ovulation. The reason was that biotechnological methods to synchronize estrus and ovulation, and enabling fixed-time insemination in pigs should be a crucial part of the reproductive management in pig farms (Hühn et al. 1996, Brüssow et al. 1996, 2009). The production of pork especially in large-scale farms had to fulfil the demands on meat production in the former German Democratic Republic (socialist East-Germany). The biotechnical methods of batch-wise reproduction management were at this time pioneering and a model for modern pig production, today. Several studies had been performed to study the role of equine chorionic gonadotropin (eCG) to stimulate follicle growth in gilts after estrus synchronization and in sows after weaning. Here we determined optimal doses and time points to ensure a sufficient number of pre-ovulatory follicles able for ovulation, releasing mature oocytes for fertilization and finally enabling litter (Brüssow and Bergfeld 1984, Bergfeld et al. 1990). These data were obtained also by diagnostic laparotomy. A tool of ovarian diagnosis including a detailed morphological description of ovarian structures was developed (Bergfeld et al. 1981, Schnurrbusch et al. 1981) and applied under farm conditions, too (Bergfeld et al. 1984 ab).

Optimal batch-wise reproduction requires also synchronization of ovulation to make fixed-time insemination possible (*reviewed*: Brüssow et al. 2009). Here, human chorionic gonadotropin (hCG) were studied to induce ovulation. In our studies, the mean time of hCG-induced ovulation within groups was 40.5h (36 – 45h) for gilts and 40h (37 – 42h) for sows, and with a mean duration of ovulation of 8h (4 – 12h) and 14h (7 – 20h) in groups of gilts and sows, respectively (Bergfeld et al. 1984 ab). These studies allowed obliging recommendation for fixed-time insemination.

Although hCG was successfully used for fixed-time insemination protocols in East-German pig production, its biological origin has bear some problems regarding collection of raw material, purity and standardization. Therefore, it was looking for alternatives.

After the synthesis of gonadotropin-releasing hormone (GnRH, Matsuo et al. 1971) and demonstrating that synthetic GnRH was able to induce ovulation in prepuberal and cyclic gilts (Arbeiter 1973, Ellendorff et al. 1973, Baker et al. 1973, Webel and Rippel 1975), the East-German pharmacy produced a synthetic decapeptide (GnRH vet "Berlin-Chemie") exclusively for veterinary use. The clinical trials started in 1976 and we demonstrated first the successful application of GnRH for ovulation induction after estrus synchronization in gilts as well for induction of puberty in gilts, thought as a preliminary step to synchronize estrus (Brüssow and Bergfeld 1979, 1981). The application of 600 and 900 µg GnRH induced an earlier (6h) and synchronous onset of ovulation compared to untreated control, and was comparable to hCG. Ongoing endocrine and clinical studies described in more detail the reproductive hormone dynamic after GnRH application (George et al. 1989) and the use of GnRH/hCG to induce synchronized ovulations (Bergfeld and Brüssow 1979).

Although used in pig farms, the decapeptide GnRH vet "Berlin-Chemie", however, required high doses and was relatively expensive for practical use. Progress in peptide chemistry enabled the synthesis of GnRH agonist with higher efficiency at lower doses. We performed several endocrine and clinical studies in pigs using different GnRH agonists (depherelin, triptorelin, goserelin) for ovulation induction and pregnancy outcome (Brüssow et al. 1994, 2001, 2007, 2011, Schneider and Brüssow 2006). However, to date depherelin (D-Phe^{6}-LH-RH, Gonavet®) has found practical application in (German) pig production to synchronize ovulation in gilts and sows.

Brief outcome; studies on GnRH and gonadotropins made it possible to regulate estrus cycle and ovulation in pigs both for in-farm reproductive management and for ET related superovulation.

Oocyte – embryo – oviduct

In 1985, my research interest was also focused on distribution and transport of oocytes and embryos within the porcine oviduct. At this time, there were only little literature data regarding these post-ovulatory events (Andersen 1927, Alanko 1965, 1973, Hunter 1974, Oxenreider and Day 1965). Furthermore, no data were available on oocyte/embryo transport after synchronization of estrus and ovulation, and following gonadotropin induced superovulation (SO). In our experiments (Brüssow 1985, Brüssow et al. 1987) we studied the distribution of oocytes and embryos, their development and the time of remaining within the oviduct after follicle stimulation with different doses of eCG (500, 1000 and 1500 IU) and the transport of fertilized and non-fertilized ova

(Brüssow and Rátky 1996), respectively. Besides the calculation of velocity of ovum transport, the developmental stages of embryos and their location within the oviduct in relation to the time after follicle growth and ovulation induction were determined. Additionally it was found that SO induction had no negative influence on distribution and development of embryos, but with influence on dissolution of cumulus investment. These data have been beneficial for subsequent ET related studies.

Since the oviduct plays a crucial role in early embryo development, we studied the protein concentration and characterized cyclic specific secretions in oviductal fluid collected by *in vivo* cannulation of the Fallopian tube during the early estrus (Wollenhaupt and Brüssow 1995). A specific 97 kD protein was detected on days 1 – 3 (8.6 – 6.8% of total proteins). This protein was detected also only in *in vivo* derived embryos (zygote to morula), but not in intrafollicular oocytes and embryos produced *in vitro* (Brüssow et al. 1998) demonstrating its exclusively oviductal origin. The cultivation of *in vivo* derived embryos (1- to 4-cell embryos, morulae and blastocysts) *in vitro* supplemented with the 97 kD protein increased *de novo* protein synthesis and improved further development (Wollenhaupt et al. 1997). The beneficial effect of oviductal secretory proteins at *in vitro* fertilization was later shown by McCauley et al. (2003), who determined decreased polyspermy, higher penetration rates and an embryotrophic effect.

Brief outcome; studies on oocyte/embryo transport within the oviduct after superovulation (SO) stimulation and on biochemistry of oviductal proteins were basics for subsequent ET related research.

Embryo transfer in swine (ETS)

In 1985 there was a need to establish ETS as a basic tool for *in vitro* related techniques (IVF, gene transfer), but also for breeding and commercial purposes in the former East-Germany. Based on previous attempts in our institute (Rommel 1977), we developed and adapted an ET system which included donor selection and SO, embryo recovery, embryo handling (IVC, embryo diagnostic, handling, transport), recipient selection and embryo transfer (Brüssow and Kauffold 1989, Brüssow 1990, Brüssow and König 1990). All efforts of ETS done at this time have to regard the former political situation, too.

All together, we established a proper SO regime for gilts and sows, elaborated a catalogue for embryo quality, studied the reproductive hormone milieu in donor and recipient gilts (Brüssow and Schneider 1993) and the influence of eCG anti-serum on superovulation response (Brüssow 1992). Further, we analyzed both the impact of the number of transferred embryos and of recipient's ovarian status on ET success (Brüssow 1990), and used ET for the propagation of adult sows (Brüssow et al. 1989). Additionally, ET assisted techniques were performed such as splitting of embryos (Brüssow and Schwiderski 1990) and gene transfer (Brüssow et al. 1990, 1991) – the first piglets with integrated bSTH were born in 1988. Basic research on ETS was also organized within the former socialist countries (Bulgaria, Czechoslovakia, GDR, Hungary, Soviet Union) and common methodical recommendations were published. Our experience and the state of art of ETS

were reviewed at the 16th Scientific AETE Meeting in Santander, Spain in 2000 (Brüssow et al. 2000).

Efforts have been done also to improve the embryo recovery techniques. First we applied a minimal-invasive endoscopic embryo recovery which allowed also repeated embryo flushing from the same donor (Rátky and Brüssow 1995, Brüssow and Rátky 1996).

Brief outcome; although, the embryo transfer in swine has not the same significance as in cattle, it is an indispensable requirement for assisted embryo techniques and with some value for special breeding in pigs.

Laparoscopy and oocyte-sperm-oviduct interaction

Meeting in 1988 Dr. Jozsef Rátky and learning to use laparoscopic technique in swine, it was not only the beginning of a fruitful collaboration of several decades to date, but also a close and creative friendship. Laparoscopy was applied to study the time of ovulation in gilts (Brüssow et al. 1990ab, 1993, Brüssow and Rátky 1993) and of follicle development during estrus cycle (Rátky et al. 1995). Furthermore, we established first laparoscopic follicle puncture and oocyte aspiration (Ovum-Pick-Up, OPU) in swine (Brüssow and Rátky 1994, Brüssow et al. 1997). Using such approach, we studied the morphology of *in vivo* derived cumulus-oocyte-complexes (COCs), their nuclear configuration, mitochondrial aggregation pattern and apoptosis (Torner et al. 1998, 2004) as well as oocyte quality during lifetime periods of sows (Brüssow et al. 2002). Laparoscopic OPU was also used for *in vitro* fertilization of *in vivo* derived porcine oocytes (Rátky et al. 2003) and for comparison of oocyte development of pigs of different breeds (Egerszegi et al. 2001, Rátky et al. 2005).

Laparoscopy as a save and minimal-invasive technique has been also applied to study (1) the involvement of follicular fluid (FF) and components of FF on ovulation and sperm migration, (2) the influence of COCs on sperm release from the oviductal sperm reservoir, and (3) to obtain low diverse embryos after single fixed-time laparoscopic intrauterine insemination (LIUI). We could demonstrate that no or only an unimportant amount of FF reaches the oviduct at ovulation, that FF is not a compulsory carrier of the porcine oocyte at ovulation, and that FF does not trigger sperm cell distribution within the porcine oviduct at ovulation (Brüssow et al. 1999ab). Studying the role of porcine COCs, we found that both cumulus-oocyte-complexes and ZP-associated hyaloronan (HA) are involved in triggering sperm release from the oviductal sperm reservoir (Brüssow et al. 2006). By means of laparoscopic insemination we could clearly show that spermatozoa, after insemination into one uterine horn, reach the contralateral oviduct via transuterine, but not transperitoneal migration (Brüssow et al. 2011). Furthermore, LIUI was successfully applied to obtain low-diversity porcine embryos for, among others, *in vitro* assisted techniques and research (Brüssow et al. 2013).

Brief outcome; laparoscopic techniques have been successfully applied to derive COCs from porcine follicles *in vivo*, to perform minimal-invasive surgery to study oocyte-sperm-oviduct interactions and to apply intrauterine insemination.

Reproductive studies on Hungarian Mangalica pigs

The Hungarian Mangalica are an ancient pig breed which is well adapted to environmental conditions, but with relatively low fecundity and has met difficulties to compete with modern breeds (Egerszegi et al. 2003a, Brüssow et al. 2005). The Hungarian Mangalica nearly disappeared and in 1995 only 170 Blond, 18 Swallow Belly and 20 Red Mangalica breeding sows remained. In 1996, a Hungarian-German research project started to propagate Mangalica by biotechnical methods and to highlight the physiological background of low fecundity. First we studied the ovarian characteristics of Mangalica and recommended respective ovarian stimulation procedures. Mangalica pigs reveal a lower number of ovulations and could only moderately stimulated with exogenous gonadotropins (Rátky and Brüssow 1998, Rátky et al. 2001). Based on an adapted stimulation protocol, Mangalica donor sows (n = 90) were superovulated, and recovered embryos (n = 815) collected in different ET trials were transferred into 43 Landrace recipients, which finally gave birth of 202 Mangalica piglets. These pigs were one nucleus for future Mangalica herds.

To study physiological backgrounds of low fecundity, we investigated intrafollicular oocyte development, uterine development during early pregnancy and endocrine processes during the estrus cycle. Altered oocyte maturation compared to Landrace sows was found in Mangalica (Egerszegi et al. 2001). Furthermore, growth restricted uterine development was observed which may influence the initial process of early pregnancy and be another reason of lower fecundity (Brüssow et al. 2004). Several studies were directed on reproductive hormone secretion in Mangalica. We described first the gonadotropin and steroid hormone release during the peri- and post-ovulatory period (Egerszegi et al. 2003b), highlighted the hormone levels of LH, leptin and progesterone in the systemic and near to the ovary circulation (Brüssow et al. 2008) and determined the steroid hormone content in the follicular fluid prior to ovulation (Egerszegi et al. 2007). All these experiments demonstrated a double higher amount of estradiol and a 5-times higher concentration of progesterone in the FF of Mangalica gilts. Furthermore, Mangalica revealed an earlier increase in estradiol secretion, and higher levels and later decrease of progesterone secretion despite lower number of corpora lutea. Additionally, Magalica expose an increased (more than 4-times) leptin secretion and considerable alterations in LH pulse secretion pattern. All these alteration as well contribute to lower fecundity in the Mangalica breed.

Brief outcome; the fatty Mangalica breed differs from others regarding ovarian response, oocyte/embryo developmental competence, uterine development and altered reproductive hormone (E2, P4, LH) and leptin secretion.

Acknowledgements

Looking back to my professional way, I am very grateful to very many colleagues and friends who went along with me, supported and guided my research over a period of nearly 40 years. Without their aid my little footprints certainly disappeared.

Immense gratitude also to all pigs; they allowed me to study their reproduction and to propagate a little by means of embryo transfer. I am touched by pigs and keep in mind the notably word of Winston Churchill – *"I am fond of pigs. Dogs look up to us. Cats look down on us. Pigs treat us as equals."*

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Commercial Bovine Embryo Transfer Activity in Europe 2013

by Hiemke Knijn, The Netherlands

Commercial Bovine Embryo Transfer Activity in Europe 2013

General

Country	Name Collector	N° of approved ET teams	N° of teams provide ET data
Austria	G Wetchy	5	4
Belgium	P Vercauteren/I Donnay	8	8
Bosnia & Herzegovina	T Markovic	1	1
Czech Republic	P Bucek	5	-
Denmark	H Callesen	12	9
Estonia	J Kurykin	1	1
Finland	M Mikkola	4	4
France	C Ponsart	20	18
Germany	H Cramer	39	17
Greece	S Foteini	2	2
Hungary	F Flink	4	2
Ireland	P Lonergan	6	3
Italy	G Lazzari	-	-
Kazakhstan	A Zhanserik	2	1
Luxembourg	M Vaessen	2	2
Moldova	G Darie	1	1
The Netherlands	H Flapper	-	-
Poland	J Jaskowski	7	7
Portugal	J Chagas e Silva	10	6
Russian Federation	Denis Knurov	7	5
Spain	J de la Fuente	21	11
Sweden	C Andersson	-	-
Switzerland	R Saner	6	2
Turkey	J Owens	2	1
Ukraine	V Madison		2

Bovine In vivo embryo production

Country	N° of flushed donors	N° of embryos collected	N° of transferable embryos	N° of transferable embryos/flush
Austria	178	2034	1320	7.4
Belgium	926	5926	4599	5.0
Bosnia Herzegovina	1	12	10	10.0
Czech Republic	241	2536	1314	5.5
Denmark	622	5666	4572	7.4
Estonia	2	13	8	4.0
Finland	425	4718	3037	7.1
France	7205	68426	38244	5.3
Germany	2573	28235	17624	6.8
Greece	1	8	5	5.0
Hungary	104	1507	901	8.7
Ireland	840	7409	4739	5.6
Italy	2067	21948	15305	7.4
Kazakhstan	353	2069	254	0.7
Luxembourg	185	2230	1347	7.3
Moldova	4	12	2	0.5
The Netherlands	4308	27720	26670	6.2
Poland	139	1215	859	6.2
Portugal	108	1252	661	6.1
Russian Federation	157	1051	766	4.9
Spain	547	4999	2958	5.4
Sweden	20	129	69	3.5
Switzerland	362	4286	2791	7.7
Ukraine	9	58	39	4.3
Total	21377	193459	128094	6.0

Bovine In vitro production

Country	N° of OPU sessions	N° of oocytes collected	N° of transferable embryos
Czech Republic	1	20	10
France	234	1882	688
Germany	1638	5036	3480
Italy	51	1081	170
The Netherlands	4391	37328	5094
Spain	265	3206	705
Russian Federation	925	12722	3565
Total	7505	61275	13712

Bovine In vitro production "Slaughtered donor"

Country	N° of oocyte donors	N° of oocytes collected	N° of transferable embryos
France	2	65	14
Italy	19	845	67
Italy (Buffalo)	52	446	34
The Netherlands	60	2661	358
Portugal	1368	16310	611
Total	1501	20327	1084

Embryo Technologies in Bovine

Country	N° of biopsied embryos		N° of sexed embryos		N° of genotyped embryos	
	In vivo	**In vitro**	**In vivo**	**In vitro**	*In vivo*	*In vitro*
The Netherlands	682	--	-	-	682	-
Total	682	-	-	-	682	-

Bovine Embryo Transfer

Country	N° of transferred embryos				
	In vivo fresh	In vivo frozen	In vitro fresh	In vitro frozen	*Total*
Austria	414	584	-	-	998
Belgium	962	3914	-	-	4876
Bosnia Herzegovina	10	-	-	-	10
Czech Republic	729	658	-	14	1401
Denmark	2420	1161	-	-	3581
Estonia		11	-	-	11
Finland	710	2263	-	-	2973
France	17833	16684	173	515	35205
Germany	6639	11326	2566	970	21501
Greece	2	4	-	-	6
Hungary	184	465	-	-	649
Italy	5510	-	100	386	5996
Kazakhstan	44	210	-	-	254
Moldova	-	2	-	-	2
The Netherlands	5770	26318	4173	703	36964
Norway	-	45	-	-	45
Poland	222	727	-	-	949
Portugal	192	410	45	29	676
Russian Federation	105	491	1503	49	2148
Spain	895	1426	721	167	3209
Sweden	28	45	-	-	73
Switzerland	645	1565	-	-	2210
Ukraine	-	115	-	-	115
Total	43314	68424	9281	2833	123852

Embryo Activities in other Species (I)

Country	Sheep		Horses	
	N° embryo produced	**N° embryo transfers**	**N° embryo produced**	**N° embryo transfers**
Czech Republic	-	13	-	-
Finland	-	-	1	1
Ireland	16	16	35	35
Italy	-	-	40 flushes 74 (OPU)	
Poland	-	-	4	4
Portugal	-	-	6	6
Turkey	-	250	-	-
Total	16	279	160	46

INVITED LECTURES

THE EUROPEAN EMBRYO TRANSFER INDUSTRY IN CATTLE – A CHALLENGE IN 1984, A SUCCESS IN 2014 - AND WELL SUPPORTED AND REPORTED BY THE AETE.

Thibier Michel -
Saint Sauveur - 64120 SAINT PALAIS - France

SUMMARY

October 1984, first formal meeting of the Association Européenne de Transfert Embryonnaire (AETE) in Lyon (France), thirty years ago…, who would have then reasonably dreamt of seeing both the European Embryo Transfer (ET) industry and the AETE up to the standard they now are 30 years later. Europe ET teams transfer a little more than 20% of the worldwide total transfers every year, hence showing its sustainability. The European evolution over the years regarding In Vivo Derived (IVD) embryos shows that it is quite parallel to that observed worldwide with perhaps a little less fluctuation from one year to another than in other continents. Referring to In Vitro Produced (IVP) embryos, although a good start was observed in Europe in the late 80's, early 90's, the numbers are still low and far from those observed in other continents.

The sustainable development of the ET industry in Europe is concomitant to the success story of AETE which has had very well attended annual meetings. AETE has been a strong support of the Industry in: communication, training and scientific information.

This sustainability of the European ET industry obviously relies on the existing market due to the benefits the farmers get from applying such a technology and hence from a good cost/benefits ratio.

In the author's opinion, there are three main technical reasons which have supported this success story. The first is that ET is the tool of choice to more efficiently implement genetic improvement programs including those deriving from the recent genomic revolution. The second is the high competence of all practitioners and people involved, together with excellent training and high degree of innovation from the field to the bench as assessed by the high standard of scientific presentations at the AETE annual meetings and of the quality of the European pioneers in this area. The third reason is the excellent approach taken by both the veterinary community and the ET industry in elaborating an original and most efficient policy to enable ET to be recognized as the safest mean of exchanging genes from farm to farm or continent to continent. The European ET industry has participated in generating and implementing such a policy based on the definition of officially approved embryo transfer or production teams.

In conclusion, the ongoing research of innovation, the excellent expertise following well designed training of the practitioners, their sense of responsibility in taking most seriously the recommendations and rules regarding the health safety and precautions taken by the officially approved embryo transfer or production teams all explain the resulting level of excellence of this industry. Clearly, the conditions are currently met to foresee that this success story will continue as such in our European continent in the near future.

INTRODUCTION

October 1984: after a kind of Repeat Breeding syndrome, the first regular meeting of the AETE was held in Lyon, thanks to the constant support of Dr Charles Mérieux and the famous Foundation Marcel Mérieux. Marcel Mérieux was one of the close collaborators of Pasteur and he was one who had a strong spirit of entrepreneur. He created the Mérieux laboratory and his son Charles, after having taken his MD degree, took over and started to develop widely his business domestically and internationally particularly in developing vaccines. After the last world war, the Foot and Mouth Disease (FMD) became a critical problem and although in the field of veterinary medicine, Ch. Mérieux invested strongly in this research in making FMD vaccines with great success leading to the eradication of the disease in many European countries. In this occasion, he worked with young veterinarians and remained very impressed with this profession. This explained why when some of us went to visit with him in the early 80's, he immediately brought his full support to the new arising Embryo Transfer industry. He well understood all the benefits this new technique, second generation of animal biotechnologies of Reproduction after Artificial Insemination, could bring to the livestock industry. So he enthusiastically helped the young scientists in getting this Association on its feet, appreciative of the European approach and remained a strong supporter the rest of his life until his death in the early 2000's. It is with this blessing that the AETE started its meeting in the Fondation M Mérieux facilities at Lyon, the first years in a somewhat loose but very friendly fashion. It is only in 1987 that the first Proceedings were published, then moving on a more professional way. The details of this saga have been reported previously by Ponsart et al. in 2009.

October 1984: who would have dreamt to see this Scientific Association and the ET industry so well and alive 30 years later? To this, there is no miracle but just the dedication of a lot of people, scientists and practitioners who dedicated their expertise to that industry and to the development of this Association. However this in itself would not have been sufficient if by the same token this technology had not been able to provide considerable benefits to the farming industry.

During those three decades, the AETE has had the merit to constantly accompany this development in supporting the industry by its own means: annual meetings, communication, interrelationship with the Brussels authorities, close integration of the young generations of scientists and practitioners giving them strong opportunities to participate to the meetings.

We will narrow our presentation to the bovine species due to lack of space and time. In a first part, we will report on the economic impact of ET in Europe and compared to the world development. In a second part, we will try to pinpoint the main technical reasons for such a successful story.

1. THE EUROPEAN EMBRYO TRANSFER INDUSTRY IN NUMBERS DURING THE PAST 30 YEARS

1.1. In Vivo Derived (IVD) cattle embryos.

The first data retrieval in Europe reported was that in 1987 reporting for 1986 with only but a few countries having reported numbers. When the data collection became better organized (in 1988) the number of countries exchanging their collected data increased with 23 countries reporting in 2013. Table 1 reports the numbers of the first four years of data retrieval. As can be seen, the numbers reported showed an increase close to 5-fold, obviously not all due to the same increase in the field but mainly due to a more consistent retrieval and widened reporting. One may notice that by the end of the 80's more than 100 000 embryos in Europe were transferred in cattle, of course almost all IVD embryos. This claimed to the world that this technology was no longer confidential but widely used by the farmers due to their inherent benefits to the livestock industry.

Table 1. IVD cattle embryo transfers in the 1986-1990 (AETE, 1987-1991) and compared to those of 2012 (IETS, 2013).

Year of reference	Year of publication (AETE Proceedings)	Number of countries reporting	Number of donors collected (A)	Number of embryos transferred (B)	B/A
1986	1987	3(*)	6 479	24 389	3.76
1988	1989	13	17 278	79 174	4.58
1989	1990	15	25 384	111 420	4.38
1990	1991	23	32 732	165 680	5.06
…..					
2012	**2013**	**23**	**23 653**	**106 463**	**4.50**

(*) France, Italy and Germany

Some decades later, not taking here into account IVP embryos (see below), the number of donors and embryos transferred have been noticeably maintained in the 90 000 for the embryos transferred –with a peak recorded in 1990 (more than 150 000 embryos) - and the mean number of embryos transferred per donor has also been quite sustained between 4.5 and 5.

How does this match with the world statistics? To answer this question, we will borrow tables, figures and numbers from the remarkable report of G Perry of the IETS (International Embryo Transfer Society) data retrieval committee (Perry, 2013).

Table 2. Number and proportion of Data Collectors by region (Perry, 2013).

Region	No of countries in region	No countries submitting data	% countries submitting data
Africa	57	2	3.51%
Asia	53	5	9.43%
Central America	31	1	3.23%
Europe	45	26	57.78%
North America	3	3	100.00%
Oceania	23	2	8.70%
South America	13	2	15.38%
Globally	**225**	**41**	**18.22%**

Table 2 shows that Europe does not do too bad a job in collecting data as more than 50 % of the European countries have reported, including 3 countries that were able to be collected by the IETS data retrieval committee.

Europe is collecting and transferring about 20% of the world IVD embryos and is so ranking behind North America but ahead of Japan and South America (Table 3). The numbers of embryos transferred per donor collected in North America and Europe which may to some extent reflect the level of technology are however very close, being respectively 4.46 and 4.50.

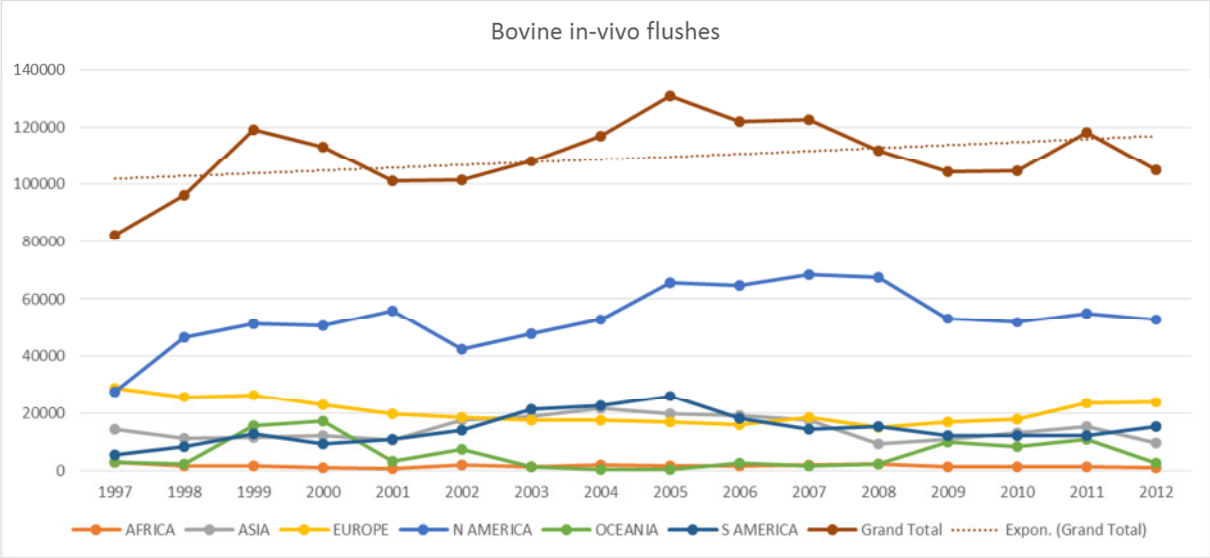
Table 3 Collection and transfer of embryos by region (adapted from Perry, 2013)

	Embryo collection		Embryo transfer	
Region	**Donors**	**% global**	**Total**	**% global**
Africa	1 107	1.09%	6 347	1.25%
Asia	9 494	14.37%	64 770	12.8%
Europe(*)	23 653	19.33%	106 463	21.05%
North America	52 701	50.87%	235 344	46.52%
Oceania	2 654	2.22%	15 050	2.98%
South America	15 274	12.12%	77 902	15.40%
Grand Total	**104 883**	**100.00%**	**505 876**	**100.00%**

(*) slight differences in Europe due to additional data collected by the IETS data retrieval committee.

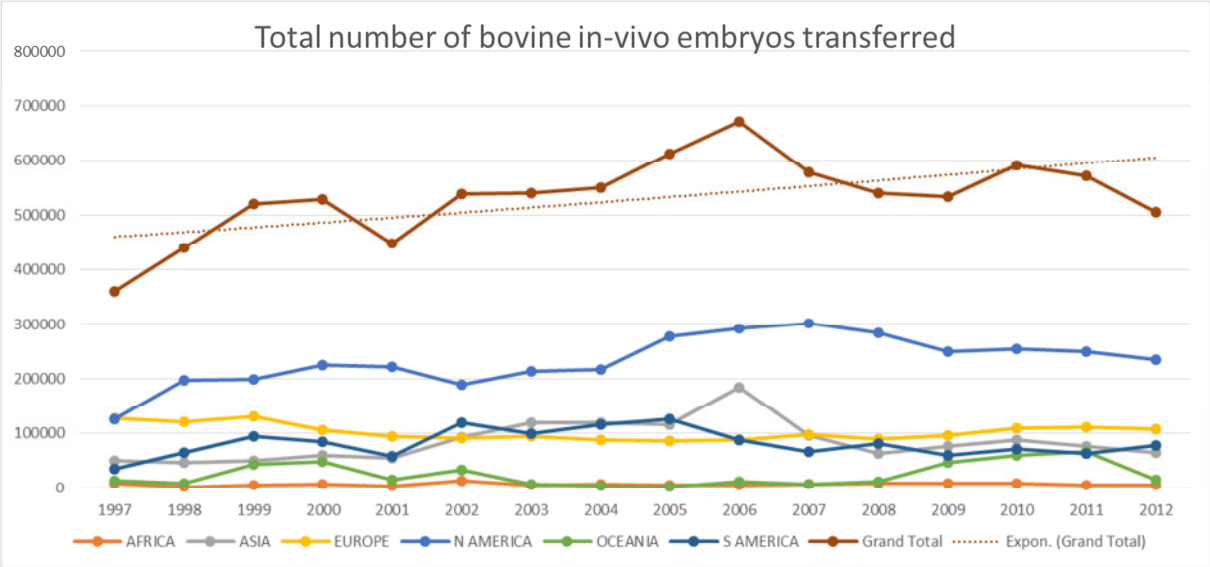
Regarding the trend over the years since 1997, Figure 1 shows a stabilization in the numbers of donors collected in Europe (yellow line) as opposed to a slight increase up to 2006 in North America (blue line) and then also a stabilization with similar trends for the two continents for those six last years.

Figure 1. Graph of IVD embryo collection per region and year (Perry, 2013)



The trend in the numbers of IVD cattle embryos transferred (Figure 2) is close to that described for the numbers of donors, indicating stabilization in both continents since 2006.

Figure 2 Total number of bovine IVD cattle embryos transferred (Perry, 2013)



1.2 In Vitro Produced (IVP) cattle embryos

AETE reported on IVP cattle embryos in 1997 (referenced year 1996) and the following years thanks to Y Heyman and his colleagues (Table 4).

Table 4. Numbers of IVP embryos collected and transferred in Europe in the late 90's (AETE, 1997-2000).

Year of reference	Year of Publication (AETE)	Number of embryos produced	Number of transferred embryos
1996	1997	7 952 (5.8%)*	6 621 (5.6%)
1997	1998	11 443 (7.3%)	7 748 (6.5%)
1998	1999	19 180 (12.0%)	14 113 (10.7%)
1999	2000	25 146 (14.7%)	13 389 (9.13%)

* The percentages refer to the percentages of IVP embryos on total (in vivo + in vitro)

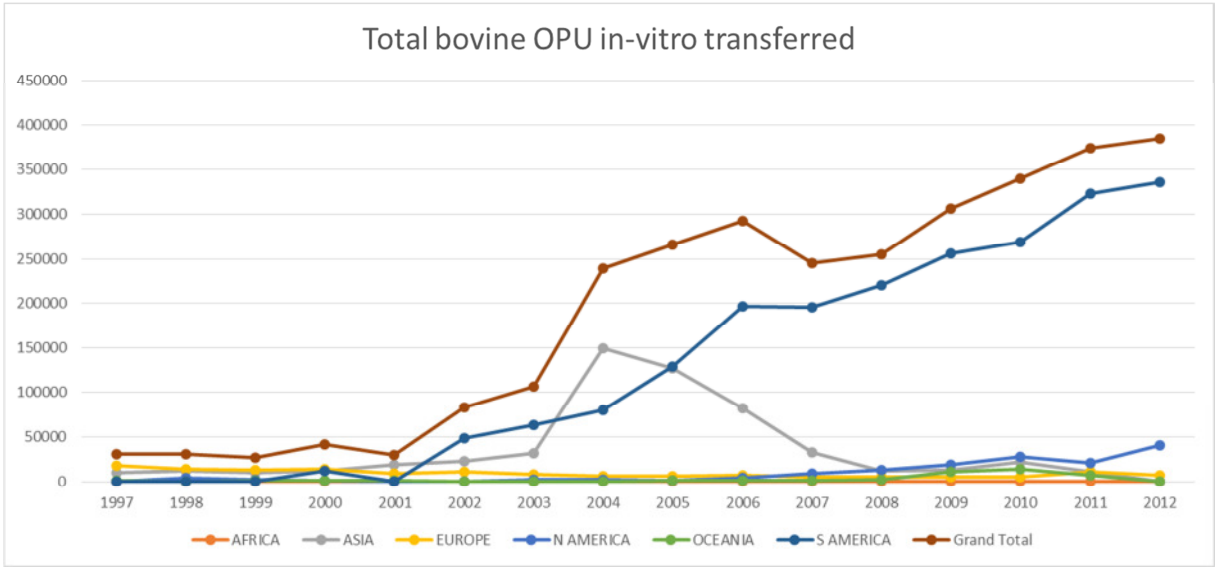
This table shows that the European ET industry successfully managed to bring to the ground embryos produced in vitro with significant numbers as soon as the mid 90's, with numbers doubling in 4 years' time and with a percentage of total embryos transferred of the magnitude of 10%.
As shown in Table 5, the worldwide total number of IVP embryos (close to 400 000) is now close to that of IVD embryos and the vast majority of those come from the South American region and particularly from Brazil. Europe does not seem quite as active as in other continents such as the two Americas and also Asia.

Table 5 Bovine in vitro embryo production (adapted from Perry, 2013)

Region	Ovum pick up		Abattoir		Total Embryos transferred
	Embryos produced	Embryos transferred	Embryos produced	Embryos transferred	
Asia	5 294	665	12 584	11 809	12 474
Europe	8 792	8 266	1 138	38	8 304
North America	74 242	40 546	200	1 916	42 462
South America	355 205	335 994	0	O	335 994
Oceania	0	125	0	0	125
Grand Total	**443 533**	**385 596**	**13 922**	**13 763**	**399 359**

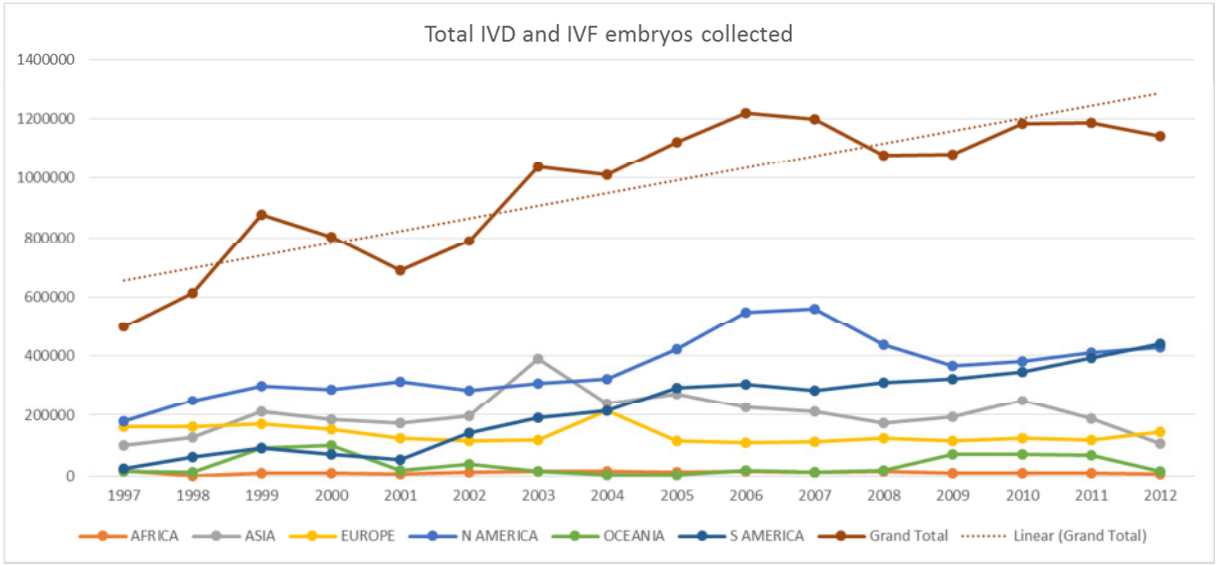
The trend over the years (Figure 3) as reported by Perry referring to OPU IVP cattle embryos is interesting in that it is likely that in the world, conversely to Europe, one will see the numbers of IVP embryos equal to those of IVD embryos before perhaps overcoming the latter in the near future.

Figure 3 Numbers of bovine OPU IVP embryos transferred (Perry, 2013)



As seen in figure 4 taking into account all the embryos transferred, both IVD and IVP all around the world, the industry is clearly growing mainly due to the IVP embryo activity in South America. Europe has not yet followed that worldwide trend.

Figure 4. Total cattle embryos transferred worldwide (Perry, 2013)



In considering those figures in a prospective approach, it is also interesting to realize that the impact of ET in the world wide cattle population is still very low (Table 6) allowing foreseeing some progress provided that the cost of this technology remains reasonable.

Table 6 Impact of AI and ET in the world wide cattle population (adapted from Thibier, 2005)

Regions	Total number of bovine females of breeding age(*)	Total first service AI	Impact ratio of AI (**) X100	Total number of IVD and IVP embryos(***)	Impact ratio of ET (****) X 100
Africa	51 577 000	870 892	1.68	6 347	0.01
North America	42 296 000	11 203 880	24.80	275 890	0.65
South America	124 460 000	1 366 678	1.09	413 896	0.33
Asia	236 850 000	58 181 005	24.56	65 435	0.02
Near East	23 433 000	1 068 991	4.55	0	0
Europe	61 750 000	37 738 142	61.11	114 099	0.18
Total	**543 276 000**	**110 429 588**	**20.32**	**875 667**	**0.16**

(*) 40% of total cattle and buffaloes)
(**) calculated from the total first service of AI / total number of females of breeding age
(***) data from Perry, 2013
(****) calculated from the total number of IVD and IVP embryos / total number of females of breeding age

1.3. European Economic impact in the international trade of embryos

It is hard to have a good estimate of the international movements of embryos for many reasons, including the non-reporting in some countries or else some illegal imports. However in trying to get such information from some countries which do have some international movements of cattle embryos, we were able to generate a set of data (Thibier and Wrathall, 2012) here reported in Table 7. These numbers are most certainly underestimated as informal information indicates that the real number of such IVP embryos internationally moved may, in some years, have exceeded 100 000.

Therefore this only gives an indication but, to some extent, this is consistent with the previous survey (Thibier 2009). As can be seen from those countries, Europe as a major importer of embryos is no surprise but it is true that data from South America were not available. Europe is not only a major exporter but also a very significant trader of bovine embryos within the European Union Member States.

Table 7. Bovine embryos exported and imported in 2010(*) (Thibier and Wrathall, 2012)

Region	Exports	Imports
Australia	4 077(**)	4 326
New Zealand	943	695
Canada	4 974	479
United States	10 280	1 794
European Union(***)	7 803	5 588
Grand Total	**27 134**	**12 882**

(*) These figures are for the year 2010 unless specifically mentioned. These data were provided by the official national agencies.
(**) For the year 2009.
(***) intra-Europe.

It is of notice that export/import numbers must be taken with caution due to many uncertainties, some countries do not publish data of this kind (particularly in Europe), some numbers from official agencies show discrepancies between practices or business records and the official data. However, from the 5 countries that reported their import/export data to the IETS data retrieval committee in 2013 (Argentina, Australia, South Africa, Canada, USA) the numbers reported (total: 34 700 IVD and 300 IVP embryos exported) grossly match with the numbers published in 2012 (Thibier & Wrathall, 2012), only Canada and the USA seem to have increased export of embryos significantly. Regarding the European countries, one might suggest that the Member countries with their AETE members try to collectively collect their import/export numbers hence showing that these European teams do also export embryos. Regrettably, it is not possible at the moment to retrieve such "official" data from the EU Commission as semen and embryo data are combined in their sheets of data.

In terms of values, Chillaud (2007) reported from a study made 8 years ago that the bulk of the cattle embryo market seems to be about 10 million euros (14.5 million US$) hence in the magnitude of 6% of the market of semen. Almost half of those embryos come from the USA (exported value in 2006: 4.7 million Euros (or 6.8 million US$) and a third from the EU (mainly France, Germany, Ireland, Italy and the Netherlands). The major importing countries are in decreasing order: EU, East Asia, Oceania and South America with respected values in million Euros of 2; 1.5, 0.5 and 0.3 million (or in US$: 2.9; 2.2;.72 and .43 million).

2. THE MAIN TECHNICAL REASONS FOR SUCH A SUSTAINABLE TECHNOLOGY IN EUROPE.

Obviously, if this technology is still quite active more than 30 years after its beginning, it just shows that the market is there and that the economic benefit the farmers get from applying it with its diverse possibilities, is real. It also quite clearly means that the results the practitioners perform every day are up to the expected high standard. We will here, according to my personal opinion, only briefly report on 3 main technical reasons for such a success in implementing this embryo transfer technology on the ground.

2.1 A technology well adapted to the needed improvement of the genetic value of the European cattle population.

It was early recognized that embryo transfer was the tool of choice in good genetic management in allowing generating particular bulls' sires with top breeding value and being the top of the pyramid of progeny and performance testing. This was particularly true for the sophisticated programs in place in Europe for dairy cattle. It has contributed to significantly improve the efficiency of such programs as judged by the mean annual improvement rate, notably in reducing

the interval between generations simultaneously to intensify the selection of candidates and improve the precision of such a selection. In this regard, it is of notice that AETE has always be a forum to exchange ideas about the best way to integrate those embryo transfers in such schemes. It was as early as 1989 that a detailed survey and simulations were reported by the invited lecturer of the 1989 AETE session, JJ Colleau on "the real impact of embryo transfer of the various animal breeding programs" (Colleau, 1989). Transfer of high value embryos became in the 1980-2005 the core of all the genetic programs and this explains also why in Europe, many ET teams were closely related to the artificial insemination centers or companies in charge of conducting the genetic improvement schemes.

When the genomic revolution occurred in the mid 2000's, it was impressive to see how quickly the ET industry embarked in such a revolution, modifying strongly the ET and dam schemes and providing the most useful service to the new schemes. With this genomic selection in effect, it is necessary to generate a larger number of candidates to evaluate in a given period of time. Whatever the strategy in place, it will be necessary to identify a large number of elite females which will then either be bred with sexed semen or from which a batch of IVP embryos would be collected in order to be later transferred on recipients. A good combination of semen and embryo biotechnologies will make it possible to act on all parameters involved to speed up the genetic gain (Ponsart et al., 2014).

2.2 A technology applied with well-trained and competent practitioners and continuous innovation.

One of the striking features in this area was, since the beginning, the high professionalism of all involved in embryo transfer. Very quickly, due to the critical importance of even the smallest of all details, the people in charge and all the staff both in veterinary clinics and artificial insemination cooperatives realized that training of excellence was absolutely necessary in order to obtain the best results possible. Good training and professionalism were key points to ensure the market would be sustainable and the farmers applying the technology keen on applying it in their own herds. In addition to training and professionalism, a third component was also retrospectively - and is still at this stage – a major feature of the ET industry, this being innovation. Innovation was constantly under scrutiny at both ends, that of daily practice and institutional research. This allowed the technology to positively evolve and the practitioners to keep abreast of discoveries of all kinds to the farmers' benefits.

In Europe, the AETE has been most instrumental in this area with the leadership of extraordinary pioneers as recognized as such worldwide by the Pioneer award of IETS (Table 8). In particular such pioneers in embryo transfer and Reproductive physiology as a whole, such as J Hahn and Ch Thibault were very supportive since the beginning, thanks to them.

Some of these IETS awardees were also recognized by the AETE and the first AETE awards were precisely given to J Hahn and Ch Thibault in 1993 and 1994 respectively. The list of all AETE awardees was published in 2009 (Ponsart et al., 2009).

In looking back at the table of contents of the AETE meetings, one has a very good idea of the evolutions of the ideas, and the techniques discussed and further implemented once back on the field following the meetings. In the first years, three topics dominated the meetings and most of the discussion in sessions or out at the coffee breaks. The first related to superovulation which was the main part of the meeting of the first year of AETE with published Proceedings and subsequent meetings. In 1987, there were 6 invited presentations on superovulation by scientists who further had a major influence on this technology worldwide, namely:

- Superovulation chez les bovins……..by J Saumande
- Induction of superovulation in cattle…….....by J F Beckers
- Improved embryo yield.. ……..…..by S J Dieleman
- Comparison of 2 to 3 days FSH treatment…….…by H Callesen
- Effect of LH on FSH induced superovulation……by D Chupin

The two other major topics treated at AETE at that time were fresh vs frozen in vivo derived embryos and modes of embryo transfer.

Ponsart et al., (2009) have reported the most "popular" topics presented at the annual meetings of AETE over the years up to 2009 and embryo transfer remains the most popular with 15 presentations over the 3 decades.

However very quickly, the Association extended its points of interest and as soon as 1988, in Lyon, I Wilmut presented a famous lecture on "Biotechnology and the bovine embryo: at present and in the future". This was way before the birth of Dolly… By the same token, it was in 1990 that two other famous lectures were given by G Brem on "State of the art, limitations and prospective of gene transfer in domestic mammals" and J P Renard on "the state of the art, limitations and prospective of cloning in domestic mammals". Those two lectures had a profound influence in the world's thought about biotechnologies in farm animals.

It is about at that time – late 80's- that in vitro production of embryos became also a major subject of oral presentations, posters, round tables and debates. The presentation by P L A M Vos and colleagues in 1990 on the methodology in use for applying what was since then called OPU was also one of the highlights of the innovation realized in Europe and presented and debated within the AETE.

In looking retrospectively, it is clear that one of the major reasons for this sustainability of ET in cattle in Europe is the constant search for innovation at both levels, practically on the animals' side and more fundamentally on the bench.

Table 8. Names of European IETS pioneer awardees

Name	Years	Names	Years
J Hahn	2013	A K Tarkowski	1991
I Wilmut	2011	Ch Thibault	1989
S Willadsen	2005	A L McLaren	1988
I Gordon	1998	E J C Polge	1987
S Winterberger Torres	1997	L E A Rowson	1985
R G Edwards	1993		

2.3 Embryo transfers: the safest mean of exchanging genes.

This statement was presented by Thibier in 1990 (Thibier, 1990) and was one of the major points claimed to the world and to the society as a whole, explaining why this critical comparative advantage from now on "allows any farmer in the world to obtain any gene that "Dame Nature" has provided the world with, at no risk health wise".

This statement results from a long chain of involvements and of strong sound research from the veterinary community in close association with the embryo transfer industry including that in Europe. Research in the early 80's showed that pathogens such as Foot and Mouth Disease virus for example behaved in a specific manner and in such a way that there was a possibility to eliminate pathogens associated with the embryo zona pellucida. Without getting into too many details which have been widely reported (Thibier 1990), it was then elaborated together with the scientists and practitioners one specific set of procedures to follow in order to secure this high level of safety. This relies on the concept of the "official embryo collection teams" further extended to the "official embryo production teams" which are officially designated and approved by the national veterinary authorities. The EU for example publishes the list of such official ET teams of which the total for the EU is of 229 to which it is added those from Norway and Switzerland: total 235. The countries with most ET teams are German, France and United Kingdom with respectively 39, 36 and 31.

This concept was somewhat opposed to what then (early 80's) was the dogma of the veterinary community to limit and prevent the extension of animal diseases. For example for AI centers, the policy relies on the fact that only specific disease free males are allowed to enter an AI center, itself free from such specific diseases. Here for ET, it was found uneconomic and impracticable in many circumstances and particularly in the European context to manage embryo donors in closed facilities such as AI centers.

A major step to allow the ET industry to work efficiently and safely was the organization of a round table in 1985 at the World Organization for Animal Health (OIE) which agreed in publishing and further implementing this concept of the "official embryo transfer team" to which is attributed all the responsibility to follow all the rules and guidelines put forward in order to prevent all association of pathogens with embryos. This was further "translated" in international guidelines on one hand and further legal Directives, laws, decrees or rules on the other hand. The former was the elaboration of the relevant chapters of the OIE Terrestrial Animal Health Code which hence on is the template of any legislation implemented domestically. Regarding the European Union, it is

with the guidelines elaborated following the 1985 OIE round table that was prepared, proposed and discussed the famous EU Directive 89/556/EEC published on 25 September 1989.

It should be here mentioned that some members of the AETE from different member countries played a key role in debating at OIE and in discussing the preparation of the directive 89/556/, exerting its expected influence as being members of the concerned industry and hence knowing what it was talked about.

And the end point, as published elsewhere (Thibier, 2011), ET has proven on the ground and for more than 30 years that indeed it is the safest mean of exchanging genes with no confirmed report of any contamination of the recipients or of the offspring from transfers of IVD or IVP embryos.

CONCLUSION

The European ET industry has shown these last 30 years its sustainability thanks to the excellent expertise of all the people involved and the favorable cost/benefit ratio to the farmers' benefits. It has mainly devoted its effort to the IVD embryos transfers taking its share of the worldwide activity of a little more than 20% with little fluctuation over the years, an additional indication of its sustainability. The IVP of embryos has not seemed so appealing to the European livestock industry and has hence fluctuated quite a bit over the years. It could rapidly increase in the near future in relation to the genomic revolution, the ET teams have the knowledge and expertise to produce such embryos.

In conclusion, the constant research of innovation, the excellent expertise following well designed training of the practitioners, their sense of responsibility in taking the most seriously the recommendations and rules regarding the health safety and precautions taken by the officially approved embryo transfer or production teams all explain the resulting level of excellence of this industry. Clearly, the conditions are currently met to foresee that this success story will continue as such in our European continent in the future.

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INSIGHTS INTO THE CELLULAR AND MOLECULAR RESPONSES OF BOVINE OOCYTES AND EMBRYOS TO ELEVATED TEMPERATURE

Z. Roth

Department of Animal Sciences, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University, Rehovot 76100, Israel

Email: roth@agri.huji.ac.il; Tel.: 972-8-9489103; fax: 972-8-9489552

Introduction

Given the intensive genetic selection for high milk production and the concomitant global climate warming, the reduced reproductive performance of lactating cows during the summer is expected to worsen in the coming years. Moreover, the effects of heat stress are not limited to the hot months; they carry over to the following cooler months, resulting in long-term effects throughout the year. Evaporative cooling is the most common strategy to maintain cows in normothermia. However, it cannot eliminate the decline in reproduction. The current review focuses on direct and indirect effects of thermal stress on reproductive functions, while providing new insights into the cellular and molecular responses of oocytes and preimplantation embryos to elevated temperature. This includes nuclear and cytoplasmic maturation, mitochondrial function and apoptotic pathways. The review also discusses some potential strategies to alleviate the effects of heat stress.

Effect of heat stress on the hypothalamic–pituitary–ovarian axis

The ovarian pool of follicles is highly sensitive to thermal stress. Given their lengthy developmental process, heat-induced alterations in small antral follicles (3–5 mm) can be expressed weeks later in impaired growth and function of medium-size (6–9 mm) and dominant follicles (Badinga *et al.* 1993, Wilson *et al.* 1998a, b). Heat stress compromises the functioning of the dominant and preovulatory follicles (Roth *et al.* 2000; de S Torres-Júnior et al. 2008), and is associated with changes in the endocrine milieu: reduced androstenedione and estradiol secretion, reduced inhibin concentration, and increased follicle-stimulating hormone (FSH) concentration in the plasma (Badinga *et al*. 1993, Wolfenson *et al*. 1995, Roth *et al*. 2000). These alterations carry over into the following seasons, as reflected by relatively low estradiol content in the follicular fluid in late summer and increased levels throughout the autumn (Roth *et al.* 2001b, 2004). Reduced estradiol concentrations during the follicular phase can lead to reduced duration and intensity of estrus and increased incidence of anestrus (Gwazdauskas *et al.* 1981). It might also affect the

preovulatory LH surge (Gilad *et al.* 1993; Wise *et al*. 1988) and disrupt the cascade of events leading to oocyte ovulation and meiosis resumption. As the oocyte achieves its developmental competence through the lengthy processes of folliculogenesis, perturbations in the physiology of the follicle-enclosed oocyte can potentially lead to its reduced competence for nuclear and cytoplasmic maturation and subsequent fertilization. Accumulated in-vivo and in-vitro evidence support this notion (Fig.1).

Moreover, the effects of heat stress are not limited to the hot months as they carry over to the following cooler months, resulting in long-term effects through the year on follicular steroidogenic capacity (Roth et al. 2001, Roth et al. 2004) and oocyte developmental competence (Roth et al., 2001a). In that respect, hormonal manipulation to enhanced turnover damaged follicle was recently tested by Friedman et al. (2011). Induction of three consecutive 9-day follicular waves with gonadotropin-releasing hormone and prostaglandin F2α (GnRh/PG) during the summer and fall improved conception rate in primiparous, in cows with a high body condition score (BCS) or low somatic cell count (SCC) than for multiparous cows or cows with a low BCS or high SCC.

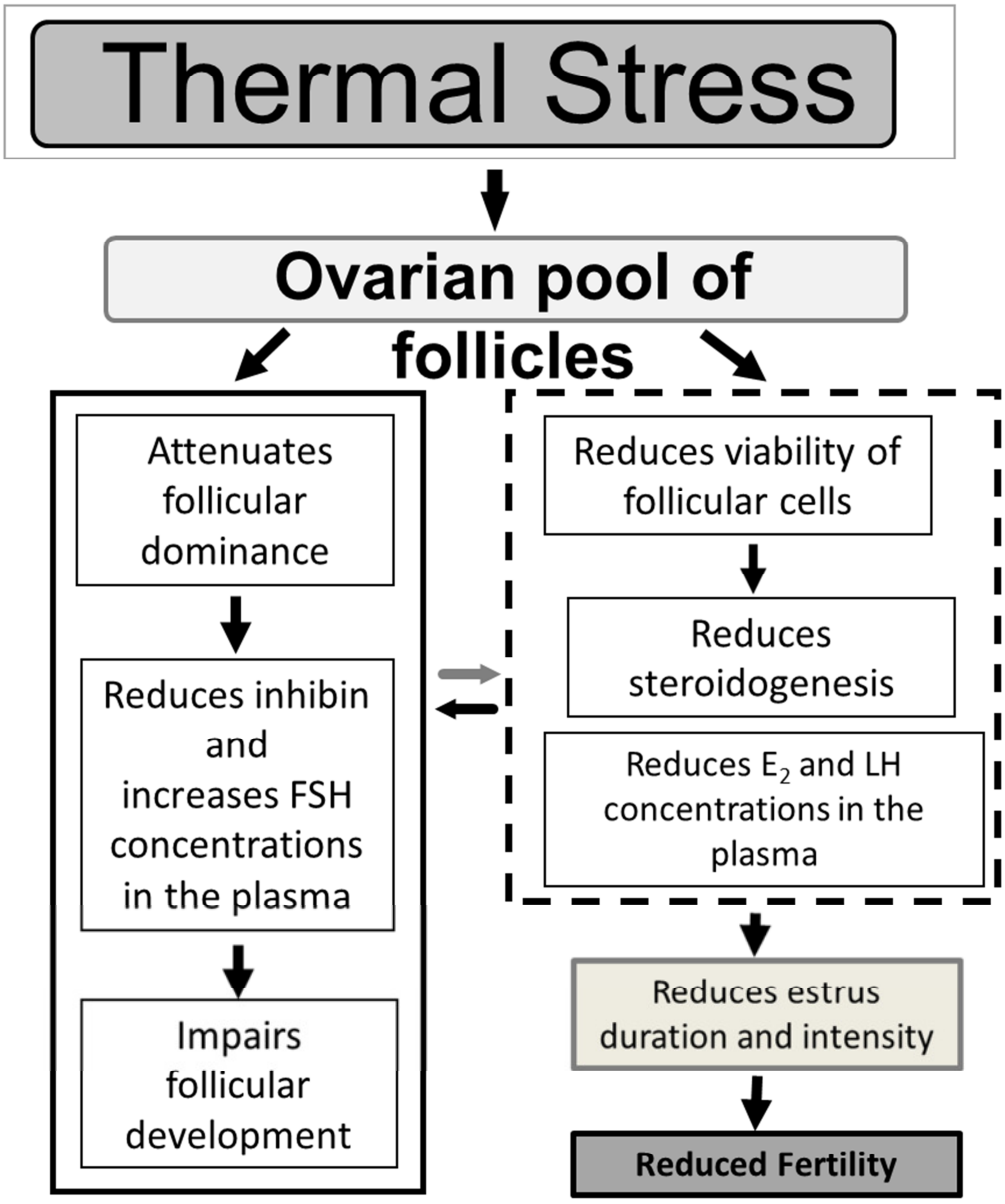

Fig. 1. Diagram illustrating the direct and indirect effects of thermal stress on the ovarian pool of follicles, reflected by impaired follicular growth (left panel) and function (right panel). The immediate effect of thermal stress is associated with alterations in the hypothalamic–pituitary–ovarian axis, expressed by reduced inhibin and estradiol concentrations and increased FSH concentration in the plasma. These alterations indicate impaired function of the dominant follicle which in turn might impair estrus behavior and the cascade of events leading to oocyte ovulation and successful fertilization.

Effect of heat stress on the oocyte nucleus

Mammalian oocytes are arrested at the prophase stage of the first meiotic division. During the maturation process, the oocyte undergoes multiple nuclear events, including resumption of meiosis, formation of the second metaphase (MII) plate and extrusion of the first polar body (Smith 2001). Exposing bovine oocytes to heat shock during maturation impairs both microtubulin and microfilaments, which are involved in nuclear and organellar transport. As a result, most heat-shocked oocytes fail to undergo maturation and are arrested at the metaphase I (MI) to MII stages (Roth & Hansen 2005; Fig. 2). Similar to the reduction reported for seasonal thermal stress, in-vitro studies have shown that exposing oocytes held at the germinal vesicle (GV) stage to heat shock reduces the proportion of oocytes that progress to MII (Payton et al. 2004; Gendelman & Roth 2012a). Heat shock has also been reported to induce perturbations of the spindle apparatus in porcine (Ju & Tseng 2004) and bovine (Ju *et al.* 2005, Roth & Hansen 2005) oocytes. These alterations most likely underlie the reduced proportion of oocytes that undergo successful fertilization and further cleave and develop to blastocysts (Roth & Hansen 2004a, b), 2005). Nevertheless, while some studies have shown a reduced cleavage rate after fertilization (Gendelman et al. 2010), others have reported that season does not affect the first cleavage division (Al-Katanani et al. 2002).

Roth and Hansen (2005) reported on heat-induced activation of apoptotic cascades through the intrinsic mitochondrial pathways. Exposing oocytes to 41ºC during maturation increased the proportion of oocytes expressing high activity of group II caspases (i.e., caspases 2, 3 and 7) and nuclear fragmentation (Roth & Hansen 2004a, b). Similarly, heat shock during maturation decreased the proportion of oocytes with low mitochondrial membrane potential and increased the proportion of those with TUNEL-positive chromatin (Soto & Smith 2009). The mitochondrial apoptotic pathway involves alterations in the phospholipid membrane (Lahorte et al. 2004) and activation of the sphingomyelin pathway (Hannun & Luberto 2000) at earlier cascade stages. Kalo and Roth (2011) showed that exposing bovine oocytes to heat shock induces externalization of phosphatidylserine (Annexin-V assay) in association with ceramide generation. The specific inhibitor desipramine hydrochloride, used to block acid sphingomyelinase, was found to inhibit ceramide formation through the hydrolytic pathway. On the other hand, ceramide can be produced de novo from condensation of serine with palmitoyl-CoA (Jenkins et al. 2002). Use of fumonisin B1 to block dihydroceramide synthase was found to inhibit ceramide formation through de-novo synthesis (Kalo & Roth 2011). In support of this, administering the antiapoptotic molecule sphingosine-1-phosphate (S1P) to modulate the ceramide–S1P

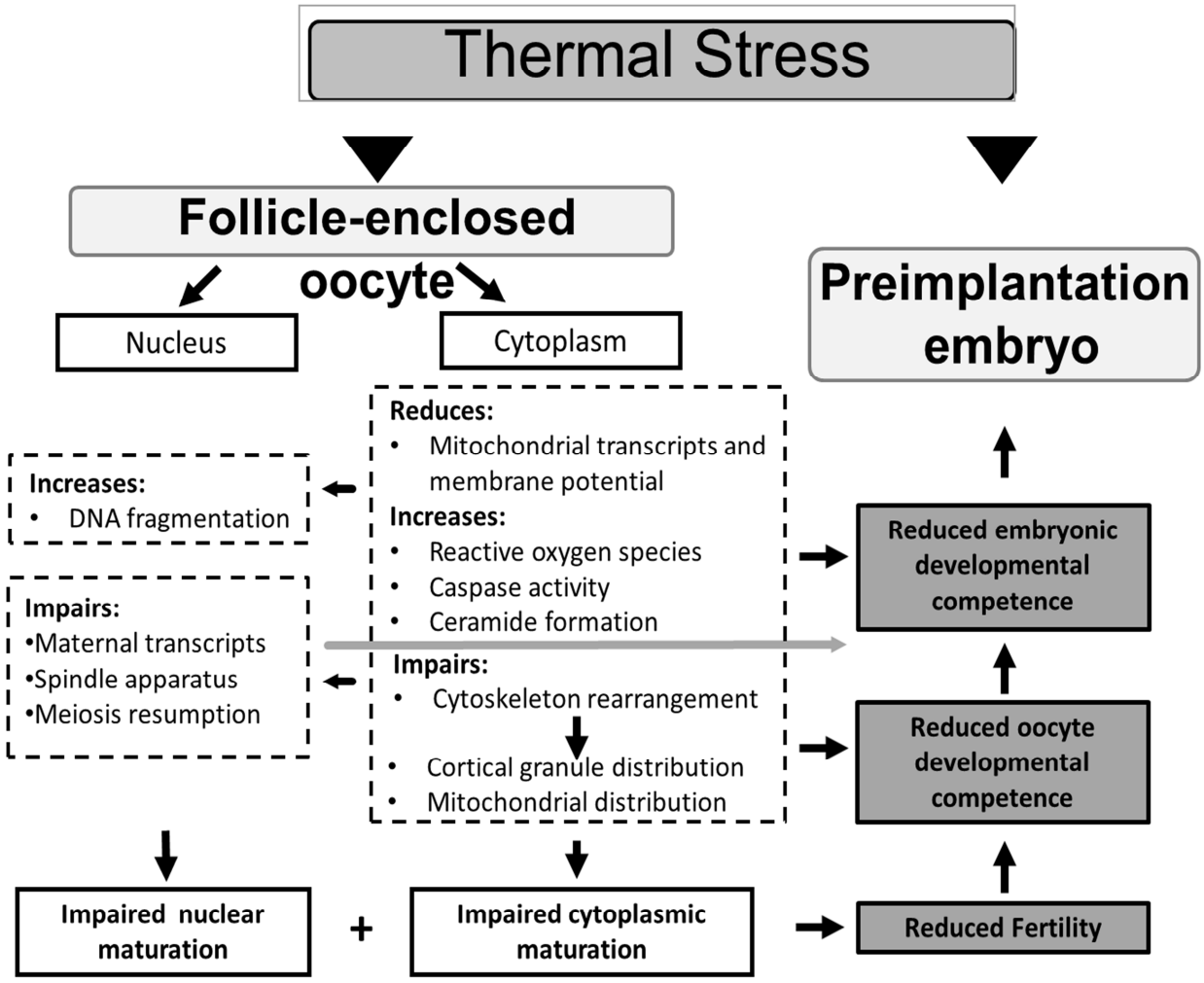

Fig. 2. Diagram illustrating the direct and indirect effects of thermal stress on developmental competence of the oocyte and preimplantation embryo. Exposing oocytes to elevated temperatures induces multiple cellular and molecular alterations in both nuclear and cytoplasmic maturation. Heat-induced perturbations in oocyte maturation are suggested to underlie the reduced fertility during the hot season. Moreover, alterations in the oocyte organelles, for example mitochondria, might subsequently reduce embryonic development. A direct effect of thermal stress on preimplantation embryos is also evident.

balance during oocyte maturation reduced the proportion of TUNEL-positive oocytes. Inhibition of apoptosis with the caspase inhibitor Z-DEVD-FMK (Roth & Hansen 2004a) or BH4 peptide (Soto & Smith 2009) reduced the deleterious effect on oocyte developmental competence. Insulin-like growth factor I (IGF-I) also plays a thermoprotective role in GV-stage and MII-stage oocytes (Paula-Lopes FF et al. 2013). Thus, various molecules have been shown to minimize the effect of heat shock in vitro, but none of them are being used in practice.

Effect of heat stress on oocyte cytoplasmic maturation

Cytoplasmic maturation consists of multiple cytoplasmic events which are essential for fertilization and early embryonic development (Eppig 1996, Brevini-Gandolfi & Gandolfi 2001, Watson 2007). These include reorganization of the cytoplasmic organelles, such as cortical granules, mitochondria, and endoplasmic reticulum, through the actions of cytoskeletal microfilament and microtubules (Ferreira et al. 2009, Yamada & Isaji 2011). In bovine, the cortical granules' distribution pattern changes from dispersed throughout the cytoplasm at the GV stage to a thin layer lining the oolemma at the MII stage (Hosoe & Shioya 1997, Izadyar et al. 1998). In that respect, Payton *et al.* (2004) reported that heat shock impairs cortical granule translocation. In addition, at the GV stage, the mitochondria are restricted to the peripheral region of the oocyte, whereas at the MII stage, they are relocated into a diffuse pattern throughout the center of the cytoplasm (Kruip et al. 1983, Stojkovic et al. 2001). Gendelman and Roth (2012b) reported that season has no effect on the number of oocyte mitochondria, but mitochondrial distribution within the oocytes differs between summer and winter. Taken together, studies indicate that heat shock induces alterations in not only nuclear but also cytoplasmic events associated with oocyte maturation (Fig. 2).

In bovine, the increase in embryonic mitochondria is initiated at the blastocyst stage (Smith et al. 2005). Given that the maternal mitochondria stored within the oocyte are the primary energy source for further embryonic growth, perturbation in mitochondrial function might lead to failure in embryonic development postfertilization. A seasonal study reported that the proportion of oocytes with high mitochondrial membrane potential is relatively low during the summer (Gendelman & Roth 2012b). Furthermore, season-induced alterations in the expression of both nuclear (*SDHD* and *ATP5B*) and mitochondrial (*MT-ND2, MT-CYB, MT-CO2*) genes have been recently documented. These genes are involved in oxidative phosphorylation and therefore, impairment in the electron-transport chain might lead to reduced ATP levels. On the other hand, addition of coenzyme Q10, a ubiquitous free-radical scavenger and a key component of the mitochondrial respiratory chain, increased the proportion of polarized mitochondria and improved embryonic development (Gendelman & Roth 2012b; Fig. 2).

Effect of heat stress on oocyte developmental competence

Seasonal studies have provided evidence that oocytes harvested from cows during the summer exhibit reduced ability to develop to the blastocyst stage after in-vitro fertilization (Al-Katanani et al. 2002; Gendelman et al. 2010). Moreover, a period of two to three estrous cycles is required for recovery from summer heat damage and appearance of developmentally competent oocytes (Roth et al. 2001a). The ratio between 2- and 4-cell-stage embryos (42 h postfertilization) is

higher in the hot (May–November) vs. cold (December–April) season, indicating a delay in the two first embryonic divisions. As early-cleaving zygotes are more competent to develop to the blastocyst stage than late-cleaving zygotes, it appears that embryos developed during the hot season have inferior developmental competence (Fig. 2).

In bovine, embryo transcription is initiated when the embryo is at the 8- to 16-cell stage. Thus, during earlier stages, the embryo depends on oocyte mRNA (Memili & First 2000). Gendelman and Roth (2012a) showed that both seasonal and induced thermal stress impair the expression of maternal transcripts (C-MOS, GDF9, POU5F1 and GAPDH) involved in oocyte maturation and early embryonic development. In particular, at the MII stage, transcripts for all of the examined genes were lower in oocytes collected during the hot vs. cold season, presumably due to higher mRNA degradation. Reduced POU5F1 levels first appeared at the MII stage, when transcription is very limited, and at later developmental stages, before (i.e. 2-, 4-, and 8-cell stages) and after (i.e. 8–16-cell stage) embryonic genome activation. In mice, POU5F1 level governs embryo fate (Niwa et al. 2000) and therefore, the low expression of POU5F1 found in bovine embryos might also explain their low developmental competence during the hot season.

Heat stress and early embryonic development

While much of the effect of heat stress involves alterations in the follicle-enclosed oocyte, preimplantaion embryos are also sensitive to elevated temperature, in a stage-dependent manner (Fig. 2). Embryos at early developmental stages are more susceptible to thermal stress, and become more resistant at later developmental stages (Hansen 2007a, b). Exposing cows to elevated temperatures between onset of estrus and insemination (Putney *et al.* 1988) or on day 1 after estrus decreased embryonic development (Ealy *et al.* 1993), but this effect declined as embryonic development proceeded. Similarly, induction of heat shock in vitro adversely affected the development of 2-cell-stage embryos, had a moderate effect on 4- to 8-cell-stage embryos, and only a limited effect at the advanced morula stage of embryonic development (Hansen 2007a). Exposing embryos to elevated temperature decreased development to the blastocyst stage when performed at the zygote stage but not when exposure occurred at the morula stage (Sakatani et al., 2012). Nevertheless, the mechanism underlying thermotolerance acquisition is not clear cut and seems to involve various factors.

Synthesis of heat shock 70 kDa protein 1A (HSPA1A) increased in zygotes upon heat exposure (Sakatani et al. 2012), and was found to be higher in 2-cell-stage embryos than in the morula (Edward & Hansen 1996; Hansen, 2013). Therefore, HSPA1A might not be the controlling factor for acquisition of thermotolerance.

A change in the balance between reactive oxygen species (ROS) generation and glutathione level has been recently suggested for bovine embryos, with ROS generation declining and the intracellular concentration of glutathione increasing as the embryo develops (Hansen, 2013).

Heat shock differentially affects embryonic development depending on breed—the effect is lower in Brahman, Romosinuano and Nelore, and higher in Angus and Holstein (Paula Lopes et al. 2003, Hernández-Cerón et al. 2004, Barros et al. 2006). In support of this, heat-induced changes in the expression of genes related to embryonic development and implantation (*PLAC8, CDX2, HSF1* and *COX2)* has been found to differ between breeds (Silva et al. 2013).

The balance between pro- and antiapoptotic factors might also play pivotal role in embryonic survival. In cattle, heat-induced apoptosis does not occur until the 8–16-cell stage and can be inhibited with caspase inhibitor, resulting in improved embryonic survival (Paula-Lopes & Hansen 2002). In agreement with this, IGF-I administration to in-vitro-derived embryos improved embryo resistance to heat shock (Jousan & Hansen 2004, 2007). Moreover, transfer of in vitro-produced embryos treated with IGF-I increased pregnancy rate in heat-stressed cows (Block et al. 2007). However, treatment of lactating cows with bovine somatotropin (bST) to increase IGF-I concentration did not have any positive effect on first- or second-service pregnancy rate during the summer (Jousan & Hansen 2007).

Given that oocytes and embryos are highly sensitive to heat stress at early stages of development, an approach based on transferring 8-day embryos to bypass the thermosensitive developmental stages has been developed and shown to improve fertility during the hot season. Embryo transfer during the summer results in pregnancy rates similar to those achieved with artificial insemination or embryo transfer in the winter (for review see Hansen 2013). One of the advantages of this methodology is escaping the harmful effect of heat stress on the oocyte and bypassing loss of pregnancies caused by earlier damage to the oocyte and preimplantation embryos. It should be noted, however, that pregnancy rate following embryo transfer was compromised when the recipient could not maintain normal body temperature (Vasconcelos *et al.* 2006). Use of an efficient cooling system to maintain normothermia in the recipient cows is thus critical, as it might alleviate the deleterious effects of heat stress on the uterus and/or corpus luteum functions.

Summary

In-vitro and in-vivo studies have explored the multifactorial effects of heat stress on ovarian function. These effects include alterations in follicular growth, steroid production and hormonal secretion, and reductions in oocyte and embryonic developmental competence. Cooling is a prerequisite for any additional strategy and should be performed over long periods of time. Recent studies have examined hormonal treatments with GnRH/PG to accelerate follicular turnover by

removing damaged oocytes. Treatments were found successful for a subpopulation of cows but had only a limited impact on the herd as a whole. The effect of heat stress on the oocyte and early-cleaved embryo can be bypassed by transferring thermoresistant embryos. However, none of these suggested approaches have yet been widely implemented on farms.

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INBREEDING, GENOTYPING AND GENOMIC SELECTION IN DAIRY CATTLE BREEDING PROGRAMS

A.P.W. DE ROOS

CRV, PO Box 454, 6800 AL Arnhem, the Netherlands

During the last decade, genomic selection has been developed and implemented in all major dairy cattle breeding programs globally. This paper describes its development and impact on the dairy cattle breeding programs, with special emphasis on inbreeding management and the role of nucleus programs.

Traditionally, dairy cattle breeding programs were based on progeny testing schemes in which bulls were selected for AI at about 5 years of age, after a first batch of daughters came into milk. Advancements in DNA technology have made it possible to genotype animals for tens of thousands of DNA markers, especially SNP (single nucleotide polymorphisms), at relatively low cost. This enabled breeding organizations and scientists to compute genomic breeding values based on large reference populations, i.e. data sets of progeny tested bulls combining phenotypes (from daughter performance information) and genotypes (from SNP genotyping). The most elite young animals are subsequently used for breeding, allowing a substantial reduction in generation interval and an increased rate of genetic improvement by more than 50%.

Although genetic selection always leads to some (intended) loss of genetic variation, the rate of inbreeding needs to be controlled to avoid detrimental effects on fitness. This is achieved by optimizing selection for (genomic) total merit index and (genomic) kinship level, i.e. using lower index thresholds for animals that are less related to the rest of the breeding population. Secondly, there is a commercial drive for AI organizations to offer a broad portfolio of AI bulls that allows farmers to avoid matings that lead to too much inbreeding. As the generation interval is much shorter in genomic selection programs, there is a risk that the rate of inbreeding, when expressed per year rather than per generation, increases too rapidly.

The development of genomic selection has had enormous effects on the role of females in breeding programs. Firstly, the genetically most elite females can be identified accurately at young age. Secondly, genetic progress can be enhanced substantially by intense selection of calves from a large group of siblings. As a result, breeding organizations have invested heavily in finding the elite females, controlling them, and maximizing their reproductive capacity. An increasingly larger proportion of the AI bulls is coming from in-house nucleus programs.

In the coming years breeding organizations and scientists will collaboratively invest in technology and on-farm data collection to develop and improve genomic evaluations of cattle for traits that help to improve the efficiency and health of dairy cows. In addition, the importance of nucleus programs will continue to grow, leading to further consolidation and investment in reproductive technologies.

NEW INSIGHT ON CRYOPRESERVATION OF OOCYTES AND EMBRYOS

TERESA MOGAS

Departament de Medicina i Cirugia Animals. Universitat Autònoma de Barcelona. Spain

Abstract. The cryopreservation of mammalian oocytes and embryos has become an integral part of assisted reproduction in both humans and veterinary species. Over the past forty years, the field has seen much progress mainly as the outcome of research in humans, cows and mice. However, the methods used to cryopreserve oocytes and embryos still have several shortcomings and intense research efforts have been dedicated to improving and optimizing methods of cryopreservation. In the animal reproduction field, vitrification techniques are quickly gaining acceptance as a viable and promising alternative to traditional slow-cooling/rapid-thaw cryopreservation protocols. Vitrification is especially useful when dealing with oocytes or *in vitro* produced or micromanipulated embryos since it is simple, rapid and does not require programable cell freezers. In addition, fewer equilibration and cryopreservation steps are needed. The key to the success of vitrification lies in procedures that minimize the formation of intra-cellular ice crystals when an oocyte/embryo and the surrounding vitrification solution are "glassified". To date, however, vitrification has had limited practical applications in veterinary reproduction. The reason for this could be the reduced developmental competence of female gametes after vitrification or the lack of an standard methodology for embryo vitrification that facilitate its use in field conditions in that the warming and transfer of the vitrified embryos required similar technical skills to the transfer of thawed slow-frozen embryos. In this way, several advances and strategies has been developed to approach vitrification/warming procedures to the veterinary species.

GERMPLASM CRYOPRESERVATION

Two basic techniques are currently used to cryopreserve oocytes and embryos: slow-rate freezing or vitrification.

The slow freezing technique, developed independently by Wilmut (Wilmut, 1972) and Whittingham et al. (Whittingham *et al.*, 1972), involves lowering the temperature of a chamber in a controlled, stepwise manner. Different modifications of this technique have been tested but they all have in common the same basic steps. Generally, the germplasm is gradually exposed to relatively low concentrations of permeating cryoprotectants (CPAs) resulting in a final concentration of 1.0–1.5 M for oocytes or 1.35–1.5 M for embryos: the aim being to reduce CPA toxicity while minimizing osmotic stress. The germplasm is then loaded into straws and placed in a programable freezer at a temperature range slightly below -0ºC (from -4 to -9ºC). Within this interval, extracellular ice formation (seeding) is induced and samples are then cooled slowly, at a rate of about 0.3–0.5ºC/min (<1ºC/min; (Mazur, 1990). Thus, using a very slow cooling rate, extracellular water crystallizes, resulting in an osmotic gradient that draws water from the intracellular compartment and the

gradual dehydration of the cell together with the gradual intracellular diffusion of additional permeating CPA through an equilibrium process. Once sufficient cellular dehydration has been achieved (by cooling to temperatures between -30 and -65ºC), the straws are plunged into liquid nitrogen (LN_2), and the remaining non solidified solution is converted into a "glassy", vitrified state. During thawing, a rapid transition of temperature is preferred to prevent recrystallization of water with the potential for ice-crystal damage. The CPA, which is now at a very high concentration in the intracellular space, can be removed via the stepwise dilution of CPA or using an additional nonpermeating CPA which will act as an osmotic buffer allowing the CPA to be removed without excessive cellular water uptake (Friedler *et al.*, 1988; De Santis and Coticchio, 2011).

The second technique is vitrification. Vitrification refers to the vitreous, transparent, ice-free solidification of water-based solutions at subzero temperatures. The physical definition of vitrification is the solidification of a solution (glass formation) at low temperatures without ice crystal formation. The phenomenon can be regarded as an extreme increase in viscosity and requires either rapid cooling rates or the use of CPA solutions, which impair ice crystal formation and increase viscosity at low temperatures. Vitrification of water inside cells can be achieved in two ways: 1) increasing the speed of temperature conduction or 2) increasing the concentration of cryoprotectant. A practical limit to attainable cooling speed exists, as does a biological limit for the concentration of cryoprotectant tolerated by cells during vitrification. Therefore, the aim of any vitrification protocol is to reach the ideal balance between maximizing the cooling rate and minimizing the cryoprotectant concentration (Liebermann *et al.*, 2002a; Smith *et al.*, 2011; Saragusty and Arav, 2013).

Although slow freezing is the most widely used cryopreservation technique, vitrification is a viable and promising alternative that is increasingly attracting attention because of its economic repercussions. Different reports comparing conventional embryo slow freezing and vitrification have described either equal or better *in vitro* or *in vivo* survival rates following vitrification (van Wagtendonk-de Leeuw *et al.*, 1997; Agca *et al.*, 1998). So far, the vitrification of oocytes and embryos has been tested in several species with good results. Among its benefits, it does not require sophisticated equipment to manage the cooling rate, it uses a small amount of liquid nitrogen, it is less time-consuming, and can be done relatively cheaply and even under field conditions with no need for special equipment (it can be performed using a simple foam box). However, vitrification is a more technically demanding process that requires more experience to master than slow freezing. Hence, the operator must accurately and quickly pipette small volumes of viscous vitrification media containing the oocyte(s) or embryo(s) onto or into the vitrification container. The technical demands of slow freezing are generally restricted to the seeding process. On the other hand, vitrification of one or a few embryos is quite convenient and considerably faster than slow freezing the same number of embryos. However, calculating the time for a larger number of embryos to be cryopreserved, the difference is minimal compared to slow-freezing.

OOCYTE VITRIFICATION

Interest in oocyte cryopreservation has been mounting because of the wide use of oocytes for procedures such as *in vitro* embryo production, nuclear transfer or gene banking. The long-term storage of oocytes has benefits such as it allows for storage of female genetic material unfertilized until an appropriate male germplasm is selected, facilitates assisted reproductive procedures and serves to preserve genetic material from animals that die unexpectedly (Ledda *et al.*, 2001). As for semen, oocyte cryopreservation is beneficial for the international exchange of germplasm, as it avoids injury and the health risks of live animal transportation (Pereira and Marques, 2008). In humans, oocyte cryopreservation offers advantages over embryo freezing including preserving fertility in women at risk of losing their fertility due to cancer treatment or chronic disease, egg donation, and postponing childbirth, and avoids religious and/or other ethical, legal, and moral concerns about embryo freezing.

These benefits have determined that research efforts have focused on improving the efficiency of oocyte cryopreservation. Thus far, survival rates of cryopreserved oocytes from small rodents and humans are high, adapting well to maintaining the vast number of gene-modified transgenic strains and their efficient use in therapies for human infertility. In contrast, in cattle there have only been limited reports of blastocyst and subsequent calf development from slow-frozen or vitrified oocytes and results remain inefficient *(Hamano et al., 1992; Saha et al., 1996a; Vajta et al., 1998; Zhou et al.*, 2010).

The loss of developmental potential after cryopreservation makes mammalian oocytes probably one of the most difficult cell types to cryopreserve. Indeed, the survival and developmental capacity of the cryopreserved oocyte are greatly impaired, probably as a consequence of morphological and cytological damage induced by the cryopreservation process. The factors most likely to affect the success of oocyte cryopreservation are the particular structural and functional characteristics of the oocyte, such as its size, cumulus-oocyte complex, maturation status, the dynamic nature of its subcellular organelles, active biochemical processes and meiosis (Thibault *et al.*, 1987; Leibfried-Rutledge *et al.*, 1989).

Maturation stage of the oocyte

The nature of cryodamage, how this damage is assessed and its implications vary according to the developmental stage of the oocyte. Oocytes collected from *in vivo* pickup or at slaughter are at the germinal vesicle (GV) stage at which genetic material is contained within the nucleus. Since there is no spindle present, GVs are assumed to be less prone to chromosome and microtubular damage during cryopreservation. However, oocytes can also be cryopreserved at the metaphase II (MII) stage of maturation. The MII oocyte has a very special structure (i.e., large size, very sensitive to low temperatures, extremely fragile, high water content, a low surface to volume ratio, presence of the spindle and other cell organelles, not optimal plasma membrane permeability to CPA and water, etc.) making the cell very sensitive to chilling and susceptible to intracellular ice formation. The spindle is crucial for the events following fertilization including the completion of meiosis, second polar body formation, migration of the pronuclei, and formation of the first mitotic

spindle. Damage (depolymerization) and/or absence of the spindle compromise the ability of the oocyte to be fertilized and undergo normal preimplantation development. In addition, hardening of the zona pellucida (ZP) due to premature cortical granule (CG) exocytosis—as a result of cryopreservation—can adversely affect the normal fertilization process. As a consequence of CPA toxicity and/or chilling injury, the spindles of oocytes cannot correctly sustain the chromosomes at the metaphase plate prior to polar body extrusion, leading to chromosome dispersion, an increased incidence of aneuploidy or polyploidy, and termination of embryonic development (reviewed in Jain and Paulson, 2006; Smith *et al.*, 2011; Diez *et al.*, 2012; Clark and Swain, 2013; Saragusty and Arav, 2013). In immunocytological studies performed at our laboratory on MII-stage cow and pig oocytes vitrified by the open-pulled straw (OPS) method, higher percentages of oocytes showing abnormal spindle and chromosome configurations were observed when compared to fresh oocytes. Changes detected in oocytes after vitrification/warming included chromosome dispersal or decondensation, microtubule depolymerization or disorganization and an altered or lack of spindle structure (Rojas *et al.*, 2004; Albarracín *et al.*, 2005a; Albarracín *et al.*, 2005b). Similarly, when oocytes were vitrified by the OPS procedure, greater oocyte percentages showed the discontinuity or absence of cytoskeletal actin structure when compared to a control group both in cattle (Albarracín *et al.*, 2005b) and pigs (Rojas *et al.*, 2004). When calf and cow oocytes were vitrified by the OPS method, significantly lower percentages of oocytes with a normal and peripheral CG distribution were observed, and most CGs occurred in the cortical area away from the plasma membrane (Morató *et al.*, 2008a).

Immature oocytes do not have an organized meiotic spindle, and so cryopreservation at this stage may be an alternative approach. Indeed, immature bovine oocytes have been cryopreserved successfully using both slow freezing or vitrification methods, giving rise to blastocysts (Küchenmeister and Kuwayama, 1997; Le Gal and Massip, 1998; Prentice-Biensch *et al.*, 2012), pregnancies and newborns (Suzuki *et al.*, 1996; Vieira *et al.*, 2002). However, rates of *in vitro* embryo development are still very low. Several authors have reported that GV oocytes are more sensitive to chilling or cryopreservation than MII oocytes, mainly due to irreversible structural damage to the oocyte membrane (Arav *et al.*, 1996) or impaired intercellular communication between the oocyte and the cumulus cells after cryopreservation of GV oocytes (Fuku *et al.*, 1995). In a study in which the efficiency of oocyte vitrification at two meiotic stages, GV and MII, was compared, we observed that oocytes vitrified at the GV or MII stage exhibited lower percentages of normal spindle configuration, actin distribution and cleavage rate than non-vitrified oocytes, although results from both meiotic stages did not differ significantly. None of the vitrification procedures yielded blastocysts, irrespective of the initial meiotic stage (Albarracín *et al.*, 2005a). Conversely, Zhow et al. (Zhou *et al.*, 2010) reported significantly higher cleavage and blastocysts rates for the vitrification of GV oocytes compared with MII stage oocytes.

Inhibition of meiosis using a variety of chemicals that interfere with the cAMP transduction pathway is thought to reproduce the period necessary for cytoplasm maturation, allowing the oocyte

to synthesize and store mRNA and protein. The use of meiotic inhibitors such as roscovitine or butyrolactone improves the developmental competence of oocytes without reducing blastocyst rates (Mermillod *et al.*, 2000; Diez *et al.*, 2005) yet these inhibitors have not served to improve oocyte survival following vitrification (Albarracín *et al.*, 2005a; Diez *et al.*, 2005). In addition, vitrified oocytes subjected to meiotic arrest have returned high oocyte degeneration rates, regardless of the timing of vitrification (Diez *et al.*, 2005).

Cytoskeleton stabilizers

Stabilizing the cytoskeleton system during vitrification could be beneficial for improving the post-thaw survival and subsequent development of vitrified oocytes. Several cytoskeleton stabilizers have been used to reduce oocyte and embryo damage during vitrification including cytochalasin B (Fujihira *et al.*, 2004; Schmidt *et al.*, 2004; Silvestre *et al.*, 2005), cytochalasin D (Vieira *et al.*, 2002) and Taxol (Park *et al.*, 2001; Kyono *et al.*, 2002; Fuchinoue *et al.*, 2004; Schmidt *et al.*, 2004; Fujihira *et al.*, 2005; Shi *et al.*, 2006). Taxol™ (paclitaxel) is a diterpenoid taxane used as an antineoplastic agent in patients with ovarian cancer, metastatic breast carcinoma, and non-small cell lung carcinoma (Rowinsky *et al.*, 1990). At lower doses, Taxol stabilizes the kinetics of spindle microtubules (Jordan *et al.*, 1993). The addition of Taxol to the vitrification solution has been found to improve the post-warming development of immature human oocytes (Kyono *et al.*, 2002; Fuchinoue *et al.*, 2004) and mature mouse (Park *et al.*, 2001), pig (Shi *et al.*, 2006) and cow oocytes (Schmidt *et al.*, 2004). When the effects of pre-treating the oocytes with 1 µM Taxol on chromosome organization, spindle morphology and CG distribution were examined, Morató et al. (Morató *et al.*, 2008a; Morató *et al.*, 2008b) observed that Taxol stabilized spindle configuration and promoted CG migration and early embryo cleavage when cow oocytes where subjected to OPS vitrification. Contrarily, Fujihira et al. (Fujihira *et al.*, 2005) observed no positive effects of Taxol on the developmental capacity of vitrified *in vitro* matured pig oocytes using cryotops.

Carrier systems

To achieve the rapid cooling rates needed for vitrification, oocytes are placed in small volumes of media containing CPAs and exposed directly to LN_2. Several carrier systems have been described and classified into two categories: surface techniques and tubing techniques. The former, among others, include the methods electron-microscope grid, nylon mesh, Cryotop, cryoloop, hemi-straw, solid surface or minimum drop size. Tubing techniques include, among others, plastic straw, OPS, closed pulled straw, Flexipet-denuding pipette or CryoTip (reviewed by Saragusty and Arav, 2013). Standard 0.25 mL plastic insemination straw, used commonly for the slow-freezing technique, was the first container tested for the vitrification of oocytes/embryos despite limited achievable cooling and warming rates. Vajta and collaborators developed the open-pulled straw, which is a regular plastic straw that is heat-softened and pulled manually, like a glass capillary, to reduce the inner diameter and a wall thickness. The OPS vitrification method has been successfully used for the cryopreservation of matured cow oocytes and embryos (Vajta *et al.*, 1998; Vieira *et al.*,

2002; Vieira *et al.*, 2008). The OPS procedure was the first method used at our laboratory for the vitrification of cow oocytes (Albarracín *et al.*, 2005b). In an effort to improve survival we examined the efficacy of other cryodevices based on the results of other authors. Thus, after vitrifying/warming human pronuclear zygotes using the Flexipet-denuding pipette (FDP) as the cryodevice, Liebermann et al. (2002b) obtained rates of 87.5% survival, 77% 2-cell cleavage and 31% blastocyst formation on Day 5. The major difference between the two cryoprocedures was the speed of cooling and warming; the FDP method providing a faster technical solution for both steps. This was achieved through the use of a smaller oocyte-containing volume (FDP 1 µL vs OPS ~2 µL) and improved heat exchange during contact of the FDP with the LN_2. During the OPS vitrification method, the larger volumes of vitrification solution cool more slowly. Further, the thicker plastic wall of the tube acts as an insulation layer between the LN_2 and the vitrification solution. However, when we compared the OPS and FDP methods for the vitrification of mature cow oocytes, we detected higher rates of clustered chromatin and an abnormal spindle in calf and cow oocytes vitrified by FDP, and no improved results in terms of cleavage and blastocyst formation compared with the OPS method (Morató *et al.*, 2008b).

The Cryotop was developed especially for the vitrification of human oocytes/embryos but has also been used successfully in domestic animals (Chian *et al.*, 2004; Kuwayama *et al.*, 2005). It consists of a fine polypropylene strip attached to a plastic handle. The solution containing the oocyte/embryo can be loaded on the strip and excess solution can be removed almost entirely by aspiration. The sample is then immersed into LN_2 for vitrification and storage. The Cryotop allows for higher cooling and warming rates than those achievable with OPS. When the Cryotop procedure was compared with the OPS method, higher rates of normal spindle and chromosome configurations were observed. Interestingly, vitrification in cryotops improved the cleavage rates of both cow and calf oocytes compared with those obtained using OPS and led to increased numbers of blastocysts (Morató *et al.*, 2008a). Other cryodevices have been used to vitrify bovine oocytes. However, no significant improvement has been achieved during the last two decades, with blastocyst yield from vitrified bovine matured oocytes commonly over 10%, even though cleavage rate could be superior to 60% using different devices and vitrification protocols (reviewed by (Hwang and Hochi, 2014).

EMBRYO VITRIFICATION

In cattle, the cryopreservation of in vivo-produced, transferable-stage embryos has been successfully resolved by the traditional slow-rate freezing method and vitrification has been unable to produce additional breakthroughs in this field. Due to the increasing worldwide economic importance of cattle, many lines of research have been devoted to improving assisted reproduction technologies such as *in vitro* produced (IVP) embryos, nuclear transfer cloning and cryopreservation. Consequently, large numbers of embryos are generated through in vitro technologies and the cryopreservation of IVP bovine embryos is a prerequisite for their large-scale

commercial use. According to a recent report by the International Embryo Transfer Society, 58.7% of the in vivo derived embryos transferred in 2012 were frozen while only 9.5% of the IVP embryos transferred were frozen. In this way, vitrification has become a viable and promising alternative to conventional approaches when dealing with in vitro produced or micromanipulated embryos and considerable success has been reported for the vitrification of IVP embryos from one cell to the hatched blastocyst stage (Vajta *et al.*, 1998).

The cryopreservation of embryos at the blastocyst stage allows for better embryo selection, which serves to maximize the implantation potential of subsequent embryo transfer and minimize the number of embryos cryopreserved. Of the numerous factors that affect cryosurvival, the age, developmental stage and quality of the embryo, as well as interactions between these factors, have been found to be essential (Mahmoudzadeh *et al.*, 1995; Carvalho *et al.*, 1996; Saha *et al.*, 1996b; Pugh *et al.*, 1998) for the freezing of IVP bovine embryos. Blastocyst stage at the time of vitrification could be a key factor influencing outcome measures. Blastocysts invariably represent an embryo that has shown its developmental potential *in vitro*. Indeed, this selection process is far more reliable than selection based on the morphology of earlier-stage embryos, and makes the transfer of blastocysts a better option. Cryopreservation of blastocysts is challenging because of its inherent characteristics, such as: 1) slow permeation of the CPA into the blastocyst largely due to its multicellular shape; 2) the zona pellucida acting as a physiologic barrier to the permeation of the cryopreservation medium; and 3) the presence of the blastocele, which may be inadequately dehydrated during cryopreservation.

Optimal embryonic stage for cryopreservation

Several studies have reported differences in survival rates or hatching percentages after the cryopreservation of blastocysts obtained after different times of *in vitro* culture. However, some of these studies have addressed the use of slow freezing methods rather than vitrification and have stressed that the duration of embryo culture affects the cryotolerance of the blastocysts produced (Hasler *et al.*, 1995; Gustafsson *et al.*, 2001; Havlicek *et al.*, 2009). For example, Gustafsson *et al.* (2001) reported that blastocysts formed on Day 7 had an almost threefold greater chance of surviving freezing and thawing than embryos frozen on Days 8–9, regardless of their developmental stage. In a more recent paper, Havlicek *et al.* (2009) recorded significantly higher survival and re-expansion rates of Day 7 embryos compared with Day 8 IVP cow embryos. Similar observations have been reported when vitrification protocols have been used as the cryopreservation method (Mahmoudzadeh *et al.*, 1995; Saha *et al.*, 1996b). Using the cryotop in the vitrification/warming protocol, we designed a study to determine the ability of 7- and 8-day *in vitro* cultured blastocysts at different stages of development (classified as blastocysts, expanded blastocysts, or hatching/hatched blastocysts) to survive the vitrification process (Morató *et al.*, 2010). Our findings were consistent with previous observations and significantly higher survival rates were recorded for vitrified–warmed Day 7 versus Day 8 blastocysts. In contrast, Dinnyes *et al.* (1999) reported the similar survival of Day 7 and Day 8 blastocysts.

In our experiments, improved cryosurvival could be correlated with a more advanced embryonic stage on a given day. When we vitrified–warmed cow or calf embryos at different developmental stages after 7 or 8 days of *in vitro* culture, higher survival rates were observed for expanded blastocysts and those in the process of hatching or those that had hatched completely compared with non-expanded blastocysts. The fact that early developing embryos are better at surviving than later developing embryos was highlighted in embryo transfer experiments in which frozen–thawed IVP bovine blastocysts cryopreserved on the day of their appearance in culture rendered significantly higher pregnancy rates when Day 7 blastocysts were compared with Day 8 blastocysts (Hasler *et al.*, 1995). The reasons for the higher survival rates of more advanced stage embryos remain to be determined. As shown for hatching and hatched pig embryos (Nagashima *et al.*, 1992; Dobrinsky, 1996), the characteristics of expanded and hatching embryos that confer cryotolerance include mature junction complexes among cells (Prather and First, 1993), small trophectoderm cells with an epithelial-like structure and a blastocele containing a large amount of liquid. In the study carried out for our research team, we observed a more pronounced and consistent decrease in blastocele volume when expanded and hatching and/or hatched blastocysts were vitrified, suggesting a more marked dehydration step achieving better embryo protection, possibly due to the release of more water from the blastocele cavity. Conversely, when the volume of the blastocele increases, the ZP becomes thinner and possibly more permeable than in early blastocysts. In contrast with our findings, it has been reported that human expanded blastocysts show relatively lower survival rates after vitrification compared with early blastocysts (Cho *et al.*, 2002; Vanderzwalmen *et al.*, 2002). These authors attributed this reduced survival to greater mechanical damage suffered by expanded compared with early blastocysts due to greater ice crystal formation in the blastocele. Embryo transfer experiments are needed to confirm that the improved *in vitro* cryosurvival of expanded and hatched blastocysts will indeed lead to higher pregnancy rates. Hasler *et al*. (1995), who undertook a large number of transfers using fresh Day 7 and Day 8 IVP cow embryos, reported that within the different age and grade categories, embryo stage failed to modify the pregnancy rate. In contrast, the transfer of large numbers of IVP cow embryos, independently of age or grade, resulted in higher pregnancy rates when early and mid blastocysts were used rather than morulae and expanded or hatched blastocysts (Hasler *et al.*, 1997).

The survival rates achieved using hatching or hatched blastocysts indicate that an intact ZP is not necessary for successful vitrification. Our results are similar to those obtained by others in studies in which biopsied embryos were slow frozen (Gustafsson *et al.*, 1994) or vitrified (Agca *et al.*, 1995) and may have practical implications for sex determination or for embryos produced by nuclear transfer (Galan *et al*. 2003; Hiraoka *et al*. 2004; Zech *et al*. 2005).

In-straw warming/dilution and direct transfer methods

For the successful application of vitrification technology to veterinary practice, embryos need to be warmed and diluted in the vitrification straw so that they can be directly transferred to

the uterus requiring the same level of skill as for artificial insemination. This in-straw warming/dilution and direct transfer method reduces the need for equipment and technical skills. On many farms, these requirements are obstacles for embryo transfer. Following the vitrification of cow embryos, several devices have efficiently served for in-straw cryoprotectant dilution including 0.25-ml plastic straws (Saha *et al.*, 1996a; Pugh *et al.*, 2000; Inaba *et al.*, 2011), OPS (Vajta *et al.*, 1999), hand-pulled glass micropipettes (Vieira *et al.*, 2007) or cryotops (Inaba *et al.*, 2011). The use of these devices for vitrification enables the warming and dilution of vitrified embryos in a single step. However, the subsequent transfer of embryos requires more than one dilution step or proper handling of the embryo carrier system during in-straw warming, determining a need for more accuracy when these techniques are to be used in the field by embryo-transfer practitioners. The present authors have designed a new vitrification device (VitTrans) that enables field-warming/dilution and direct embryo transfer. The advantages of this device are that warming is easily and rapidly accomplished, and embryo transfer can then be directly performed to recipient females in field conditions. In addition, there is no need for a microscope employing the 0.5-ml straw used as the protective cover for the device.

The VitTrans device comprises a carrier where the embryo is loaded, a hard plastic handle and a cover straw to protect the device from mechanical damage during storage that functions as a 0.5-ml straw for sample dilution and embryo transfer. The handle has an inner channel into which warming solutions are introduced to dilute the CPA and transport the embryo to the cover straw for transfer. This step is performed using a regular syringe connected to the handle via a Luer connector (Figure 1). When we evaluated the effectiveness of the new devise developed in terms of in vitro survival after in-straw cryoprotectant (0.5 M sucrose) dilution of vitrified in vitro-produced bovine embryos, significantly lower percentages of re-expanded embryos were recorded after 3 h of culture compared to the control (warming was performed by transferring the tip of the device into a well containing the warming solution at 38.5ºC). However, when warming was performed at 45ºC, embryo percentages developing to the expanded/hatched blastocyst stages after 24 h of culture did not differ from controls (65% and 61.5 %, respectively) (Morató and Mogas, 2014).

Recently, Rodriguez-Villami *et al.* (2014) developed a new method whereby embryos are vitrified by the solid-surface system using Fyberplugs attached to a 0.5-ml plastic straw containing the diluting solution. During warming, straws are warmed and gently agitated to mix the embryo drop and the diluting solution. This method reduces the chances of manipulation errors or variations during the warming process. However, as the authors described, the diluting solution takes approximately 15 to 30 seconds to warm, which may result in slower warming and dilution than directly plunging the vitrified embryos into the warming solution. Recently, several studies have emerged supporting the hypothesis that warming rates are far more important than cooling rates (Seki and Mazur, 2008; Seki and Mazur, 2009; Mazur and Seki, 2011). The VitTrans device may circumvent these issues, as the whole device (carrier plus cover straw) is introduced into a water bath and as soon as the cryoprotectant solution is injected in the device's handle, the embryos are

flushed into the cover straw while CPA are diluted away. Once this has been accomplished, the VitTrans device is removed and the straw is ready for embryo transfer. However, the major limitation of the VitTrans is direct contact between the medium containing the embryos and the LN_2, which may be a source of contamination (Fountain *et al.*, 1997). This limitation may be avoided by vitrifying the embryos in a small volume of decontaminated LN_2, and then placing the original sample in the 0.5ml-straw and hermetically sealing the whole VitTrans device (with its cover) before plunging into LN_2 for storage (Bielanski and Hanniman, 2007; Bielanski and Vajta, 2009). In this way, the VitTrans combined with in-straw dilution may be used for direct transfer and this could be a simply, rapid on-farm alternative to traditional slow rate freezing for the cryopreservation and transfer of IVP cow embryos. These proposals, however, still require verification by embryo transfer.

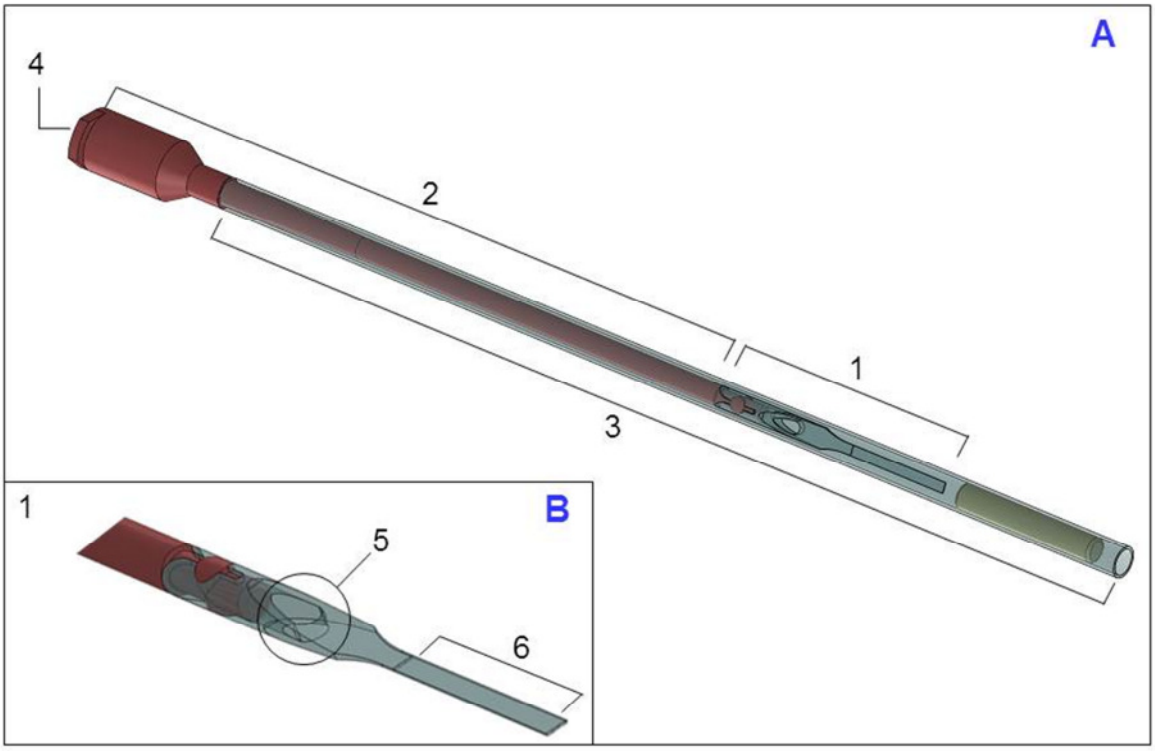

Figure 1. A: *The device VitTrans* comprises a carrier where the embryo is loaded (1), a hard plastic handle with an inner channel (2) into which warming solutions are introduced to dilute the cryoprotectant and transport the embryo to the cover straw (3) for transfer and *a Luer connector (4) for connecting the device to the warming solution source.* The cover straw (3) is attached to protect the device from mechanical damage during storage. During warming, the cover straw functions as a 0.5-ml straw for sample dilution and direct embryo transfer. B: *Closer view of the end of the device (1) where the output mouth of the inner channel (5) and the embryo attachment support (6) are observed.*

CONCLUSIONS

The cryopreservation of gametes and embryos has become essential for the cost-effective, long-term conservation and widespread dispersion of animal genetic resources. Cryopreservation also has applications in the management of infertility in both human and animals. The simplicity and economic benefits of vitrification could provide an efficient and practical method for the cryopreservation of cow oocytes and embryos, and markedly improve domestic animal breeding programs. Further, oocyte vitrification can be used to bank the gametes of genetically valued laboratory animals and is foreseen to have a significant impact on conservation biology. However, despite all the efforts made in the past 20 years, the vitrification of bovine oocytes has yet to provide convincing results of widespread application, mainly due to their complex structure and sensitivity to chilling. Hence, work is needed to improve oocyte survival after vitrification either by modifying protocols or modifying certain oocyte traits to increase their resistance to cryopreservation. Finally, although vitrification has proved to be an adequate technique for the cryopreservation of in vitro produced embryos with acceptable pregnancy rates, the method has not been widely adopted by embryo transfer practitioners for commercial use in cattle. It is therefore essential that research yields more consistent results to develop a "universal" vitrification method protocol suitable for the in-straw warming and field transfer of embryos.

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SHORT COMMUNICATIONS

SPINDLE CONFIGURATION AND DEVELOPMENTAL COMPETENCE OF *IN VITRO* MATURED BOVINE OOCYTES EXPOSED TO SUCROSE PRIOR TO CRYOTOP VITRIFICATION

Arcarons N[1], Morató R[2], Spricigo J[1], Ferraz Mamm[1], Mogas T[1]

[1]Departament de Medicina i Cirurgia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona. Cerdanyola del Vallès, Spain. [2]Departament de Biologia, Facultat de Ciències, Universitat de Girona. Girona, Spain

It has been previously described that a simple treatment with medium containing elevated sucrose concentrations increases the cryotolerance and developmental competence of *in vitro*-matured porcine oocytes after vitrification and parthenogenetic activation (Lin *et al*. *Reproduction, Fertility and Development*, 2009, 21, 338–344). This work was designed to study whether the exposure to increased concentrations of sucrose prior to vitrification improves cryotolerance of *in vitro*-matured bovine oocytes. In Experiment 1, a total of 392 bovine *in vitro*-matured oocytes were exposed to four different sucrose concentrations (from 375 to 808 mOsm) for 1 h. In Experiment 2 (n=354), and according to the results obtained in the first experiment, oocytes were exposed to 375 mOsm sucrose solution, vitrified and warmed. Non-treated oocytes were used as controls. In both experiments, oocytes were fixed after treatment and microtubule and chromosome distribution was analyzed by immunocytochemistry. In experiment 3 (n=728) *in vitro* embryo development was evaluated after vitrification of oocytes with or without sucrose (375 mOsm) pre-treatment. All statistical analyses were conducted with the IBM SPSS 19 for Windows (IBM corp.; Chicago, Illinois). ANOVA was performed to analyze differences in meiotic spindle configuration cleavage rates and blastocyst yield. When data do not accomplish the tests of normality a Kruskall-Wallis test was used for comparisons. The level of statistical significance was set at $p < 0.05$. After exposure to 375 mOsm sucrose, similar percentages of oocytes showing normal chromosome distribution were obtained compared to control group (71.8% and 85.0%, respectively). Groups treated with higher sucrose concentrations (443 to 808 mOsm) triggered significantly lower proportions of normal spindles, showing higher percentages of oocytes with dispersed or decondensed chromosomes and microtubules. After vitrification/warming, significantly higher percentages of normal chromosome configuration were observed when oocytes were exposed to 375 mOsm sucrose prior to vitrification (34.2%) compared to control vitrified oocytes (23.3%). However, both percentages differed significantly from control group (81.7%). Cleavage and blastocyst rates were higher in the non-vitrified groups (62.6% and 16.1% vs 67.7% and 17.2%, control and sucrose treated groups; respectively) compared with vitrified groups (26.6% and 1.3% vs 33.0% and 1.2% in control vitrification and sucrose-treated vitrification groups; respectively). In conclusion, treatment with 375 mOsm sucrose before vitrification did not induce adverse changes in the metaphase of bovine oocytes. Besides, sucrose treatment prior to vitrification did not have positive effects on embryo development of bovine oocytes matured *in vitro* in contrast with the results observed in porcine species.

Notes

IMPROVING *IN VITRO* MATURATION AND PREGNANCY OUTCOME IN DAIRY CATTLE UNDER COMMERCIAL CONDITIONS USING A NOVEL OOCYTE SHIPPING AND MATURATION SYSTEM DEVOID OF CO_2 GAS PHASE

Barceló-Fimbres M[1], Campos-Chillón LF[2], Verstegen J*

[1] *MOFA global, ICB, 2633 State Rd 78, Mt Horeb, WI, USA*
[2] *Animal Sciences. California Polytechnic State University, San Luis Obispo, CA.*

The present work evaluated the benefit of a novel shipping and maturation media (SMM) that does not require a CO_2 gas phase for maturation and subsequent embryonic development of Oocyte Pick Up (OPU) bovine Cumulus Oocytes Complexes (COC). Stimulated non-lactating Holstein (n=10) and Jersey (n=10) donors with 6 pFSH injections (Pluset®, Mofa Global, Wi, USA) were used for 102 OPU sessions. COC were aspirated in the farm and from each donor some OPU sessions were delivered the same day within three hours after collection for IVM in the conventional gas bicarbonate equilibrated medium system (Control) while the rest of the COC were placed in a dry portable incubator at 38.5 ºC, delivered the next day allowing 24 h maturation in SMM (BoviPro®, Mofa Global, Wi, USA). COC were fertilized using commercial semen for each breed, and embryos were culture in BBH7 medium (BoviPro®, Mofa Global, Wi, USA) at 38.5 ºC in 5%O_2, 5%CO_2, 90%N_2 atmosphere and transferred (ET) 7 d after fertilization. At day 35 pregnancies were diagnosed by ultrasonography. Data were analyzed by ANOVA using GLM, percentages were transformed using arcsin square root, and pregnancy rates were analyzed by GenMod using SAS statistical software (Cary, NC, USA). Similar COC numbers were recovered for maturation treatments (P> 0.1). COC matured in SMM had similar cleavage (P> 0.1) but grater blastocyst rates than control group (P< 0.01; Table 1). After ET, SMM had similar pregnancy rates than control (P< 0.1); however, SMM had more transferred embryos per OPU session which resulted in 2.2 more pregnancies per OPU session (P< 0.01; Table 1). Number of COC recovered of Holstein vs. Jersey was similar (P> 0.1). Holstein COC had superior embryonic development (cleavage and blastocyst rates; P< 0.01) but similar pregnancy rates compared to Jersey (P> 0.1; Table 1). We conclude that COC matured in SMM had greater oocyte competence than control and in commercial settings. SMM resulted in greater embryonic development, similar pregnancy rates, but more transferable embryos and pregnancies per OPU session than the conventional maturation system.

Table 1. Least square means (±SE) of embryonic development and pregnancy outcomes of embryos from OPU derived COC matured in SMM or Control.

Variables	COC recovery	Cleavage (%)	D7 Blastocyst/ oocyte (%)	Transferred embryos/ OPU session	Pregnant/ Total ET (%) 35 days	Pregnant per OPU session
Maturation						
Control	8.6±2.0	70.7±3.7	36.7±4.1[a]	3.4±1.2 [a]	35/60 (58.3)	2.1 ± 0.7 [a]
SMM	11.0±1.1	72.9±2.2	48.5±2.2 [b]	7.3±0.7 [b]	296/550 (53.8)	4.3 ± 0.4 [b]
Breeds						
Holstein	10.5±1.9	83.8±3.5 [a]	62.6±4.4 [a]	6.7±1.2 [c]	112/206 (54.4)	3.7 ± 0.7
Jersey	11.1±1.2	71.5±2.2 [b]	42.1±2.6 [b]	4.9±0.7 [d]	219/404 (54.2)	2.7 ± 0.4

Values without common superscripts in the same column differ ([a,b] P < 0.01, [c,d] P < 0.05).

*: *Corresponding author: jverstegen@mofaglobal.com*

Notes

EVALUATION OF DIFFERENT SERA SOURCES FOR IN VITRO MATURATION IN NOVEL SHIPPING AND MATURATION MEDIUM DEVOID OF CARBON DOIXIDE A OF BOVINE EMBRYOS

Barceló-Fimbres M, Verstegen J*

MOFA global, ICB, 2633 State Rd 78, 53572 Mt Horeb, WI, USA

Fetal Bovine Serum (FBS) is the most common source of sera for in vitro maturation (IVM) of bovine embryos; however, there are other sources as estrus cow serum (ECS) or preovulatory follicular fluid (pFF) highly used for porcine IVM. The present work evaluated the benefit of different sera sources for a novel shipping and maturation medium (SMM, BoviPro®, MOFA Global, Wi, USA), that does not require a CO_2 gas phase for IVM and subsequent embryonic development of slaughterhouse bovine Cumulus Oocytes Complexes (COC). The SMM was supplemented with 10% of FBS, ECS or pFF. A total of 691 COCs were used for different treatments and placed in a dry portable incubator at 38.5 ºC allowing 24 h maturation, then fertilized in IVF medium (BoviPro®, MOFA Global, WI, USA) and culture in BBH7 medium (BoviPro®, MOFA Global, Wi, USA) at 38.5ºC in 5% O_2, 5%CO_2, 90% N_2 atmosphere for 7 d. A total of 6 replicates were done. Data were analyzed by ANOVA using GLM, percentages were transformed using arcsin square root, and if the ANOVA was significant ($P< 0.05$) means were separated by Tukey's procedure, using Statistix 10 software (Tallahassee, FL, USA). No differences in cleavage rates were found between treatments ($P> 0.1$); however, for day 7 blastocyst rate ECS was higher compared to FBS and pFF ($P<0.01$). There was no difference among treatments at day 7 total embryo production rate where all day 7 blastocysts plus morulae were added ($P> 0.1$). For blastocyst stage rate, there were no differences in the proportion of stage 6 (blastocyst), 7 (expanded blastocyst), and 8 (hatched blastocyst) for any treatment ($P> 0.1$); however, lower proportion of stage 5 (early blastocyst) were observed for FCS compared to ECS ($P< 0.05$), and lower proportion of stage 4 (morulae) for ECS compared to pFF ($P<0.05$; Table 1). We found differences in overall embryonic stage between treatments, where ECS embryos were more advanced in stage than pFF ($P> 0.05$; Table 1). We conclude that either ECS or pFF can be used as sera replacement for the conventional FBS. However, ECS was superior in blastocyst production than FBS and pFF, but not for total embryo production. This is due to ECS stimulated embryonic development with less proportion of morulae produced.

Table 1. Means (±SEM) of embryonic development of COC matured in different treatments.

Treatment	Oocytes No.	Cleavage (%)	D7 Blastocyst (%)	D7 Total embryos (%)	Stage (%) 8	7	6	5	4	Embryonic Stage *
ECS	226	89.2±2.6	40.6±4.2 ^{a}	45.7±5.6	2.3	48.1	21.4	18.3 ^{a}	9.9 ^{a}	6.2±0.1 ^{a}
FBS	234	84.9±3.7	31.3±3.9 ^{b}	38.8±4.4	0.0	43.5	30.2	7.0 ^{b}	19.3 ^{a,b}	6.0±0.1 ^{a,b}
pFF	231	88.7±2.8	30.0±3.2 ^{b}	39.0±2.5	0.0	39.3	22.1	12.9 ^{a,b}	25.7 ^{b}	5.8±0.1 ^{b}

Values without common superscripts in the same column differ (^{a,b} $P < 0.05$).
* Overall embryonic stage (4= morulae, 5=early blastocyst … 8=hatched).

*: *Corresponding author: jverstegen@mofaglobal.com*

Notes

VERO CELLS CONDITIONED MEDIUM IMPROVES EMBRYONIC DEVELOPMENT DURING *IN VITRO* CULTURE OF BOVINE EMBRYOS

Barceló-Fimbres M[1], Guillemin Y[2], Gouze J[2], Mtango N[1], Verstegen J[1*]

[1]*MOFA global, International Center for Biotechnology, 2633 State Rd 78, 53572 Mt Horeb, WI, USA*
[2]*GenBiotech, 280 rue de GOA, 06600 Antibes France*

Beneficial effects on embryonic development using co-culture with Vero cells during *in vitro* culture has been previously reported and related to Vero cells growth factors/cytokines secretion into the medium (Human Reprod 1600–5, 1998); however, co-culture increases the probability to develop microorganisms contamination during culture. In this study, we aimed to overcome this problem by culturing bovine embryos in conditioned medium derived from Vero cells culture (CM) and added at different percentages on day 0 of *in vitro* culture. Vero cells (GenBiotech, France) were cultured for 24 h then the medium was removed filtered and frozen until its use. A total of 701 slaughterhouse bovine Cumulus Oocytes Complexes (COC) were matured and fertilized *in vitro* under standard conditions. After fertilization, zygotes were allocated to four different treatments (0 (control), 5, 10, and 20% of CM v/v) for *in vitro* culture in BBH7 medium (BoviPro®, MOFA Global, Wi, USA) at 38.5ºC in 5%O_2, 5%CO_2, 90%N_2 atmosphere for 7 d. A total of 8 replicates were done. Cleavage rates were assessed at 2.5 d, blastocyst rates and embryonic stage at 7 d, and blastocyst total cell number at 7.5 d after fertilization. Data were analyzed by ANOVA using GLM, the percentages were transformed using arcsin square root. If the ANOVA was significant ($P< 0.05$) means were separated by Tukey's procedure, using Statistix 10 software (Tallahassee, FL, USA). No differences in cleavage rates were found between treatments ($P> 0.1$; Table 1); however, the addition of conditioned medium at all levels increased the blastocyst production at day 7 of culture (between 11 to 19 percentage points) compared to the control group ($P< 0.01$; Table 1); likewise, CM addition was superior in total embryo production (day 7 morulae plus all blastocysts) ($P< 0.01$; Table 1): between 12.5 to 15 percentage points higher than control group. No differences were found between treatments for blastocyst total cell number at 7.5 d of culture ($P> 0.1$; Table 1), and overall embryonic developmental stage ($P> 0.1$; Table 1).

In conclusion, we found a superior embryonic development when Vero cells conditioned medium was added at day 0 of *in vitro* culture; the positive effect on total embryo production was observed even at 5% of conditioned medium.

Table 1. Means (±SEM) of embryonic development of bovine embryos.

Treatment	Oocytes No.	Cleavage (%)	Blastocyst day 7 (%)	Total embryos day 7(%)	Total cell number	Blastocyst Stage *
Control	159	88.6±2.4	33.8±3.1[a]	43.8±4.8[a]	153.8	6.03
CM 5%	179	88.9±2.2	52.8±3.8[b]	58.8±3.8[b]	164.4	6.10
CM 10%	178	90.2±1.4	49.4±2.7[b]	58.8±3.2[b]	172.4	6.08
CM 20%	185	84.8±2.8	45.0±2.8[b]	56.3±2.8[b]	141.5	5.81

Values without common superscripts in the same column differ ([a,b] $P < 0.01$).
* Overall blastocyst stage (5=early… 8=hatched).

*: *Corresponding author: jverstegen@mofaglobal.com*

Notes

EFFECTS OF CYCLIC AMP REGULATORS DURING OOCYTE *IN VITRO* MATURATION ON BOVINE EMBRYO DEVELOPMENT AND QUALITY IN PREPUBERTAL AND ADULT DONORS

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*

Prepubertal animals are used to shorten the generation interval, accelerate genetic gain and to prolong the reproduction period. *In vitro* embryo production systems have been applied in prepubertal females, however, oocyte developmental competence is lower than in the adult counterparts. *In vitro* maturation (IVM) systems using cyclic AMP modulators and 30h IVM, have been suggested to improve bovine blastocyst yields from adult donors. The present study evaluated the effects of the cAMP modulators forskolin, 3-Isobutyl-1-methylxanthine (IBMX) and cilostamide during IVM on oocyte maturation and embryo development and gene expression in bovine prepubertal and adult females. Oocytes from adult (lactating cows) or prepubertal donors (6-10 mo. old) were retrieved via Ovum Pick-up (OPU). After OPU, COCs were submitted to IVM in the following three groups: TCM24 (24h IVM, standard protocol), TCM30 (2h pre-IVM culture (forskolin-IBMX) and 30h IVM adding cilostamide), DMSO30 (2h pre-IVM culture and 30h IVM with DMSO/ vehicle control). After IVM, oocytes were fertilized and zygotes were cultured *in vitro* to assess embryo development. Maturation, cleavage and blastocyst rates were recorded. The mRNA abundance of the developmentally important genes *SLC2A8, DNMT3B, BCL-XL, EGR1* and *PRDX1* was determined by RT-qPCR in single blastocysts. *In vivo* blastocysts were retrieved and used for gene expression analysis. Maturation rates (prepubertal donors: 63.4±6.3%, 66.0±6.8%, 73.7±4.1%, cows: 81.6±6.1%, 79.7±5.2%, 79.3±6.7%, for TCM24, cAMP30 and DMSO30 respectively, $p > 0.05$), cleavage rates (prepubertal donors: 63.4±4%, 54.9±5.1%, 52.1±3.6%, cows: 56.1±4.7%, 57.8±5.6%, 51.6±4.4%, for TCM24, cAMP30 and DMSO30, respectively, $p > 0.05$) and blastocysts/presumptive zygotes rates (prepubertal donors: 26.2±5.3%; 19.6±2.8%; 16.2±2.0%; cows: 27.5±3.0%; 28.1±2.7%; 21.5±2.8% for TCM24, cAMP30 and DMSO30, respectively), were not different among *in vitro* treatments. The mRNA relative abundance of the *EGR1* gene was down-regulated in all *in vitro* produced blastocysts compared to their *in vivo* counterparts. Gene expression profiles for *SLC2A8, DNMT3B, BCL-XL* and *PRDX1* genes were similar in all groups. The present results showed similar embryo production patterns in prepubertal and adult donors. The use of DMSO during *in vitro* maturation did not affect embryo developmental rates. The gene expression profile of *EGR1* was similar to previous observations in blastocysts obtained from oocytes of slaughtered animals (Bernal et al, 2014), thus confirming its usefulness as embryo quality marker. Results indicate that oocyte developmental capacity in prepubertal bovine females can be comparable to the adults equivalent with and without cAMP modulators addition.

Notes

IMPACT OF INTRAFOLLICULAR SULFATED STEROIDS ON FOLLICULAR CELLS AND THE OOCYTE´S DEVELOPMENTAL CAPACITY IN CATTLE - FIRST RESULTS -

Blaschka C, Stinshoff H, Poppicht F, Wrenzycki C

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

Steroid hormones are regulators in the fine-tuned mechanism of follicular development. Their concentration and property can be modulated via different processes. Sulfoconjugation of steroids via sulfotransferases (SULT) changes them from being hydrophobic to hydrophilic molecules, thereby preventing them from diffusing freely across the lipid bilayer and necessitating a transport system like the solute carrier family 10 (SLC10; for example, the sodium-dependent organic anion transporter [SOAT; SLC10A6] transports primarily sulfated steroids). In addition, estrogen sulfoconjugates can no longer bind to the estrogen receptor, rendering them biologically inactive. Steroid sulfatase (STS) is the enzyme responsible for the removal of the sulfonate moiety from sulfoconjugated steroids transforming them to the free active ones. Data about the concentration of sulfated steroids in follicular fluid and the expression of the related enzymes are small in horses and still missing in cattle.

Follicles of bovine abattoir-derived ovaries were categorized according to their size. Follicular fluid was collected via aspiration and analysed for the presence of steroids and their sulfated counterparts via LC-MS/MS. The results are shown in the following table.

Follicle size	**E2** (ng/ml)	**P4** (ng/ml)	**T** (ng/ml)	**E2S** (ng/ml)	**E1S** (ng/ml)	**PREGS** (ng/ml)	**CHOLS** (ng/ml)
3-5 mm	15.0 ± 9.1	118.8 ± 97.0	16.3 ± 2.0	0.5 ± 0.5	1.0 ± 0.0	0.7 ± 0.2	50.2 ± 11.4
6-8 mm	43.7 ± 19.8	118.2 ± 54.5	11.6 ± 8.8	0.1 ± 0.1	0.3 ± 0.1	1.0 ± 0.3	69.7 ± 12.8
9-14 mm	144.1 ± 54.8	97.1 ± 54.3	8.8 ± 2.5	1.7 ± 0.1	0.2 ± 0.1	1.0 ± 0.2	64.8 ± 12.4
>15 mm	94.3 ± 88.5	108.4 ± 95.6	8.6 ± 6.8	2.1 ± 0.1	0.2 ± 0.1	1.1 ± 0.2	35.8 ± 14.3

E2: 17β-estradiol; P4: progesterone; T: testosterone; E2S: 17β-estradiol sulfate; E1S: estrone sulfate;
PREGS: pregnenolone sulfate; CHOLS: cholesterol sulfate

Furthermore, it was possible to detected androstendione, estrone, androsterone and 17OH-pregnenolone. In addition, transcripts of the steroid metabolizing and transporting enzymes (SULT1E1, STS, SLC10A6) were determined in cumulus cells from immature bovine COC via RT-qPCR.

These data indicate that only small amounts of sulfated steroids are present in bovine follicular fluid. However, the related enzymes are present at the mRNA level. Further studies are underway to analyse the protein level.

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Notes

MICROARRAY ANALYSIS OF ANTIGEN-DEPENDENT B CELL ACTIVATION GENE EXPRESSION IN BITCHES WITH PYOMETRA

Bukowska D[1], Kempisty B[2,3], Zawierucha P[2,3], Ziolkowska A[2], Ciesiolka S[2], Piotrowska H[4], Jopek K[2], Wozna M[1], Antosik P[1], Brüssow KP[5], Nowicki M[2], Jaskowski JM[1]

[1]Institute of Veterinary Sciences, Poznan University of Life Sciences, Poland , [2]Department of Histology and Embryology, Poznan University of Medical Science, Poland,[3]Department of Anatomy, Poznan University of Medical Science, Poland,[4]Department of Toxicology, Poznan University of Medical Sciences, Poland, [5]Institute of Reproductive Biology, Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany

The canine pyometra is defined as a complex disease associated with activation and proliferation of immune specific cells, B and T cells, as well as synthesis and activation of immune and pro-inflammatory molecules. Although all of these mechanisms are well recognized in several human immune diseases and cancers, the possible role or dysfunction of these molecules in dogs with pyometra still require investigations.

This study was aimed to examine antigen-dependent B cell activation gene expression (CD4, CD28, CD40, CD80, Fas, HLA-DRB1 and IL10) in a total of 24 mixed-breed bitch uteri with pyometra and 20 healthy controls. Using canine RNA microarray assays (Affymetrix) altogether 17,138 different transcripts were analyzed.

A significant increase was found of CD28, CD40, HLA-DRB1 ($P<0.001$), and CD4, CD80, Fas and IL10 ($P<0.01$) in the group of bitches with pyometra, as compared to controls. In the affected group an increased share of CD4, CD28, CD40, CD80, Fas, HLA-DRB1 and IL10, (13.6-, 2.8-, 2.9-, 5.6-, 3.3-, 4.4-, and 6.5-fold increase, respectively) was also detected.

It is suggested that an increased expression of B cell-specific immune response molecules may be associated with recruitment of immunologically specific cells in bitches with pyometra as well as with activation of pro-inflammatory proteins as a consequence of exposure to foreign antigens due to bacterial infection.

Notes

SPERMATOZOA IMPROVE MITOCHONDRIAL STATUS OF MATURE BOVINE OOCYTES

Ctvrtlikova-Knitlova D, Jeseta M, Hanzalova K, Hulinská P, Milakovic I, Machatkova M

Veterinary Research Institute, Brno, Hudcova 70, 621 00, Czech Republic

Bovine oocytes used for in vitro fertilization are characterized by the level of both nuclear and cytoplasmic maturation. One of the most important parameters of cytoplasmic maturation is mitochondrial status of oocytes. Mitochondrial clusters are formed during maturation as large circular agglomerates around the peripheral network of endoplasmic reticulum and their association with ATP production has been verified. The objective of this study was to assess the influence of spermatozoa on mature bovine oocytes in terms of mitochondrial cluster formation. The oocytes were isolated from ovaries of slaughtered cows and selected according to the cytoplasm and cumulus morphology. Only those assessed as suitable for IVF were matured for 24 hours using a standard protocol. Subsequently, the oocytes were transferred into IVF-Talp medium and either inseminated with spermatozoa of a standard bull or cultured only in the medium. Equal parts of oocytes were collected at 24 h after maturation and at 6, 12 and 18 h after insemination or cultivation. The oocytes were stained with Sytox Green and Calnexin antibody for visualization of nuclear status and mitochondria and ER network, respectively, and examined using confocal microscopy. Almost one half of the oocytes (49.2%) showed mitochondrial cluster formation after maturation. The proportion of unpenetrated oocytes with clusters did not change at 6h after insemination (47.5%) or cultivation in medium (47.1%) but it decreased significantly in oocytes penetrated with spermatozoa (12.5%). At the 12h interval, the significantly higher ($P \leq 0.01$) proportion of oocytes with clusters was found in unpenetrated oocytes (62.1%) than those cultured in medium (16.7%) or penetrated with spermatozoa (1.1%). This difference disappeared at the 18 h interval, when the proportion of oocytes with clusters was reduced in unpenetrated oocytes and it did not statistically differ from that of oocytes cultured in medium (35.1 vs 21.1%). Our results confirmed a specific influence of spermatozoa on mature bovine oocytes in terms of their mitochondrial status. The unpenetrated oocytes kept mitochondrial clusters for 12 hours after co-cultivation with spermatozoa. At this time, mitochondrial clusters were disintegrated in oocytes cultured without spermatozoa or in those which were fertilized.

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Notes

EFFECTS OF DIFFERENT LEVELS OF *SATUREJA MACRANTHA* EXTRACT ON MICROSCOPIC PARAMETERS OF FROZEN-THAWED HOLSTEIN BULL SPERM

Daghigh Kia H[1], Shahbazzadeh R[1], Ashrafi I[1], Ghafari I[2], Hosseinkhani A[1]

[1]*Department of Animal science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran*
[2]*Animal Breeding Center of North and Northwest of Iran, Tabriz, Iran*

Oxidative stress is one of the various causes, which affect sperm physiology and can lead to infertility. During spermatogenesis, sperms lose a considerable amount of cytoplasm thus only small amounts of neutralizing ROS (reactive oxygen species) remain in the cells, which is insufficient for inhibition of lipid peroxidation during cryopreservation. Therefore, there is a requirement for additional exogenous antioxidants during dilution of semen. Herbal antioxidants have been tested to successfully freeze semen of domestic animals. *Satureja Macrantha* belongs to the family of *Mentha* which have phenolic compounds such flavonoids, phenolic acids and di-terpene – phenolic and they have high antioxidant activity. Flavonoids and phenolic compounds have several pharmacological properties including antioxidant, anti-inflammatory and free radical neutralizing.

The purpose of this experiment was to evaluate the effect of different levels of *Satureja Macrantha* extract on total motility, viability and membrane integrity of sperm cells after freeze-thawing process. Semen samples were collected by an artificial vagina, twice a week from three mature bulls that were kept under identical nutritional conditions. In order to eliminate the individual effects of bulls, the semen samples were pooled together and were diluted with citrate - egg yolk extender and different levels of *Satureja Macrantha* extract (2, 4, 8, 12, 16, and 20 mL/dL) were used. The results showed that the total mobility was significantly higher in diluent containing 4 mL/dL of *Satureja Macrantha* extract than the control group ($P< 0.05$). No significant differences were observed between treatments 2 and 8 mL/dL compared to the control group. On the other, total motility was significantly reduced in 12, 16 and 20 mL/dL treatments compared to other levels ($P< 0.05$). The sperm viability, were significantly affected in the all treatments compared to control groups except groups treated with 2 and 8 mL/dL extract ($P< 0.05$). The higher rate of viability was found in 4 mL/dL group ($P< 0.05$). Treatments with 12, 16 and 20 mL / dL had the lowest percentage of viable sperm. The results of HOST test showed that the number of sperm with twisted and swollen tails is significantly higher in treatment 4 mL/dL compared to control group ($P < 0.05$). Addition of extract 12, 16 and 20 mL / dL to semen diluent had a significant negative effect on sperm membrane integrity ($P < 0.05$). Based on the results of this survey, usage of 4 mL/dL *Satureja Macrantha* extract in citrate - egg yolk diluent had the best protective effects on freeze-thawing Holstein bull sperm.

Keywords: *Satureja Macrantha*, Oxidative Stress, Bull Sperm, Semen Parameters

Notes

THE EFFECT OF NON-ESTERIFIED FATTY ACIDS DURING IN VITRO CULTURE ON DNA METHYLATION OF BOVINE BLASTOCYSTS

DESMET K.[1], VAN HOECK V.[2], MERCKX E.[1], SIRARD M.-A.[3], BOLS P.E.J.[1], LEROY J.L.M.R.[1]

[1]Gamete Research Centre, Laboratory of Veterinary Physiology and Biochemistry, Department of Veterinary Sciences, University of Antwerp, Belgium
[2]Laboratório de Fisiologia e Endocrinologia Molecular, Departamento de Reprodução Animal, Faculdade de Medicina Veterinária e Zootecnia, University SaoPaulo, Pirassununga, SP, Brasil
[3]Centre de recherche en biologie de la reproduction, Département des Sciences Animales, Faculté des sciences de l'agriculture et de l'alimentation, Université Laval, Québec, Canada

High producing dairy cattle undergo a period of negative energy balance during which the concentration of non-esterified fatty acids (NEFA) increases in serum and follicular fluid. This metabolic disorder is simultaneously associated with subfertility. Elevated NEFA concentrations during embryo culture have adverse effects on the developmental competence of the embryo and alter gene-expression. Some of these differentially expressed genes (*HIST1H1C*, *HIST1H2BN*) are related to the compaction of chromatin and thus to epigenetic mechanisms. Changes in these epigenetic markers may induce pertinent changes in gene expression influencing further embryonic development or even later life. We hypothesized that high NEFA concentrations during early pre-implantation growth alter DNA methylation in embryos.

A total of 1412 bovine oocytes (4 replicates) were matured and fertilized following standard procedures. Zygotes were cultivated for 6.5 days under 1) physiological NEFA conditions (mixture of 23 µM palmitic acid (PA), 28 µM stearic acid (SA) and 21 µM oleic acid (OA)) (BASAL) or 2) elevated NEFA concentrations as under lipolytic conditions (mixture of 230 µM PA, 280 µM SA and 210 µM OA) (HIGH COMBI). Cleavage and blastocyst rate were determined at day 2 and day 7,5 after fertilization, respectively. A selection of 10 blastocysts per treatment per replicate was analyzed for DNA methylation patterns using the EmbryoGENE Bovine microarray platform (fold-change > 1.5 and P-value ≤ 0.05). Epigenetically modified pathways were examined by Ingenuity Pathway Analysis.

The cleavage and blastocyst rate were significantly decreased due to elevated NEFA concentrations ($P < 0.01$). The microarray data revealed a total of 1314 differentially methylated genes of which 528 hypermethylated genes and 786 hypomethylated genes in blastocysts under BASAL conditions compared to HIGH COMBI conditions. The five most important altered pathways were cell death and survival, lipid metabolism, carbohydrate metabolism, molecular transport and embryonic development. Previous research revealed that maturing oocytes under high NEFA conditions for 24 hours resulted in a five times lower number of genes with an altered DNA methylation pattern.

We conclude that embryonic exposure to elevated NEFA concentrations not only reduce the embryo development but also alter the DNA methylation profile in embryos that do survive. Especially genes associated with metabolism and cell fate are affected which may lead to an altered embryonic or fetal development or even postnatal health. The developmental stage during and/or the duration of the NEFA exposure could influence the number of genes that were differentially methylated.

Notes

THE EFFECT OF OVULATION AND TRANSFER SITES ON REPRODUCTIVE PERFORMANCE OF PROLIFIC AND NONPROLIFIC EWES

Emsen E[1], Kutluca Korkmaz M[2]

[1]*Ataturk University, Department of Animal Science, 25240, Erzurum, Turkey*
[2]*Ataturk University, Ispir Hamza Polat Vocational School, 25900, Ispir, Erzurum, Turkey*

Inequality of ovarian function occurs in various species. Although the occurrence of ovulation has been shown to be random with respect to which ovary contained the previous CL in primates (Hartman, 1932; Morse and van Wagenen, 1936) and many other mammals (Brambell, 1956) ovulation consistently alternates between the ovaries, and in others ovulation may predominate from one ovary. Inequality of ovarian function may or may not lead to inequality of function between the right and left horns of the uterus, depending upon differences in embryo mortality between the two sides. In this study the relative activity of the right and left ovaries of sheep showing single and multiple ovulations was examined. Loss of percentage of embryos arising from single and twin ovulations in the two ovaries were studied with both pregnancy rates and litter size at birth after twin fresh embryo transfer in prolific and nonprolific ewes. Based on estrus detection, 13 prolific (Romanov crossbred) and 24 fat tailed native breeds of recipients were evaluated for embryo transfer. At the scheduled time of embryo transfer one technician performed laparoscopic examinations of the ovarian structures. The location and number of CL were recorded. Pregnancy rate of recipient ewes was assessed by ultrasound scanning 50 days after transfer. Embryo survival rates were confirmed with lambs born form twin embryo transfer.

The number of CL on the right ovary (2.5) significantly ($P<0.05$) differed from those observed either on the left (0.9) or on both ovaries (2.1). Ewes recorded with a single ovulation had a similar litter size at birth while pregnancy rates were higher (19%; $P>0.05$) in multiple ovulated ewes. Pregnancy rates were effected neither from CL nor transfer sites. However, ~~there was~~ a relatively higher (24%) pregnancy success was achieved when ovulation occurred in both ovaries. Site of ovulation played an important role in twin transferred embryo survivability. Embryo mortality was 25% when ovulation occurred on both ovaries and ~~it was~~ increased to 40% in ewes where ovulation was recorded on the left ovary. However, when ovulation has been detected on the right ovary there was an embryo survival rate of 100%. While there was no significant difference for pregnancy rates when embryos have been transferred into the right or left uterine horns, there were 30% higher embryo survival rates when embryo transfer has been done contro-lateraly to the ovulation side into the left uterine horn.

It was concluded that multiple ovulations occurs mostly in right ovaries. A single ovulation can maintain luteal function after fresh twin embryo transfer. However, relatively higher pregnancy rates in fresh twin embryo transfer can be achieved by choosing recipient ewes with multiple ovulations. Embryo survival was not affected by the number of ovulation sites. Transferring embryos into left site of uterine horns favored pregnancy and embryo survival rates.

Notes

ROYAL JELLY TREATMENTS IN LIVESTOCK FOR ASSISTED REPRODUCTIVE BIOTECHNOLOGIES

Emsen Steinman B[1], Emsen E[1]

[1]*Ataturk University, Department of Animal Science,25240, Erzurum, Turkey*

Royal Jelly (RJ) is a glandular secretion produced by honeybees that has attracted the attention of nutritionists and physicians due to the longevity and fertility that it confers to the honeybee queen. This unusual property of RJ has spurred a possible connection to fertility and we know that RJ has always been used as a stimulator of fertility. RJ is effective in improving hormonal equilibrium and fertility in male and female (Lewis, 2005). The presentation aims at summarizing RJ treatments which have been done in different reproductive studies.

The RJ treatments were based on different applications such as, oral, intramuscular and intravaginal. A series of studies have assessed RJ on ewes for improving reproductive performance. Several fatty acids from RJ has found to mimic estrogens (Suziki et al., 2008). Different studies in ewe have proved similar comparative results on the intravaginal administration of RJ and of progesterone, improving the estrus response and gestation rates (Huseina nd Kridli, 2002). RJ had similar effects likewise as the equine chorionic gonadotropin, by inducing the estrus and improving pregnancy rates either in natural mating or laparoscopic artificial insemination (Husein and Haddad, 2006; Kridli et al., 2003; Gimenez-Diaz et al., 2012).

However, the oral administration of RJ has not been effective in improving the estrus in sheep for shortening anestrus period or triggering puberty in sheep (Kridli and Al-Khetib, 2006; Molla and Kridli, 2003). In another study, orally or intramuscular RJ treatments (250 mg/day/ewe) in conjunction with exogenous progesterone (12 d) were equally capable of improving oestrus response and pregnancy rate in fat tailed ewes (Husein and Kridli, 2002). Musa et al., (2013) used royal jelly as an alternative to fetal bovine serum in cell culture using cell proliferation assays and live cell imaging. They found that RJ does not exhibit similar ability like FBS to facilitate cell growth. However, Salazar-Olivo and Paz-Gonzalez (2005) reported that protein fractions of RJ differentially stimulated the cell growth of Tn-5B1-4 insect cells. The mitogenic effect of RJCP was higher than the mitogenic ability of FBS, the usual protein supplement for insect cell cultures.

Previous reports have indicated that the production of pro-oxidants during sperm cold and liquid storage is one of the inevitable processes and therefore recommended the antioxidant supplementation in semen extenders (Michael et al., 2009). On the other hand, the antioxidant effect of RJ has been previously demonstrated in various studies such as on cisplatin-induced testis damages and scavenging ability against free radicals such as superoxide anion radical (Nagai and Inoue, 2004 and Silici et al., 2009). Moradi et al. (2013) used RJ as an antioxidant agent for semen storage and reported that the protective effects of the RJ supplementation on ram sperms viability, motility, and plasma membrane functionality in liquid storage processes. Moreover, the protective effects of RJ likely related to its antioxidant property, which could be provided at proper concentrations. They underlined that it should be taken into account that the concentration of RJ must be adjusted based on the duration of storage time. It also has been demonstrated that the RJ administration to the heat-stressed male rabbits resulted in a remarkable reduction of their “summer infertility” and improved their physiological status (Elnagar, 2010).

In total, multiple studies have been performed which argue a beneficial effect of Royal Jelly on reproduction parameters.

Notes

EFFICIENCY OF EQUINE EMBRYO PRODUCTION BY OVUM PICK UP AND ICSI IN DIFFERENT BREEDS

Galli C[1,2,3], Duchi R[1], Crotti G[1], Turini P[1], Colleoni S[1], Lazzari G[1,3]

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

Equine embryo production by ultrasound guided transvaginal oocyte recovery from live donor mares (Ovum Pick Up, OPU), Intracytoplasmic Sperm Injection (ICSI) and in vitro embryo culture is becoming an established procedure in equine assisted reproduction although only a relatively small number of clinics are presently offering this technology as a service. Here we report the outcome of 3 consequent years of OPU-ICSI service provided to breeders by our laboratory.

Most of the animals referred to us as OPU donors belong to three breeds/categories: Arabian, Quarter and Warmblood therefore only the data from these donors are included in the present abstract and are summarised in Table 1. A total number of 367 OPU-ICSI procedures were performed on 132 donor mares giving rise to 181 transferrable embryos. On average 2.78 OPU/ICSI procedures were performed on each donor mare and the average embryo production was 1.27 per donor and 0.49 per OPU/ICSI. We did not observe large differences between the 3 categories in the number of follicles aspirated and oocytes recovered nor in the percentages of oocytes that reached metaphase II and were injected. By contrast cleavage rate and blastocyst rate were significantly lower in Arabian mares (44.56% and 2.42%) compared to Quarter (58.11% and 6.44%) and Warmblood mares (62.61% and 7.86%) (Chi square test, $p<0.05$).

Table 1. Efficiency of OPU-ICSI in different breeds/categories.

Breed/category	n. donors	n. OPU	n. follicles	n. oocytes	% recovery	n. MII injected	% MII	n. cleaved	cleavage rate %	n. embryos	% / oocytes
ARABIAN	56	214	3726	2607	69.97	1526	58.53	680	44.56a	63	2.42a
per OPU			17.41	12.18		7.13		3.18		0.29	
QUARTER	22	43	611	404	66,12	265	65.59	154	58.11b	26	6.44b
per OPU			14.21	9.40		6.16		3.58		0.6	
WARMBLOOD	54	110	1683	1170	69,52	813	69.49	509	62.61b	92	7.86b
per OPU			15.30	10.64		7.39		4.63		0.84	
TOTAL	132	367	6020	4181		2604		1343		181	

In conclusion this clinical dataset indicates that the efficiency of the OPU-ICSI technology is influenced by the breed/category of the donor mares.

Notes

OVUM PICK UP AND IN VITRO EMBRYO PRODUCTION FOLLOWING PROPYLENE GLYCOL DIET IN HEIFERS DIFFERING IN THEIR AMH PROFILES

Gamarra G[a,b], Ponsart C[a], Lacaze S[b], Le Guienne B[a], Humblot P[c], Deloche M-C[a], Monniaux D[d], Ponter AA[h,i]

[a] UNCEIA Département Recherche et Développement, 13 rue Jouet, 94704 Maisons-Alfort cedex, France [b] MIDATEST, Domaine de Sensacq cidex 55A, 64230 Denguin, France [c] Division of Reproduction, Department of Clinical Sciences, Faculty of Veterinary Medicine and Agricultural Sciences, SLU, PO Box 7054, SE 75007 Uppsala, Sweden [d] INRA, UMR85 Physiologie de la Reproduction et des Comportements, 37380 Nouzilly, France [h] Université Paris Est, Ecole Nationale Vétérinaire d'Alfort, UMR 1198 Biologie du Développement et Reproduction, 94704 Maisons-Alfort cedex, France [i] INRA, UMR 1198 Biologie du Développement et Reproduction, Domaine de Vilvert, F-78350 Jouy-en-Josas, France.

The aim of this study was to test whether the daily oral administration of propylene glycol (PG) could improve *in vitro* embryo production in superovulated growth-restricted heifers (600 g/day) differing in their AMH profiles. Sixteen Holstein heifers were grouped according to AMH concentrations: low (L = 1-80 pg/mL; n=7) or high (H: >150 pg/mL; n=9).

Oestrus was synchronised, and Ovum Pick-Up (OPU) performed following superovulation, on Day 5 of the oestrous cycle. COCs were matured and fertilized *in vitro*. Embryos were cultured in SOF for 7 days. Heifers received a single daily drench of 400 mL of water (Control) from Day 1 to Day 9 of the first synchronized oestrous cycle followed by 400 mL of PG from Day 1 to Day 9 of the second synchronized oestrous cycle. A series of blood samples were collected from the jugular vein on Day 7 of each cycle to monitor the kinetics of plasma insulin, glucose and β-hydroxybutyrate (BHB) concentrations, in relation to the drench. Blood samples were also collected to measure insulin-like growth factor1 (IGF1) on Days 0, 2, 5, 7 and 9 and progesterone (P4) concentrations on Days 2, 5 and 9 of the oestrous cycle. Ovarian ultrasonography was performed on Day 2 and on Day 5 to count follicles and estimate their size.

PG increased insulin and glucose and reduced BHB concentrations in both groups of heifers compared to Control. It also increased IGF1 concentrations on Days 5 and 7 in AMH L heifers and on Days 2, 5 and 7 in AMH H heifers, and reduced P4 concentrations on Days 5 and 9 of the oestrous cycle in all heifers compared to Control. Administration of PG significantly increased the number of: small follicles (2-3 mm) and total follicles (2-8 mm) on Day 2 of the cycle in all heifers and medium follicles (4-8 mm) and total follicles (2-8 mm) on Day 5 in AMH H heifers. PG also increased the number of grade 1 oocytes in AMH H and increased the developmental kinetics of embryos at 48 h post-insemination in both groups. PG improved the *in vitro* embryonic development rate (total number of embryos/number of fertilized oocytes) in all heifers compared to Control (AMH L: Control, 37.9% *vs.* PG, 50.0%, $P<0.05$; AMH H: Control, 36.4% *vs.* PG, 48.3%; $P<0.05$). In AMH H, the number of grade 1 blastocysts on Day 7 was increased by PG when compared to Control (Control, 5.2 ± 1.0 *vs*. PG, 8.9 ± 1.0 $P<0.01$) while there was no difference between treatments in AMH L heifers (Control, 1.9 ± 1.1 *vs*. PG, 3.2 ± 1.1 $P>0.05$).

These results indicate that short-term oral PG supplementation affects circulating concentrations of metabolites and metabolic hormones and is effective in improving *in vitro* embryo production after superovulation and OPU more markedly in high AMH compared to low AMH heifers.

Notes

IN VITRO EMBRYO PRODUCTION FROM OOCYTES FERTILIZED WITH UNSORTED OR X-SORTED SPERM AND ISSUED FROM SUBFERTILE HIGH GENETIC MERIT COWS SUBMITTED OR NOT TO A 48 h COASTING PERIOD FOLLOWING FSH STIMULATION

Gamarra G[1,2], Lacaze S[1], Ponsart C[3], Mouneyses M[1], Marquant Le Guienne B [2]

[1] *MIDATEST, Domaine de Sensacq, 64230 Denguin, France*
[2] *UNCEIA, R & D Department, 13 rue Jouët, 94704 Maisons-Alfort, France*
[3] *DVM, PhD, 101 rue de la Justice, 91800 Boussy-Saint-Antoine, France*

High genetic merit cows may be sidelined from breeding schemes because of reproductive disorders. *In vitro* production of embryos (IVP) issued from ovum pick up (OPU) can be an alternative to bypass infertility problems as experienced in humans and thus accelerate genetic progress (Duszewska *et al*., 2012, J Anim Feed Sci, 21, 217–233). It has been shown previously that OPU-IVP methods may be used to produce embryos from infertile cows (Guyader-Joly C et al, 2009, 25th A.E.T.E meeting, p. 176). The aim of this work was to evaluate if *in vitro* embryo production from high genetic merit subfertile cows could benefit to the breeding scheme under commercial conditions, at the Biotechnology MIDATEST Station located in Denguin, South West, France.

Holstein Cows (n=16) from 3.5 to 13 years old with different reproductive pathologic problems (repeated breeding, failure in *in vivo* embryo production, embryo mortality, permanent cysts, oviduct infection) were used in OPU-IVP program. Donor cows were stimulated with decreasing doses pFSH twice daily during 3 days (Stimufol®, total dose: 350 µg of pFSH). OPU was performed 48 hours after the last FSH injection in the "Coasting" group vs 12 hours after the last FSH injection for "No-Coasting" group using a 240 Parus Vet ultrasound scanner (Pie Medical) with a 7.5 MHz annular-array probe. Oocytes were *in vitro* matured in M199 supplemented with FCS, FSH/LH, estradiol and EGF for 22 hours at 38.5°C. They were fertilized with frozen – thawed unsorted or x-sorted sperm in TALP medium using different bulls (n=42) without any previous IVP testing. Presumptive zygotes were cultured in SOF medium (Minitub®) 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 once to 6 times (3.75 ± 2.6) for each donor cow. Grade1 blastocysts and expanded blastocysts (excellent and good quality, according to IETS classification) were recorded on days 6.5 and 7. Viable embryos were frozen or transferred as fresh. Embryo production was analyzed with ANOVA and blastocyst yield was analyzed by Chi-Square.

From 60 OPU sessions, a total of 703 oocytes (11.7±7.6 per session) were processed for *in vitro* maturation, and 403 Grade 1 embryos were produced (6.8 ± 5.6 per session). The mean embryo development rate (total number of grade 1 embryos / number of oocytes put in maturation) was 58.2 ± 23.1 %. The results in both coasting or no-coasting groups and the effect of fertilization using unsorted or x-sorted sperm are presented in Table 1. The embryonic development rate was significantly higher when using unsorted semen to fertilize the oocytes compared to x-sorted semen ($P<0.05$). On the other hand the coasting period had no significant effect neither on the number of collected oocytes nor on the embryonic development rates.

Table 1. Oocyte collection and *in vitro* embryo production in donor cows submitted or not to a 48 h coasting period and effect of fertilization with unsorted vs x-sexed sperm.

Donor cows	Coasting		No-Coasting	
In vitro Fertilization	X-Sorted	Unsorted	X-Sorted	Unsorted
Total no. fertilization sessions (FS)	11	9	9	33
Mean no. IVM oocytes /FS	10.5±4.7	12.1±7.3	11.3±6.8	11.4±7.0
Mean no. G1 embryos/FS	5.0±3.7	8.2±5.7	5.3±3.6	7.0±5.2
% G1 embryos/IVM oocytes	47.4[a] ±22.6	67.9[b] ±14.8	47.1[a] ±32.1	61.7[b] ±21.1

[a,b] $P<0.05$

In conclusion, our work confirmed the efficacy of OPU-IVP techniques to produce grade 1 embryos using x-sorted or unsorted sperm in subfertile high genetic merit cows.

Notes

CORRELATION BETWEEN FOLLICULAR DIAMETER AND CONCEPTION RATE IN HOLSTEIN COWS SUBMITTED TO TAI IN SEMI-ARID CONDITIONS

Guido SI[1], Guido FCL[2], Santos Filho AS[1], Oliveira JCV[1], Bartolomeu CC[3]

[1] *Agronomic Institute of Pernambuco – IPA, Brazil.*
[2] *Autonomous Veterinary Doctor, Brazil.*
[3] *Department of Veterinary Medicine / Federal Rural University of Pernambuco-DMV/UFRPE, Brazil*

The study was conducted at the Experimental Station of São Bento do Una (EESBU / IPA), Pernambuco, Brazil (Latitude 08 31 '35" and longitude 036 27 '34.8") in order to evaluate the correlation between follicular diameter and conception rate (CR) in Holstein cows kept in semi-arid conditions with temperature and humidity index (THI) 63.5. 244 cyclic cows were used, between 90 and 120 days in milk with an average production of 24.8 kg/milk/day and age ranging from 36 to 108 months. The cows were kept in a semi-intensive system, receiving a diet composed of cactus pear (Opuntia ficus-indica Mill), sorghum silage (Sorghum bicolor (L) Moench) and protein concentrate with 24% of crude protein and mineral supplement and water ad libitum. All cows were subjected to gynecological examination by rectal palpation, being randomly distributed into two treatments T1 and T2: At T1 (n = 112) cows received an intravaginal device (1.9 g progesterone) on D0 associated with 2mg of estradiol benzoate (BE). On D7, 0.530 mg of cloprostenol and 300 IU of eCG at device removal on D8. On D9 it was administered 1mg of EB and on D10 TAI was preceded 54 hours after device removal. The T2 cows (n = 132) received the same protocol as in T1 except for 400 IU of eCG. All cows in both treatments (T1 and T2) underwent ultrasonography at D9 to evaluate the follicular diameter and were inseminated with conventional semen of bulls of the same breed. The data were subjected to chi-square test and analysis of variance and means were compared by t-test at 5% by the statistical program SPSS 16 for Windows, and was also checked the Pearson Correlation of follicular diameter and CR at 30 days. Pregnancy diagnosis was performed by ultrasound on day 30 after TAI.

At D9 follicular diameter ranged from 6.1 to 16.3 mm in T1 and from 6.8 to 23.2 mm in T2 ($p = 0.0001$). CR was 28.6% (32/112) in T1 and 63.6% (84/132) in T2 ($p = 0.0118$). A correlation between follicular diameter and CT for both groups T1 ($r = 0.9268$) and T2 ($r = 0.6928$) was identified.

Therefore, it can be concluded that in this experiment follicular diameter at D9 of protocol presented correlation with CR on the 30th day. As well, the dose of eCG used in the T2 provided superior results.

Notes

FIRST BIRTH AFTER TRANSFER OF SEXED AND CRYOPRESERVED WELSH PONY BLASTOCYSTS IN FRANCE

Guignot F[1], Reigner F[2], Blard T[2], Orbinot J[1], Barriere P[2], Yvon JM[2], Mermillod P[1], Caillaud M[3]

[1] *UMR INRA-CNRS-Université de Tours-IFCE, PRC, F-37380 Nouzilly, France*
[2] *UE1297, UEPAO, INRA, F-37380 Nouzilly, France*
[3] *IFCE, la Jumenterie du Pin, F-61310 Exmes, France*

Embryo cryopreservation and transfer is a powerful tool for genetic selection and has extensive economical and clinical applications, particularly if the embryo is genotyped before transfer. The first foal resulting from a biopsied and vitrified embryo was obtained with an embryo at pre-capsule stage (Troedsson et al., AniRep, 2010). Unfortunately, equine embryo cryopreservation was problematic at capsule stage, and particularly with embryos > 300 µM in diameter. In fact, at embryo collection, generally D7 after ovulation in field conditions, large blastocysts with capsules were often obtained. In 2011, Choi et al (Theriogenology) demonstrated that blastocoele fluid suction could circumvent this difficulty and allow the cryopreservation of large equine embryos. We have already tested this technique on pony embryos and obtained early pregnancies (Reigner et al, CRYO, Berlin, 2012).

The aim of the present study was to test the viability up to term of D7 Welsh pony embryos after biopsy by cell aspiration for sex determination, blastocoele fluid suction, vitrification and transfer to equine recipients in field conditions.

Nine expanded blastocysts, 166-422 µm in diameter, grade 1 and 2, were collected at D7 after ovulation from Welsh pony mares. Approximately 2-4 trophectoderm cells and 70% of the blastocoele fluid were aspirated using a glass pipette attached to a Piezo drill. Biopsied embryos were immediately vitrified using the OPS procedure. Briefly, embryos were placed in culture medium: modified synthetic fluid (mSOF) + 20% foetal calf serum (FCS) + 19 mM glucose for 5 min at 38.5 °C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. They were then vitrified in 2 steps: 1.5 M ethylene glycol (EG) for 5 min and 7 M EG supplemented with 0.6 M galactose for 30 sec. One embryo was then loaded per straw. For warming, embryos were deposited in culture medium with decreasing sucrose concentration (0.2 – 0.1 and 0.0 M) for 5 min each and immediately transcervically transferred to mares. Pregnancies were ultrasonographically monitored until an embryonic heartbeat was detected (D30) and maintained to term. Sex diagnosis was carried out on biopsied cells by PCR amplification of ZFX/ZFY and SRY sequences. Three equine control (not biopsied) embryos were collected and fresh transferred.

All twelve embryos were transferred. Two biopsied embryos lost their capsule at warming. They did not give pregnancy. Among the seven remainders, five were pregnant at D14 and 4 have an embryonic heartbeat at D30. The first foals were born the 18th of May. Sex diagnosis on biopsied cells was accurate (two males identified and born). Two others were born the 26th of May (two females as predicted). 100% of accuracy. The 4 foals are alive and healthy. All 3 control embryos have given a pregnancy at D30 and have been voluntarily stopped.

A grant from IFCE (Institut français du Cheval et de l'Equitation) was received to perform this experiment.

Notes

THE EFFECT OF CALCIUM SALT OF FATTY ACIDS WITH DIFFERENT PROFILES DURING THE FLUSHING PERIOD ON REPRODUCTIVE PERFORMANCE OF IRANIAN *AFSHARI* EWES

Hosseinkhani A, Askari AH, Daghigh Kia H

Department of Animal science, University of Tabriz, Tabriz, Iran

Nutritional supplementation or flushing prior to mating has been reported to increase ovulation and lambing rates in many breeds of sheep. Dietary lipid can increase the number and size of ovarian follicles in estrous cyclic ewes by increasing the circulating progesterone and cholesterol concentrations. Several experiments have been showed that the linoleic acid (LA, ω_6) and a-linolenic acid (ALA, ω_3) are necessary for numerous processes, such as growth, reproduction and brain development. The present study was conducted to evaluate the effects of feeding CSFA with different profiles of fatty acids on reproductive performance in flushing period of *Afshari* ewes.

Forty-eight Iranian *Afshari* ewes were allocated in four groups (n=12) to study the effects of CSFA with different profiles of fatty acids in flushing period on reproductive performance. The ewes in each group were fed the same basal ration. In addition each group received a group specific supplementation of the basal ration. Group A: barley grain; group B: 5% of CSFA with flaxseed oil (as source of ω_3); group C: 5% of CSFA with sunflower oil (as source of ω_6) and group D: control (only received basal diet). It was shown, that all flushing treatments improved fertility and lambing rates compared to the control group (125, 150 and 116.7 vs. 83.3 percent respectively). In the flaxseed supplemented group 18 lambs were born whereas the birth of 10 lambs was observed in the control group. These groups represent the highest and the lowest number of progeny respectively (χ^2= 10.50, P <0.05). The flushing ration containing flaxseed oil had the highest levels of blood progesterone and cholesterol compared to the other groups (P <0.01).

Key words: CSFA, flushing, lambing rate, Afshari ewe

Notes

CLINICAL OR ULTRASOUND EXAMINATION FOR SELECTION OF RECIPIENT HEIFERS

Jaskowski JM, Gehrke M, Urbaniak K, Wozna M, Rybska M, Jozwiak P, Jaskowski BM, Boryczko Z

Institute of Veterinary Sciences, Poznan University of Life Sciences, Poland

Clinical or – recently - ultrasound examination of genital tracts of recipient heifers before embryo transfer has become a significant method to successfully pre-select animals. The functional structures of ovaries, mainly the corpus luteum (CL) seem to play a crucial role when assessing the reproductive tract.

The aim of this experiment was to evaluate the usefulness of clinical and ultrasound examination of heifers for selection of recipients on the day of embryo transfer. In total, 490 heifers were used in the study. Only those females were selected for the experiment that showed standing heat 7-8 days before. 473 recipient heifers fulfilled these criteria. All recipients were allocated to the following groups. Good (A) recipients – animals showing increase in size of ovaries, growing structure of CL could be easily palpated; questionable (B) recipients - animals having asymmetric ovaries, one ovary was greater compared to its counterpart; insufficient (C) recipients – both ovaries of the heifers were small; bad (D) recipients – heifers with ovarian cysts or big follicles; and group E - others.

According to the first step of examination 51.6% of heifers were classified as Good (A) recipients, whereas 25.2, 28.9, 2.1 and 0.6% of animals were assessed according to the groups B, C, D and E, respectively. In the second step all animals were examined using ultrasonography. A portable ultrasound machine (iSkan Draminski) equipped with 7.5 MHz probes was used during the experiment. Animals were assessed as good recipients when a CL > 17 mm in size was detected. In total, 303 (64.1%, $p<0.0191$) animals were classified as good recipient heifers, the remaining ones as “unsuitable”. The accuracy of the clinical examination of CL in group A (good recipients) was 95.1%. After ultrasound examination 26.6% of animals
from group B (clinically questionable recipients) were re-grouped and accorded to category A.

In conclusion, the clinical examination is a more restricted method to choose good embryo recipients than using ultrasound evaluation including the visualization of ovarian structures. The use of ultrasound for examination of ovaries seems to be a much more reliable method in particular evaluating and selecting questionable recipients.

Notes

NON-ESTERIFIED FATTY ACIDS AFFECT SPERM BINDING CAPACITY OF BOVINE OVIDUCT EPITHELIAL CELLS IN TWO *IN VITRO* CULTURE SYSTEMS

Jordaens L, Valckx S, Merckx E, Andries S, Bols PEJ, Leroy JLMR

Laboratory for Veterinary Physiology and Biochemistry, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium

Early post-partum negative energy balance in high-yielding dairy cows is characterized by up-regulated lipolysis and a rise of non-esterified fatty acids (NEFAs) in blood as well as in follicular fluid (FF). This has been associated with poor reproductive performance, as it affects oocyte and pre-implantation embryo quality. However, the effects of elevated NEFAs on the oviductal micro-environment and physiology remain largely unknown. To study Bovine Oviduct Epithelial Cell (BOEC)-quality and -functionality *in vitro,* their affinity for sperm attachment can be used. In order to observe the effect of NEFAs on *in vitro* BOEC-sperm binding, and to establish a BOEC-culture that mimics the *in vivo* oviduct-sperm interactions most closely, we studied BOEC-sperm binding in two specific cell culture systems: 1) a short term suspension culture of BOEC-explants (BOECe), and 2) a 2-compartment (apical and basolateral) polarized cell culture system (PCC) with BOEC-monolayers (BOECm) in hanging inserts.

Therefore, 4 ipsilateral bovine oviducts at day 3-5 of the estrous cycle were selected in a local slaughterhouse. After mechanical isolation, BOECs were pooled and transferred into their respective culture systems. In **experiment 1,** BOECe were selected according to ciliary activity and size (surface area < 20 000µm^2) and divided in 4 treatment groups (10 BOECe per treatment): Control (0µM NEFA), Basal (72µM NEFA), Moderate (360µM NEFA) and High (720µM NEFA). In **experiment 2,** BOECm confluency was recorded (TER > 700Ω.cm^2 at day 9) after which NEFAs were added unilaterally (by alternating 72µM and 720µM NEFA-medium between the 2 compartments) or bilaterally at both concentrations. In the 2 experiments, NEFAs (NEFA combi of 3 predominant NEFAs in blood and FF: Oleic, Palmitic and Stearic Acid) were added for 24h. Subsequently the NEFA-medium was discarded, the cells were washed and coincubated with 1x10^6 spermatozoa/ml in Sperm-TALP. After 30 minutes, unbound sperm cells were washed away and BOECs with bound sperm cells were fixed in 4% PFA. The bound sperm cells per surface area (sp/0.05mm^2±SD) were counted using light microscopy.

Results in experiment 1 showed a significantly reduced BOECe-spermbinding in the Moderate and High NEFA treatment groups, of 59.19% and 68.05% respectively, when compared to binding in the control group (*P<0.05*). In experiment 2, bilateral NEFA-exposed BOECm (31.28±6.16 sp/0.05mm^2) showed a significantly reduced sperm binding affinity compared to the control (97.90±10.76 sp/0.05mm^2; *P<0.05*), and unilateral exposed monolayers tended to be more affected by apical (39.95±19.30 sp/0.05mm^2) than by basolateral NEFA-contact (68.55±15.38 sp/0.05mm^2; *P=0.051*).

BOECe sperm binding due to *in vivo*-like sperm-cilia interactions was negatively affected by elevated NEFAs, indicating a diminished BOEC-functionality. BOECm tended to dedifferentiate during culture and sperm binding was established by microvilli rather than cilia. Interestingly, the PCC allowed a more physiologically relevant BOECm-NEFA contact, in which BOECm tended to be affected more by apical than by basolateral NEFA-exposure. This suggests that BOECm are capable of partially buffering the effects of basolaterally administered NEFAs and thereby guard over the oviductal lumen and the processes taking place therein. Though, this needs further investigation, in an optimized experimental setting combining the advantages of both BOECe- and BOECm-culture.

Notes

STUDY ON CONNEXIN GENES AND PROTEINS EXPRESSION AND CELLULAR DISTRIBUTION IN RELATION TO REAL-TIME PROLIFERATION OF PORCINE GRANULOSA CELLS

Kempisty B[1,2], Ziołkowska A[1], Ciesiołka S[1], Piotrowska H[3], Jopek K[1], Antosik P[4], Bukowska D[4],Rybska M[4],Wozna M[4], Jaskowski JM[4], Nowicki M[1], Jeseta M[5], Brüssow KP[6], Zabel M[7]

[1]Department of Histology and Embryology, Poznan University of Medical Science, Poland,[2]Department of Anatomy, Poznan University of Medical Science, Poland,[3]Department of Toxicology, Poznan University of Medical Sciences, Poland, [4]Institute of Veterinary Sciences, Poznan University of Life Sciences, Poland , [5]Department of Genetic and Reproduction, Reproductive Biotechnology Unit, Veterinary Research Institute, Brno, Czech Republic, [6]Institute of Reproductive Biology, Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany, [7]Department of Histology and Embryology, Wroclaw Medical University, Poland

In the present study, porcine GCs were isolated from the follicles of puberal gilts and then cultured in a RTCA system for 168 h. The expression levels of Cx36, CX37, Cx40 and Cx43 mRNA were measured by RQ-PCR analysis, and differences in the expression and distribution of Cx30, CX31, Cx37, Cx43 and Cx45 proteins were analyzed by confocal microscopic visualization.

We found an increased expression of Cx36, Cx37, and Cx43 mRNA in GCs before in vitro culture (at 0 h IVC) compared to all analyzed time periods of IVC (24, 48, 72, 96, 120, 144 and 168 h; $P<0.001$). On the other hand, the expression level of Cx40 transcripts was higher after 24 h of IVC compared to 0 h and the other times of IVC ($P<0.001$). Similarly to mRNAs, the expression levels of Cx31, Cx37 and Cx45 proteins were higher before (0 h) compared to after 168 h of IVC. The expression of Cx30 and Cx43, however, did not vary between the groups. In all, the proteins were distributed throughout the cell membrane rather than in the cytoplasm both before and after IVC. After 24 h of IVC, we observed a significant increase in the proliferation of GCs (log phase). We found differences in the proliferation index between 72-96 and 96-140 h within the same population of GCs.

A decrease in the expression of Cx mRNAs and proteins following IVC is associated with a breakdown in GJCs, leading to a decrease in their activity and a disruption of their bi-directional communication system function. Additionally, after the initial 24 h of IVC, the development of porcine oocytes seems to base on previously accumulated templates rather than on signals transferred from granulosa cells via GJCs. GCs proliferation is distinct from GJC activity and possibly both processes are regulated by different mechanisms.

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Notes

COMPARISON OF THE EFFECTS OF THE mTORC1-INBIBITOR RAPAMYCIN AND THE mTORC1/C2-INHIBITOR TORIN2 ON MEIOTIC PROGRESSION IN BOVINE OOCYTES

Kreißelmeier M[1], Mayer S[1], Wrenzycki Ch[2], Tomek W[3]

[1]BVN Neustadt/Aisch, Germany, [2]Faculty of Veterinary Medicine, Justus-Liebig-University Giessen, Germany, [3]Leibniz-Institute for Farm Animal Biology, Institute for Reproductive Biology, Dummerstorf, Germany

Post-transcriptional control of gene expression at the level of translation is an important cellular function in normal development. In this context, it has emerged that fully grown oocyte utilizes only transcripts synthesized during earlier development. Therefore, translational control is a hallmark for meiotic maturation of oocytes. At the onset of the first meiotic division, nuclear envelope breakdown occurs, chromosomes condense, and a bipolar spindle is formed from microtubule organizing centers. During meiosis I, the spindle migrates from the center of the oocyte to the cortex and the oocyte undergoes asymmetric division resulting in a large egg competent for fertilization and a relatively small polar body. mTor (mammalian target of Rapamycin), a Ser/Thr protein kinase, which, among others, regulate translational repressors is implicated in these processes. It became evident that at least two mTor complexes exist which possess different major regulator proteins. These are mTorC1 containing Raptor and mTorC2 bound to Rictor. mTorC1 is Rapamycin sensitive, whereas mTorC2 complex is resistant to Rapamycin treatment. In the present study, we have used two different mTor inhibitors, first Rapamycin (impairs mTorC1 function) and second Torin2, an active site inhibitor which blocks mTorC1 and C2. The results show that Torin2 (3µM final concentration) irreversibly arrests oocytes in the metaphase I state. Furthermore, the phosphorylation and therefore the inactivation of the translational repressor 4E-BP1 was repressed, but in a reversible manner. In contrast, Rapamycin had an effect only at higher concentration. At 10µM, it inhibits spindle migration and asymmetric division. However, effects of Rapamycin on 4E-BP1 phosphorylation could not be established so far. In conclusion, our results indicate that Rapamycin and Torin2 are valuable tools to investigate meiotic progression more in detail, with special attention to acquire developmental competence.

Notes

INTRACELLULAR CALCIUM SIGNALLING IN PORCINE GROWING OOCYTES AND OOCYTES THAT HAVE FINISHED GROWTH PHASE

Kuzmina T[1], Denisenko V [1], Alm H [2], Torner H [2], Brüssow K-P [2]

[1]All-Russian Research Institute for Farm Animal Genetics & Breeding, 189620 St.Petersburg-Pushkin, Russia
[2]Leibniz Institute for Farm Animal Biology, 18196 Dummerstorf, Germany

Population of donor oocytes used in cell reproductive technology is heterogeneous. Brilliant cresyl blue (BCB) staining has been used for selection of oocytes from several mammalian species. It has been demonstrated that BCB staining improves the homologous in vitro penetration (hIVP) efficiency in particular in pigs (Roca et al. 1998). Our previous studies showed that rbST stimulates the release of Ca^{2+} from IP3-sensitive intracellular stores, GTP - from IP3-insensitive (Denisenko et al. 2007). Moreover, GTP forms a bond and fosters transition of Ca^{2+} between these two types of intracellular stores (Ghosh et al. 1989). The additional Ca^{2+} release from intracellular stores under the common action of growth hormone and GTP serves as a sign of the transition between the different intracellular Ca^{2+} depots. The aim of the present study was to evaluate the fluctuation of Ca^{2+} in porcine growing oocytes (BCB^-) and in oocytes that have finished growth phase (BCB^+) after rbST and GTP supplementation in vitro.

Before the ***measurement*** of membrane-bound calcium, compact cumulus oocyte complexes (COCs) were incubated in BCB solution for 90 min. Treated oocytes were then divided into BCB^- (colorless cytoplasm, increased G6PDH) and BCB^+ (colored cytoplasm, low G6PDH) regarding their ability to metabolize the stain. Thereafter, BCB^+ and BCB^- oocytes were treated for 10 min with rbST, GTP or rbST+GTP. Intensity of fluorescence of membrane-bound calcium was determined with a fluorescent microscope (excitation: 380-400nm, emission: 530 nm) using 40 µM chlortetracycline (CTC). Data were analyzed by Student's t-test.

Our results demonstrate (Table 1) that rbST and GTP separately stimulated Ca^{2+} exit from intracellular stores in BCB^+ oocytes. The common action of rbST and GTP activates additional Ca^{2+} exit from intracellular stores in BCB^+ oocytes. In BCB^- oocytes rbST and GTP also stimulated Ca^{2+} exit from intracellular stores; however, an additional effect of rbST+GTP on Ca^{2+}exit from intracellular stores in BCB- oocytes was not observed.

Table 1. Effect of rbST and GTP on the release of Ca^{2+} from intracellular stores of BCB^+ and BCB^- porcine oocytes.

Treatments	Fluorescence intensity of complexes of Ca^{2+} + CTC + membrane in oocytes (A.U. ± SEM/oocyte) (n oocytes)	
	BCB^+	BCB^-
Control	0.93 ± 0.058^{a} (70)	0.85 ± 0.048^{e} (68)
10ng/ml rbST	0.66 ± 0.057^{b} (33)	0.46 ± 0.035^{f} (31)
10 µM GTP	0.66 ± 0.040^{c} (35)	0.40 ± 0.025^{g} (36)
10ng/ml rbST+10 µM GTP	0.46 ± 0.028^{d} (68)	0.41 ± 0.016^{h} (67)

$^{a:c,c:d,e:f,e:g}$ $P < 0.001$, $^{a:b,b:d}$ $P < 0.01$ (Student's t-test)

Our results demonstrate significant differences in the mechanisms of pathway of calcium signaling in BCB^+ and BCB^- porcine oocyte and show the ability to determine the completeness of the growth phase by measuring calcium after rbST and GTP supplementation.

Notes

A USEFUL METHOD FOR EVALUATION OF HORSE EMBRYOS AFTER DIFFERENT MANIPULATIONS

Lebedeva L, Nikitina I, Sidorova N

The All – Russian Research Institute for Horse Breeding, 391105
Rybnoe district Ryazan region, Russia

In experimental work with embryos in mammals, particularly horses, it is very important to evaluate the level of viability of embryos after various manipulations. Currently, there are five basic ways to assess the quality of embryos: 1) morphological assessment by light microscopy, 2) in vitro culture, 3) transfer to recipient, 4) histological analysis (light and electron microscopy) and 5) staining of embryos by special dyes. The more exact the methods are the more laboratory skills, time, efforts and financial input are needed. The present study focused on the methodology of embryo staining. The viability of embryos from laboratory and farm animals were tested by staining with different dyes such as trypan blue, Evans blue, Hoechst 33342, FITC (fluorescein isothiocyanate), PI (propidium iodide), DAPI (4,6-Diamidino- 2 -phenylindole), etc.

In this experimental work Evans blue was used, which allows to image and distinguish live and dead cells in the embryo. However, it is difficult to estimate the level of cell damage in intact stained embryos in Dulbecco's PBS because of its round shape. Therefore, embryos were put on a glass slide in a drop of Dulbecco's PBS medium and covered with a coverslip, to flatten the embryo. In this form, dead (blue) and live (non-stained) cells in the embryo are easily distinguishable under the light microscope. When membrane/capsule of the embryo burst under the coverslip the cell mass leaves the capsule and blastomeres become better visible.

6.5-8.5 day horse embryos of good and excellent quality were divided into 5 groups for treatment: 1) effect of the cryoprotectant on the embryo without freezing (n = 13), 2) conventional slow cooling (n = 5), 3) vitrification (n = 12), 4) hypothermia (24 hours at 5ºC, n = 14), and 5) control (fresh embryos, n = 5). In each group, the embryos were divided into two sub-groups according to the size ($\leq$ 350 μ and $\geq$ 350 μ). After treatment (Group 1-5) embryos were stained with Evans Blue (0.05 % in Dulbecco PBS) for 15 min, washed in PBS for 10 min, then they were placed on the slide, covered with a coverslip and the percentage of stained (dead) and unstained (live) cells in the embryo was visually evaluated using a light microscope (X 56). Embryos in the different groups which revealed the best results were transferred into recipients (n=8).

In total, 57 embryos were used in all experimental groups. The results showed that the percentage of stained (damaged) cells in the groups (1,2,3,4) was higher when age/diameter of embryos increased (from 1% to 95%). The freezing process had the most damaging effect on the cells of the embryos (30-95% in large embryos). In control embryos (group 5) there were no stained cells visible. Embryos having the highest percentage of live cells were used for transfer, 8 embryos were selected for this step: 4 vitrified embryos (group 3) resulted in 2 pregnancies (50%), 2 cooled embryos (group 4) resulted in 1 pregnancy (50%), 2 fresh embryos (as control, group 5) resulted in 2 pregnancies (100%).

Thus, using Evans blue dye for ***live-dead*** determination provides a simple, fast and inexpensive method. This kind of evaluation of embryo quality after different manipulations allows to accelerate the experimental work with horse embryos and to select the best embryos for being transferred to recipient mares at the final stage of experiments.

Notes

EVALUATING BOVINE BLASTOCYST WITH FLUORESCENT NUCLEAR STAINING AND MANUAL COUNTING OF BLASTOMERES

Lindgren I, Humblot P, Laskowski D, Sjunnesson Y

SLU, Department of clinical sciences, UPPSALA, SWEDEN

Staining of nuclei is widely used for evaluating embryos as the apoptotic index and amount of blastomeres are correlated to the quality of the embryo. In this study we compared one nuclei stain and one in-situ apoptotic detection kit to evaluate bovine blastocysts. We also compared two manual blastomere counting techniques (confocal and fluorescent microscopy) in day 8 blastocysts.

Bovine day 8 blastocysts produced *in vitro* were used (batches *n=6*, blastocysts *n=193*). Blastocysts fixed in paraformaldehyde were stained with the TUNEL (Click-it TUNEL assay, Invitrogen, Stockholm, Sweden) and a nuclei dye (DRAQ5, BioNordika, Stockholm, Sweden) according to protocol from the manufacturer. Extensive DNA-fragmentation was induced for the apoptotic staining by incubating embryos with DNase I. Different permeabilization protocols were tested; Incubation with 0.25 % and 0.5% Triton X 100 for 20 (according to protocol), 40 and 60 minutes. Extended time of the reaction of fluorescent marking of DNA were also tested for 20 (according to protocol) and 45 minutes. Hoechst 33342 was used for counterstaining with DRAQ5 to test the specifics of the nuclear stain. After staining images were taken through confocal and fluorescent microscope. The blastocysts were scanned in 8 levels using the confocal microscope (height 40 µm), of which scans numbered 2, 4 and 6 were selected for manual count of nuclei (C). The blastocysts were also counted by using the fluorescent setting in which the center of the blastocyst was taken in focus and one image/blastocyst was used for counting the number of nuclei (F). The effect of the method, staining batch (n=6) and their interaction on the number of nuclei labeled were analyzed by analysis of variance (SAS-PROC GLM). When appropriate, multiple comparisons between LS means were performed while using the Scheffe option. Results are presented as LS means±SE. P-values<0.05 were considered as statistically significant.

The TUNEL-kit evaluated in the study did not possess the qualities for penetrating multiple cell-layers including the ICM, independent of different permeabilization techniques tested. DRAQ5 staining fully overlapped Hoechst 42333 and did not photobleach after 7 weeks of storage. There was a main effect of method (F *vs.* C, $p<0.001$), staining batch ($p<0.001$) and also an interaction between method and staining batch ($p<0.01$) on cell count. The number of nuclei using method C was significantly higher than F (116.4±2.0 and 93.9±1.6 respectively, $p<0.001$). The difference between methods was not related to the number of cells of embryos. There was a variation between staining batches ($p<0.001$) for both methods (minimum number of nuclei 97.4 ± 4.2 and the maximum 125.7±2.9). Interaction between method and staining batch was significant illustrating some variation in the difference between methods depending on batches. However, despite this variation (minimum difference=9 nuclei, maximum difference=38 nuclei) higher numbers of nuclei by using method C was found in all of the batches.

The TUNEL kit evaluated in the study was not efficient when working with blastocysts with multiple cell-layers. Better results were obtained with the nuclei stain. Higher nuclei counts were detected when using a confocal than with a fluorescent method ($p<0.001$).

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Notes

EFFECT OF BOVINE OVIDUCTAL FLUID ON *IN VITRO* BOVINE EMBRYO PRODUCTION

Lopera R[1], Hamdi M[1], Maillo V[1], Lloreda V[1], Núñez C[1], Coy P[2], Rizos D[1]

[1]*Dept. of Animal Reproduction, INIA. Crta de la Coruña Km 5.9, Madrid 28040, Spain*
[2]*Dept of Physiology, Veterinary Faculty, University of Murcia, Murcia 30071, Spain.*

Oviductal fluid is essential for mammalian reproduction as it provides the microenvironment for fertilization and early embryo development. In a preliminary experiment, when SOF was supplemented with bovine oviductal fluid at 5, 10 or 25% (v/v) concentration, the blastocyst development rate was very low (>7% at Day 7). Therefore, the aim of the present study was to evaluate the developmental capacity of bovine zygotes and the quality of the produced blastocysts when cultured *in vitro* with low concentrations of bovine oviductal fluid (bOF). bOF was collected by aspiration from the oviducts of slaughtered heifers in the early luteal phase. Presumptive zygotes were produced *in vitro* from oocytes derived from ovaries of slaughtered heifers and cultured in groups of 20-25 in 25 µL droplets in SOFaa (C-) or SOFaa + 5% FCS (C+) or in SOFaa supplemented with bOF (0.62, 1.25, or 2.5%). Cleavage rate was assessed on Day 2 and blastocyst development on Day 7, 8, 9 of culture (Day 0: day of fertilization). A representative number of blastocysts on Days 7/8 was used for quality evaluation through (a) differential cell count and (b) survival after vitrification /warming. Embryos cultured in the absence of serum exhibited a delay in the kinetics of blastocyst development; at Day 7, both groups without FCS (bOF and C-) had fewer blastocysts (ranging from 12.7±2.6 to 19.3±3.2%) compared with C+ group (24.3±2.1%). However, at Day 9 the blastocyst yield was similar in all bOF groups (ranging from 23.5±2.3% to 27.2±2.6%) compared with C+ (29.0±1.8%) and significantly higher than C- (18.4±1.8%) (P<0.05). In relation to blastocyst quality, 48 h after vitrification/warming, embryos from bOF 1.25%, 0.62% and C- groups survived significantly higher than C+ (61.4±2.5, 60.2±6.3%, 54.0±4.9% and 28.4±3.5%, respectively) (P<0.05). This difference was even higher at 72 h, 53.9±2.3%, 55.5±5.5%, 54.0±4.9% and 23.7±3.2%, respectively (P<0.05). Furthermore, total cell number of the embryos cultured in bOF 1.25% and 0.62% groups were significantly higher than C+ and C- (162.3±6.9 and 163.8±6.8 vs. 138.5±6.2 and 128.5±7.3, respectively) which was associated with increased TE cell number of both bOF groups (ranging from 125.4±6.9 to 124.7±6.1). Culture with 2.5% bOF had no effect on either blastocyst yield or quality. In conclusion, the use of low concentrations of bovine oviductal fluid (bOF) in *in vitro* culture has a positive effect on the development and the quality of the produced bovine embryos in absence of FCS.

Notes

PROTEIN DETECTION AND LOCALIZATION FOR ACTIVIN RECEPTORS IN PREPUBERTAL GOAT OOCYTES AND EMBRYOS

Morató R[2], Hammami S[1], Roura M[1], Catalá MG[1], Bonet S [2], Paramio MT[1], Izquierdo D[1]

[1]Departament de Ciència Animal i dels Aliments, Facultat de Veterinària, Universitat Autònoma de Barcelona. Spain. [2]Department of Biology, Biotechnology of Animal and Human Reproduction (TechnoSperm), Institute of Food and Agricultural Technology, University of Girona. Spain.

Inhibins and activins are classically defined by their regulation of FSH secretion from pituitary gonadotrophic cells. Since its isolation in 1985, several studies concerning the distribution and possible physiological roles of activin in the reproductive tract have been made. In addition to its role in the biology of the sperm and oocyte, a number of studies have suggested a role for activin and its receptors in early embryo development. Immature and mature oocytes, cleaved embryos and blastocysts from prepubertal goats were examined for the presence of activin IIA and IIB receptors (ActRII-A and ActRII-B). The localization of activin type II receptors were investigated by western blotting assay (experiment 1) and immunocytochemistry (experiment 2). When western blotting assay for ActRIIA and ActRIIB was performed, the results of these experiments provided evidence that activin receptors are synthesized during oocyte maturation and embryo development. In oocytes, no signal for both activin receptors type II was detected by immunocytochemistry. In cleaved embryos, positive immunostaining for both activin receptors was detected in all cells of the embryos. In blastocysts, whilst the staining of the ActRII-A was seen in almost every cell, staining for the ActRII-B was more variable. Specifically, for ActRII-A, the expression of ActRII-A was evenly distributed in the two cell lineages of blastocyst, the inner cell mass and the trophectoderm. In contrast, the ActRII-B immunosignal was present mainly in the inner cell mass. Control experiment performed without primary antibody confirmed that non-specific background signals were negligible. These results indicated that *in vitro* matured oocytes, embryos and blastocysts have receptors for activin. The present study is the first to identify the presence of activin type II receptors (ActRII-A and ActRII-B) in prepubertal goat oocytes and embryos up to blastocyst stage. Therefore, it could be a key factor in understanding the differences between competent and incompetent oocytes.

Notes

CRYOTOLERANCE OF PORCINE *IN VITRO* PRODUCED BLASTOCYSTS RELIES ON BLASTOCYST STAGE AND LENGTH OF *IN VITRO* CULTURE PRIOR TO VITRIFICATION

Morató R[1], Castillo-Martín M, Yeste M, Bonet S[1]

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

The feasibility to accurately select viable embryos is relevant to improve pregnancy rates and avoid futile transfer attempts. For this reason, the aim of our study was to assess whether the cryotolerance of *in vitro*-produced embryos could be influenced by the length of *in vitro* culture and size of blastocoelic cavity prior to vitrification using the pig as a model. With this purpose, we analyzed the cryoresistance and DNA fragmentation of blastocysts at different stages of development as derived on day 5 and 6 of *in vitro* culture. Blastocysts were subsequently vitrified, warmed and subsequently cultured for 24h. Re-expansion rates were recorded at 3 and 24h, and total cell number and DNA fragmentation were determined at 24h. Day 6 blastocysts showed the highest rates of survival after warming, which indicates high quality compared with day 5 blastocysts. Higher re-expansion rates were observed for expanded blastocysts and those in the process of hatching when compared to early blastocysts. Total cell number and DNA fragmentation were affected by blastocyst stage, vitrification/warming procedures and length of *in vitro* culture, as middle expanded and hatching/hatched blastocysts from day 6 presented higher percentages of cells with fragmented DNA than fresh blastocysts and blastocysts vitrified at day 5. Our findings suggest the cryotop vitrification method is useful for the cryopreservation of blastocysts presenting a high degree of expansion, particularly when vitrification is performed after 6 days of *in vitro* culture. Furthermore, these results show that a faster embryo development underlies higher blastocyst cryotolerance and evidence that blastocoelic cavity expansion prior to vitrification is a reliable index of *in vitro*-produced embryo quality and developmental potential.

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Notes

ULTRASOUND MONITORING OF FETAL AND PLACENTAL GROWTH AND VASCULARISATION IN THE RABBIT

Mourier E[1], Richard C[1], Chavatte-Palmer P[1]

[1]*INRA, UMR1198 Biologie du Développement et Reproduction, F-78350 Jouy-en-Josas, France*

The rabbit is a good animal model for biomedical research on pregnancy, particularly because of its hemochorial placentation close to human. Compared to rodents, the size of the rabbit allows the use of the same ultrasound equipment as in human medicine, but detailed biometric values are so far lacking. The aim of this study was to determine techniques and average values for the monitoring of fetal and placental growth and vascularisation in the rabbit, based on approaches used for the monitoring of human pregnancies.

10 conscious New Zealand does were examined without tranquilization using a GE Voluson E8 equiped with a 18MHz 3D transducer. Ultrasound examinations of 5 conceptuses each time were performed on day (D) 5, 7, 14, 21 and 28 post breeding. B mode, Doppler and 3D-Doppler were used.

On D5, pregnancy diagnosis can be performed with 74% success (embryo diameter being about 1mm). Diameter of the embryo was determined on D7 (6.13mm±1.07 ; n=10 does). On D14 (n=10 does), it was possible to measure fetal size (head: length 8.19mm±0.99 and width 4.28mm±0.38; body: length 11.23mm±1.10 and width 4.96mm±0.47) and heart rate using fetal aortic Doppler (219bpm±8). Fetal head and body (head: length 16.2mm±1.9 and width 9.3mm±1.7; body: width 12.9mm±1.0) were quantified as well as umbilical cord Doppler on D21 (n=6 does). Finally, placental volume was examined on D28 (n=4 does) (real volume: 5,25mm^3±2.09; mean grey: 46.4±4.4) and 3D Doppler indices were measured (Vascular Index: 3.6±4.0; Flow Index: 35.3±4.7; Vascular Flow Index: 1.4±1.8).

Ultrasound monitoring with B mode, Doppler and 3D are a useful tool to evaluate embryo-feto-placental growth and vascularisation during pregnancy in a non-invasive manner to study endpoints relevant to human medicine.

Notes

EFFECT OF CYSTEAMINE DURING IN VITRO MATURATION IS DEPENDENT ON THE BULL USED FOR IVF

Mullaart E, Knijn HM, Dotinga F, Flapper H, Schouten-Noordman J, Merton JS

CRV BV, PO Box 454, 6800 AL Arnhem, The Netherlands
Erik.Mullaart@CRV4all.com

Glutathione (GSH) plays an important protective role in relation to reactive oxygen species (ROS) generated by normal oxidative metabolism in the cell. The presence of cysteamine during in vitro maturation may facilitate the synthesis of GSH by immature oocytes. In a previous study we have shown a positive effect of the presence of cysteamine during in vitro maturation of OPU-derived oocytes on subsequent in vitro embryonic development (Merton et al., Rep Fert Dev 2006). Since higher concentrations of intracellular GSH was shown to improve male PN formation (Wang et al., Anim Reprod Science 2007), this might be one of the explanations for a higher embryo production when cysteamine is presence during maturation. From this perspective, the question rises whether the effect of cysteamine is bull dependent. Therefore this study aimed to investigate the possible interaction between the presence of cysteamine during maturation and the bull used for IVF.

Immature Cumulus-Oocyte-Complexes (COCs) were obtained twice weekly by ultrasound guided transvaginal oocyte collection (OPU). COCs were matured in vitro in TCM199/FCS/LH/FSH supplemented either with or without cysteamine (0.1 mM) in a 2X2 Factorial experimental design (week/treatment). Subsequently, matured oocytes were fertilised with frozen-thawed gradient-separated semen (n=17 bulls) and cultured for 7 days in SOFaaBSA. Number of COCs per treatment was 3941 and 3292 for control and cysteamine, respectively. On a bull level, this varied between 60 and 700 COCs. Results were analysed by Chi-square analyses.

The overall result shows that the presence of cysteamine during in vitro maturation significantly affected Morula + Blastocyst rate at Day 7 (21.7% and 33.3 % for control and cysteamine, respectively. The increase in percentage points of production was significantly affected by the bull used for IVF and varied between 1% and 42% (Figure 1).

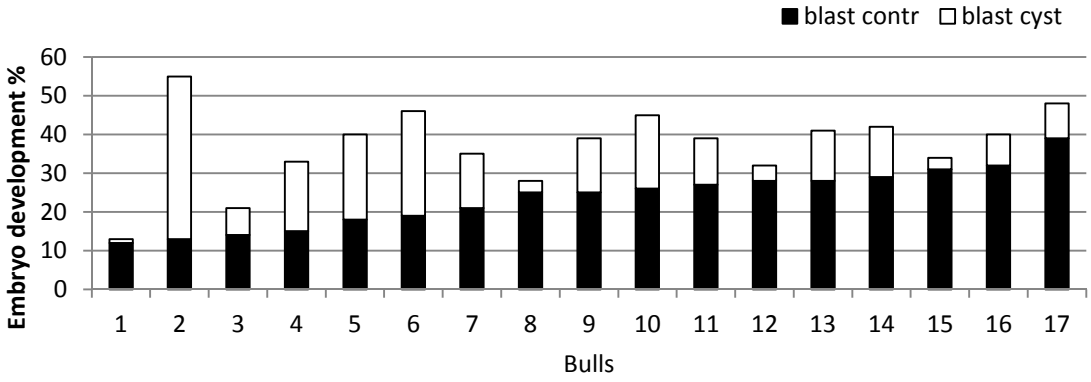

Figure 1: Interaction between the presence of cysteamine during maturation of OPU-derived bovine oocytes and the bull used for IVF (n=17) on subsequent in vitro embryonic development.

These results show that the positive effect of the presence of cysteamine during in vitro maturation of OPU derived COCs on in vitro embryonic development is affected by the bull used for IVF. Variation in sensitivity of bulls for suboptimal levels of ROS during fertilization could have caused this difference. Further research is needed to understand this process and to elucidate a possible correlation with the in vivo fertility of bulls.

Notes

SUPEROVULATION IN MEAT TYPE DORPER SHEEP WITH SINGLE FSH COMBINED WITH eCG INJECTION

Ocak S[1], Emsen E[2]

Middle East Sustainable Livestock, Biotechnology and Agro-Ecology Research and Development Centre, Zirve University, Gaziantep, 27260, Turkey[1]
Ataturk University, Department of Animal Science, 25240, Erzurum, Turkey[2]

Superovulation in sheep is usually achieved after several FSH injections in a time-consuming protocol, and the half-life of FSH is very short. In cattle and sheep there are reports in which FSH is dissolved in polyethylene glycol to prolong the half-life of FSH. It is desirable to reduce the number of treatments required to induce superovulation in sheep.

In this study we examined whether single FSH dissolved saline solution and combined with eCG injection could replace a standard multiple FSH injection protocol for superovulation treatment. We also studied different doses of FSH traditionally applied in decreasing dosages. Three years old primiparous Dorper donor ewes weighing 60-65kg on average received a CIDR for 12 days. In Experiment 1, 21 ewes received six decreasing doses of 3, 2, 2, 1, 1, 1, 1 ml Folltropin (Folltropin-V; Vetrepharm, Canada) i.m. starting 60h before CIDR withdrawal. In Experiment 2, 21 ewes were treated with total 14 ml Folltropin with decreasing doses (3, 3, 2.5, 2, 1.5, 1ml). Donor ewes were injected 400 I.U. eCG at CIDR removal. In Experiment 3, a single injection of 10ml Folltropin and 500 I.U. eCG was given to 13 ewes. Embryos were collected on Day 6 post insemination. In Experiment 1 and 3, percentage of animals which responded to the superovulation protocols were similar (77%) and which was relatively lower than animals treated with higher dose of FSH in experiment 2 (90%). The percentage of ewes which responded to superovulation treatment in the Experiments 1, 2 and 3 resulted (more than 3 CL) in 100%, 94% and 99%, respectively. The number of recorded CL was 10.43±1.31, 9.05±1.19 and 12.76±1.64 for animals in Experiments 1, 2 and 3, respectively. The number of recovered embryos were found higher ($p>0.05$) for a single injection of FSH treatment (8.34±1.9) compared to multiple injections in Experiment 1 (6.28±1.26) and 2 (5.50±1.24). However, embryo recovery rates were lower (29.9%) for the group injected with a single FSH dose than those observed in experiment 1 (49.3%) and 2 (41.4%). These findings suggest that FSH injected once provide a more practical approach of FSH administration and promise similar embryo yields compared to the traditional method using multiple FSH injections of decreasing doses.

Notes

THE INCREASED QUALITY OF CLONED PIG EMBRYOS RECONSTRUCTED WITH CELL NUCLEI OF ADULT BONE MARROW-DERIVED MESENCHYMAL STEM CELLS UNDERGOING TRICHOSTATIN A-DEPENDENT EPIGENOMIC TRANSFORMATION[1]

Opiela J, Samiec M, Romanek J

NATIONAL RESEARCH INSTITUTE OF ANIMAL PRODUCTION; Krakowska 1; 32-083 BALICE/KRAKÓW, Poland;

In recent years, there has been increased interest in potential utility of Mesenchymal Stem Cells (MSCs) in both tissue engineering and regenerative medicine. These cells can also be applied in the preclinical and clinical research programs aimed at xenotransplantation of tissues and organs. In the current experiment, we focused on investigating the effect of epigenomic modulation on the blastocysts quality and DNA fragmentation of developed porcine cloned embryos reconstructed with the cell nuclei of adult bone marrow-derived MSCs subjected to a non-specific inhibitor of histone deacetylases, trichostatin A (TSA)-dependent epigenomic transformation.

MSCs were isolated from the pig bone marrow and cultured as described in Opiela et al. (Cellular Reprogramming, 2013). Epigenomic transformation of MSCs was performed by their exposure to 50 nM TSA for 24 h. SCNT was performed as described in Samiec and Skrzyszowska (Theriogenology, 2012). The TUNEL was applied to estimate the total cell number, number of apoptotic cells and death cell index (DCI) in two groups of in vitro cultured cloned pig embryos. TUNEL analysis was performed using a Deadend Fluorometric TUNEL System™ (Promega, USA). Differences with a probability ≤ 0.05 were considered to be significant. Statistical analysis was performed using ANOVA followed by Tuckey's post hoc test.

In total we analysed 57 blastocysts: 31 TSA-treated and 26 control blastocysts. The mean number of nuclei per blastocyst developed from TSA-treated MSCs was higher (47.97±27.43) than control blastocysts (27.77±15.82). The highly significant difference ($P<0.005$) was noted between both groups. No significant differences were noted in the number of apoptotic nuclei and DCI between both analysed blastocyst groups.

This study demonstrated that TSA-dependent epigenomic modulation of MSCs being used as nuclear donor cells for SCNT highly significantly increases the total cell number of in vitro cultured cloned pig embryos.

[1] *The project was funded by the Polish National Science Centre resources allocated on the basis of decision number DEC-2011/03/D/NZ9/05537.*

Notes

INCREASING SPERM CONCENTRATION HAS A POSITIVE FFECT ON FERTILIZATION RATES OF VITRIFIED-WARMED MATURE BOVINE OOCYTES

Ortiz-Escribano N, Van Soom A

Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

It has been hypothesized that the presence of cumulus cells around the oocyte reduces the penetration of cryoprotectants and leads to an inadequate protection during the vitrification. Therefore we chose to remove the cumulus cells before vitrifying mature oocytes, knowing that cumulus-free oocytes have lower fertilization rates (Tanghe *et al.*, 2003). The aim of the present study was to determine if increasing the sperm concentration improves fertilization rate and embryo development of vitrified denuded oocytes.

Bovine oocytes (n = 836) were matured *in vitro* (TCM199+ 20% FBS) for 22 hours. After maturation, all oocytes were denuded by vortexing. Denuded oocytes were vitrified in 15% EG+ 15% DMSO + 0.5M sucrose (Kuwayama *et al.*, 2005) and stored in liquid nitrogen for one week. Both freshly matured and vitrified-warmed matured oocytes were then randomly assigned to four groups: 1) Fresh fertilized with$1x10^6$spz/ml; 2) Fresh fertilized with 6x 10^6spz/ml; 3) Vitrified-warmed fertilized with $1x10^6$spz/ml and; 4) Vitrified-warmed fertilized with $6x10^6$spz/ml. After 21 hours, half of the presumptive zygotes were fixed in 4% paraformaldehyde and stained with Hoechst and the rest was cultured in SOF medium. Cleavage rate was recorded at 45 hours post insemination and blastocyst rate was assessed on day 8 post insemination. Development data were analyzed using binary logistic regression. Differences at $p<0.05$ were considered significant.

Embryo development was significantly impaired after oocyte vitrification compared to fresh oocytes ($p<0.05$). When the denuded oocytes were inseminated with $6x10^6$spz/ml, the penetration rate was significantly higher ($p<0.05$) both in fresh (62.3% vs. 34.4%) and vitrified oocytes (32.4% vs. 14.3%). However, we did not observe a significant difference in the cleavage rate of fresh (46% vs. 38.6%) and vitrified oocytes (33.6% vs. 26.1%). With regard to blastocyst formation, the higher sperm concentration tended to increase embryo development of vitrified-warmed oocytes (8.6% vs. 3.5%) ($p=0.11$).

In conclusion, the results of the present study suggest that an increase in the sperm concentration has a positive effect on the fertilization and subsequent embryo development after vitrification of mature oocytes. Further studies are focusing on improving fertilization rates of vitrified-warmed oocytes by using cumulus components.

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Notes

DOES THE WAY IN WHICH *COXIELLA BURNETII* IS PRODUCED AFFECT THE BINDING OF THE BACTERIA TO THE ZONA PELLUCIDA FOLLOWING *IN VITRO* INFECTION OF GOAT EMBRYOS?

Pellerin JL[1], Alsaleh A[1], De Souza-Fabjan JM[2],
Mermillod P[2] , Corbin E[2], Nascimento P[2], Bruyas JF[1],Fieni F[1]

[1] *LUNAM University, Oniris, (Nantes-Atlantic National College of Veterinary Medicine, Food Science and Engineering), Department of research into the Health Sanitary and Biotechnology of Reproduction,France,*
[2] *INRA, UMR7247 Physiologie de la Reproduction et des Comportements, F-37380 Nouzilly, France*

Previous experiments using in vitro infection have shown that *Coxiella burnetii* adheres strongly to the zona pellucida (ZP) of caprine embryos produced *in vitro* or *in vivo* (Alsaleh *et al.,* 2013). The washing procedure recommended by the IETS for bovine embryos failed to remove it. The *C. burnetii* strain, CbC1phase I, used in these experiments was originally isolated from the placenta of an aborted goat; it was multiplied in chicken embryos and purified by dilution and differential centrifugation. Nevertheless, after purification, egg proteins could still be present in the media used to infect the goat embryos and thus interact with the ZP and the bacteria. The goal of this study was to determine if the same binding property was observed with *Coxiella burnetii* produced using alternative techniques.

A total of 250 ZP-intact 8- to 16-cell embryos, produced *in vitro* from ovaries collected at slaughter, were infected with 10^9/ml *Coxiella burnetti* (strain CbC1) produced in one of 4 different ways. 1) Ovoculture-Coxiella [3 batches of 10 embryos]. 2) Ovoculture-Coxiella purified in sucrose gradient [6 batches of 10 embryos]. 3) Cell culture-Coxiella [8 batches of 10 embryos].4) Wild Coxiella obtained from an aborted goat [8 batches of 10 embryos]. After overnight incubation at 37°C in 5% CO_2, the embryos were recovered and washed in batches, in 10 successive baths of PBS with 5% FCS, in accordance with the IETS guidelines.

The 10 wash baths were collected separately and centrifuged for 1 hour at 13,000 g. The presence of *C. burnetii* was determined by PCR in each batch of embryos and in the pellets of the 10 wash baths (Table 1).

Table 1: Detection of *Coxiella burnetii* (CB) in successive embryo washing baths and batches of 10 infected ZP-intact eight- to 16-cell embryos after 10 wash cycles by PCR, according to the method of production of CB and *in vitro* infection (10^9/ml) of the embryos.

Origin of *C. burnetii*	Number of embryo batches tested	Last positive wash	N° of positive batches of embryos
Ovoculture	3	10	3/3
Ovoculture purified	6	6	2/6
Cell culture	8	10	8/8
Wild	8	10	8/8

Coxiella-DNA was detected in embryo batches after ten washes irrespective of the origin of Coxiella burnetii used for infection. Therefore, egg proteins, which could be present following ovoculture, do not play a major role in the binding of the bacteria to the ZP. Further studies are needed to investigate whether this same property is observed after *in vivo* infection of the embryos and to elucidate the attachment mechanisms.

Notes

INFLUENCES OF IN VITRO CULTURE CONDITIONS ON PROGESTERONE LEVELS AND THE QUALITY OF RESULTING EMBRYOS

Poppicht F[1], Burmester-Kintrup N[2], Schlüter N[2], Stinshoff H[1],
Kassens A[3], Wilkening S[2], Piechotta M[2], Wrenzycki C[1]

[1]*Clinic for Veterinary Obstetrics, Gynecology and Andrology, Justus-Liebig-University Giessen, Germany*
[2]*Clinic for Cattle, University of Veterinary Medicine, Hannover, Germany*
[3]*Unit for Reproductive Medicine, University of Veterinary Medicine, Hannover, Germany*

Alterations of in vitro maturation conditions influence the oocyte and thus the quality of the resulting embryo. Progesterone (P4) is a key factor in female reproduction and plays a role in follicular growth, ovulation and corpus luteum formation. The aim of the present study was to determine the P4 production/metabolism of bovine COC during in vitro maturation (IVM) employing different oxygen concentrations.

In the first experiment, bovine COC were subjected to either 5% or 20% O_2 during IVM for 24 h [with or without oil overlay]. In the second experiment, IVM media were supplemented with different progesterone concentrations (<50 ng/ml, 150 ng/ml, 300 ng/ml or 450 ng/ml) and COC were in vitro matured for 24 h using 20% O_2 [without oil overlay]. Tissue culture medium supplemented with BSA (FAF) as well as eCG and hCG served as basic maturation medium.

P4 concentrations in the IVM media were measured via a radioimmunoassay (RIA). No P4 was detectable in the basic maturation medium. After 24 h of IVM, P4 concentrations were significantly higher in maturation medium stemming from COC matured without oil overlay and also higher that of the group from 20% O_2 compared to that of the other groups (20% O_2 with oil: 0.4 ± 0.2 ng/ml, 20% O_2 without oil: 8.8 ± 1.5 ng/ml; 5% O_2 with oil: 0.2 ± 0.1 ng/ml; 5% O_2 without oil: 3.9 ± 0.8 ng/ml; respectively).

A decline of P4 concentrations was observed after 24 hours of maturation in all medium groups of P4 supplementation(<50 ng/ml, 150 ng/ml, 300 ng/ml, 450 ng/ml ⇨ 17.9 ± 3.8 ng/ml, 33.1 ± 2.2 ng/ml, 88.0 ± 32.2 ng/ml, 98.0 ± 43.2 ng/ml).

These data show that the maturation conditions (O_2 concentration and oil overlay) affected the progesterone concentration in the IVM medium. Furthermore, P4 concentration was altered depending on the starting concentration. This should be taken into consideration when performing IVM.

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Notes

IMMUNOCHARACTERIZATION OF EQUINE ADIPOSE-DERIVED STEM CELL BY QUANTITATIVE IMAGE FLOW CYTOMETRY

Queiroz LMV[1]; Bonilla L[2]; Malard PF[1]; Verstegen JP[2*]

[1]*BIOCELL, Laboratory of Animal Stem Cells, Brasília-DF, Brazil*
[2]*MOFA Global, International Biotechnology Center, Mt Horeb, WI, USA*

Adult stem cells have been widely investigated with a particular interest for therapeutic use in regenerative medicine. Equine adipose-derived mesenchymal stem cells (Eq adMSCs) are a potential cell source for autologous cell therapy due to their capacity to self-renew and differentiate in specialized cell types or to modulate oxidative stress, secrete cytokines and growth factors. At the International Society for Cellular Therapy meetings (2013) human MSCs were defined as aplastic-adherent cells expressing specific surface markers, including: CD105, CD90, CD73 or CD44 and CD29. Although similar definitions have been published for veterinary cells, no consensus has been reached for the identification and characterization of adMSCs for veterinary applications. The aim of this study was to characterize immunophenotypic properties of Eq adMSCs and to characterize the presence of intra-cytoplasmic proteins responsible for their non-differentiation stage. Eq adMSCs were obtained from the subcutaneous fat tissue of 18 adult horses. Briefly, fat tissue was minced, washed in PBS Buffer, digested for 30 min with a solution containing 1 mg/ml collagenase I (Sigma-Aldrich) and 0.1 mg/ml BSA (BSA fraction V). The resulting cell suspension was filtered through a 100 µm and 40 µm cell strainer (BD) and collagenase I activity was inactivated by a double volume of PBS. After centrifugation, the cells were re-suspended in Dulbecco's modified Eagles medium, low glucose (D-MEM) containing 10% FBS and antibiotics, plated at a density of 2.5×10^4 cells/cm^2 and cultured for 8 days in 5% CO_2 at 38.5°C. After 24hrs, non-adherent cells were washed off and the ADSCs were cultured until 90% confluence was obtained. The Eq adMSCs were detached with trypsin, first passaged and maintained until 90% confluence. The Eq adMSCs were then detached and cryopreserved in a DMEM freezing medium containing 10% FBS and 5% DMSO (Sigma-Aldrich). At passage 1, a cell sample from each horse was evaluated for immunophenotypic characterization. The following mesenchymal stem cell surface markers were evaluated: (CD29-RD1, CD44-FITC, and CD105-AF648), haematopoietic (CD34-FITC) as well as cytoplasmic proteins markers (SOX2-AF488 and OCT3/4-AF488) by quantitative imaging multicolor flow cytometry (ImageStream MK II-Millipore). At the same passage, osteogenic differentiation was induced and evaluated morphologically and cytologically using Alizarin O Red to identify extracellular calcium deposition. Following quantitative imaging flow cytometry, isolated equine adipose-derived stem cells of first passage were found to be CD44 (95.21±10.4%), CD29 (99.00±0.93%), CD105 (97.37±2.90%), SOX2 (79.10±25.84%) and OCT3/4 (79.82±14.99%) positive and CD34 (0.37±0.13%) negative. All cell culture differentiated in osteogenic tissue. Our results provide fundamental information to enable control and identification of Eq adMSCs production using specific protein markers and the unique quantitative imaging flow cytometry.

*: *Corresponding author: jverstegen@mofaglobal.com*

Notes

INTEREST OF THE BUSERELIN TO IMPROVE HEAT SYNCHRONISATION TREATMENT OF RECIPIENT DAIRY HEIFERS WITH NORGESTOMET IMPLANT

Quinton H[1], Morel A[1], Charreaux F[1]

1. EVOLUTION, CS 30621, 35706 RENNES Cedex 7

For 25 years in the west of France, implants of norgestomet with prostaglandins alpha (24 or 48 hours before the implant removal) have been used to synchronize the heat of the recipient heifers: Initially, CRESTAR® was used excluding the injectable estrogen as recommended, and since 2006 CRESTAR SO® was administered due to the prohibition of estrogens in European countries. Since 2013, MSD commercializes a new more expensive package CRESTAR PACK® which contains the norgestomet implant and an injectable Buserelin solution which is recommended for use at the time of implant insertion.

The purpose of the present study was to determine whether the use of this enclosed Buserelin really improves the technical results in the field of embryo transfer. Indeed, we have supposed that in this case, the synchronisation of heat and the quality of the oocyte are less important than in the case of AI.

Methods:

Two trials were conducted under field conditions between January and July 2013 in 73 farms on 571 dairy heifers.

The Control Protocol (CP) was based on the use of a norgestomet implant during 9 to 11 days associated with a prostaglandin injection (Cloprostenol, ESTRUMATE®) 24 hours before implant removal

On each farm both protocols (with/without GnRH) were use in order to maintain comparability.

In the first trial (P1), 132 heifers were synchronized using the Control Protocol and compared to 162 heifers following the treatment of CP1 protocol (buserelin application was done simultaneously to implant insertion).

In the second trial (P2), the Control Protocol was used in 127 heifers and compared to the CP2 protocol applied to 157 heifers receiving Buserelin 36 hours after implant removal. For all heifers, the day and the quality (net or uncertain or not seen) of heat were registered. Then, 9 days after implant removal:

- Quality of all recipients was evaluated by transrectal palpation (great or good or bad or unusable)
- Blood samples for measurement of progesterone concentration were done on 152 heifers (58 CP, 49 CP1, 45 CP2)
- 366 heifers were transferred with a fresh or a frozen embryo.

Pregnancies were confirmed by ultrasonography between day 30 and day 60.

The results show no significant differences between protocols concerning heat quality and blood progesterone concentration. But we observed a significant worst proportion of recipients which were assess as unsuitable for embryo transfer in the CP group compared to the CP1 (- 8 pts) and a significant better pregnancy rate in the CP2 group than in the CP group (+ 13 pts). In Conclusion, the addition of Buserelin seems to be beneficial to both GnRH application protocols, during implant insertion and 36 h after removal. Regardless of the economic aspects, it could be interesting to use the Buserelin twice. Another experiment is needed to confirm this hypothesis.

Notes

USING GENOMIC EVALUATION OF BOVINE EMBRYOS AS A BREEDING TOOL IN A COMMERCIAL PROGRAM

Reichenbach M[1], Jung S[2], Fries R[2], Wolf E[3], Gschoederer C[1], Scherzer J[1], Reichenbach H-D[4]

[1]Bayern-Genetik GmbH, 85586 Grub, Germany, [2]Chair for Animal Breeding, Technische Universitaet Muenchen, 85354 Freising, Germany, [3]Chair for Molecular Animal Breeding and Biotechnology, Ludwig-Maximilian University, 85764 Oberschleissheim, Germany, [4]Bavarian State Research Center for Agriculture, Institute of Animal Breeding, 85586 Grub, Germany

Within the last decades some major hereditary defects developed within the Fleckvieh population in Bavaria and Austria. The most influencing ones are dwarfism (DW), stunted growth (FH2), thrombopathia (TP), zinc-deficiency-like syndrome (ZDL) and bovine male subfertility (BMS). The aim of our study was to develop a reliable procedure to analyze bovine embryos to detect gender, polled status and hereditary defects within 24 hours to select healthy embryos for transfer of valuable heterozygous cows or AI-bulls. Over a 6 month period, Fleckvieh cows (n=6) and heifers (n=3) were used for superovulation (n=15) performed at 5-6-week intervals. The animals received a progesterone intravaginal device (PRID-delta®) at a random stage of estrous cycle (d0) and were superstimulated with FSH (Pluset®) administered twice daily in decreasing doses (heifers, total=8.5 ml; cows, total=13.5 ml), from d5 to d8. The donors received two injections of cloprostenol (Estrumate®) on d7 and d8. The PRID was removed on d8. Fixed-time AI was performed 36, 48 and 60 h after PRID removal, without estrus detection. Embryos were recovered 7d after the 1st AI. A total of 121 embryos were biopsied by a single operator with a steel blade attached to a micromanipulator. Immediately after treatment, the biopsied cells (3 to 20) were transferred into 1µl TE buffer to a 500 µl reaction tube and embryos were cultured in synthetic oviduct fluid (SOF) supplemented with 5% ECS, 40 µl/ml BME and 10 µl/ml MEM until genomic results were available. DNA from biopsies was *in vitro* amplified *via* whole genome amplification. 5'-exonuclease assays were used to obtain genotypes for detection of genetic defects. Polled status and gender was determined using PCR. On average 8.07 embryos were biopsied per embryo recovery, corresponding 95.3% of the total number of embryos. Most of the micromanipulated embryos were morulae (63.6%) followed by blastocysts (19.8%) and early blastocysts (16.5%). Six embryo samples (5.0%) totally failed for analysis, possibly due to a loss of samples. In successful analyses, gender was undetermined in two embryos; remaining embryos were 54.9% female and 45.1% male. Polled status could be analyzed in 99.1% of the embryos. From all analyzed hereditary defects (115 samples x 5 defects = 575 runs) 96.7% could be reliable detected. The transfer of biopsied embryos cultured for 24 hours (n=12) led to 67.0% pregnancies. Altogether, these results confirm that this analytical procedure for the detection of sex, polled status and hereditary defects from biopsied embryos is fast and reliable.

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Notes

IMPROVING TWINNING RATE BY ASSISTED UTERINE EMBRYO MIGRATION IN SARDA EWE – A PRELIMINARY STUDY

Rodríguez A*, Porcu C*, Gallus M, Pilichi S, Dattena M

AGRIS-Sardegna (DIRPA) 07040 Olmedo (SS) Italy.

In sheep intrauterine migration of embryos into the uterine horn contralateral to an ovary containing multiple ovulations occurred in nearly 90% of ewes examined. In fact it is reported that overcrowding adversely affects embryo survival. Standard embryo transfer (ET) techniques consist of transplanting two embryos into one uterine horn which constrains embryos to make the critical process of migration. To avoid this problem the present experiment attempts to improve twinning rate modifying standard ET protocols by transplanting one embryo for each uterine horn. This procedure was called *"Assisted uterine embryo migration"*.

Twenty four recipient ewes with similar age and lambing experience were synchronized during non-breeding season by intravaginal progestagen-impregnated sponge (Crono-gest Sponge®, Intervet, Holland) for 14 days. On the day of sponge withdrawal animals received a 350 IU IM injection of PMSG (Folligon®, Intervet, Holland). Eight days after sponge withdrawal and a fasting period of 24 hours on the last day embryo transfer was performed in sheep. Once the existence of at least two corpora lutea was confirmed, irrespectively of the side of ovulation, in vivo produced vitrified/warmed embryos (n=48) were transferred, in pairs, randomly: one for each uterine horn, Assisted Migration Group (n=12) and both embryos into the same horn, Standard ET Group (n=12). The straws containing the vitrified embryos were warmed in a water bath at 37°C and the contents expelled into a Petri dish, mixed in 0.5 M Sucrose for 3-5 min and moved into a drop of H-TCM199 plus 20% FBS before the transfer. At day 28 of gestation, the pregnancy, the embryo survival and the twining rates were determined by transrectal ultrasound scanning. (Aloka Co., 7.5 MHz).

The pregnancy rate in both groups was similar as reported for this species. However, due to the low numbers of animals per group it is assumed that the significance of differences could not be statistically confirmed but only became obvious as a general tendency, i.e. the survival rate of embryos (17/24=71% vs 14/24=58%) and the number of animals that were carrying twins (8/9=89% vs 6/10=60%) were higher in the Assisted Migration Group.

**both authors contributed equally to this work*

Notes

HIGH HYDROSTATIC PRESSURE IMPACT ON CRYOPRESERVATED PORCINE MESENCHYMAL STEM CELLS QUALITY- PRELIMINARY RESULTS[2]

Romanek J, Opiela J, Smorąg Z

NATIONAL RESEARCH INSTITUTE OF ANIMAL PRODUCTION, *Krakowska 1,* 32-083 BALICE, *Poland*

High Hydrostatic Pressure (HHP) treatment has been reported to improve the cryosurvival of gametes or embryos in certain mammalian species. Cells after HHP treatment overexpress resistance proteins, therefore they can quicker adapt to next adverse factor (Pribensky et al. 2011).

The aim of this study was to examine the influence of varied HHP values on porcine mesenchymal stem cells (MSC) on 1) proliferation rate, 2) survival rate and level of apoptosis 3) phosphatidylserine (PS) exposure and 4) BAX protein expression in cells.

The MSC were isolated and cultured as described by Opiela et al. (Cell Reprogram. 2013). Before cryopreservation MSC were subjected to HHP 20MPa, 30MPa, 40MPa, 50MPa, 60MPa for 1 h at 24°C. Cells were stored in liquid nitrogen. After thawing cells were subjected to i) trypan blue staining to analyze proliferation rate and survival rate directly after thawing and after 8 days of *in vitro* culture; ii) PS exposure was estimated by ApoDETECT Annexin V-FITC Kit (Invitrogen, USA) according to the manufacturer's instructions and iii) BAX protein expression by western-blotting as described by Opiela et al. (Ann. Anim. Sci, 2013). BAX protein expression was estimated immediately after HHP treatment. The statistical analysis was performed by Tukey's post-hoc One-way ANOVA test.

Results regarding MSC subjected only to HHP:

No significant difference was noted in the BAX protein expression in any of analyzed groups (three independent replications).

Results regarding MSC subjected to HHP before cryopreservation:

The significant difference ($P<0.05$) was observed in proliferation rate between MSC subjected to 40 MPa HHP and control group (three independent replications). The high significant difference ($P<0.001$) was noted between MSC viability immediately after thawing in cells subjected to 60MPa HHP and control and significant difference ($P<0.05$) was noted between MSC subjected to both 40MPa and 50MPa HHP and control (six independent replications).

No significant difference was noted in survival rate after 8 days of *in vitro* culture in all analyzed groups (three independent replications). No significant difference was observed in PS exposure in MSC subjected to all analyzed HHP values and control cells (three independent replications).

MSC subjected to 40MPa, 50MPa and 60MPa HHP showed increased survival rate. Also in all analyzed groups, HHP treatment does not have any influence on early stage of apoptosis in cryopreservated MSC. In long-term culture, HHP does not have any impact on cells viability but 40MPa HHP increases proliferation rate. Moreover, HHP treatment by itself does not induce apoptosis in MSC.

[2] *The project was funded by the Polish National Science Centre resources allocated on the basis of decision number DEC-2011/03/D/NZ9/05537*

Notes

EFFECT OF LINOLEIC ACID (LA) AND LINOLENIC ACID (ALA) RATIO AT IVM ON EMBRYO DEVELOPMENT OF PREPUBERTAL GOAT OOCYTE

Roura M, Catala MG, Hammami S, Izquierdo D, Paramio MT

Dep. de Ciència Animal i dels Aliments, Fac. de Veterinària, Universitat Autònoma de Barcelona, Barcelona, Catalonia, Spain.

Follicular environment affects oocyte competence for embryo development. Previous studies showed that Fatty Acids play an important role in oocyte and embryo development (Reprod Fertil Dev, 2012: (24)59-67). Polyunsaturated Fatty Acids (PUFAs) such as Linoleic (LA,n-6) and Linolenic (ALA, n-3) acids added during IVM have shown an effect on in vitro embryo production (Biol Reprod, 2009:(81)1064-1072; Reproduction 2010:(139)979-988). The aim of this study was to test the effect of supplementation with different concentrations and ratios of LA:ALA (200:50, 100:50 and 50:50 µM) in IVM media (TCM-199 + 5 µg/mL FSH + 5 µg/mL LH + 1 µg/mL E_2 + 100 µM Cisteamine + 0.6 % BSA w/v). The control groups were IVM with BSA or with 10 % of FBS. IVM-oocytes were IVF with fresh semen. Table 1 and Table 2 show the results of total fertilization, cleavage and blastocyst rates recorded at 17 h, 48 h and 8 days respectively.

Table 1. Nuclear stage of IVM-IVF prepubertal goat oocytes matured with different LA:ALA ratios.

Treatment (LA:ALA)	Total oocytes	No-fertilized Oocytes (%)	Total Fertilized oocytes	
			2PN (%)	PS (%)
Control FBS	53	9 (16.98)	40 (75.47)a	4 (7.55)b
Control BSA	57	9 (15.79)	45 (78.95)[a]	3 (5.26)[b]
200:50	55	23 (41.82)	1 (1.82)[b]	32 (58.18)[a]
100:50	60	9 (15.00)	44 (73.33)[a]	7 (11.67)[b]
50:50	56	7 (15.00)	46 (82.14)[a]	3 (5.36)[b]

[a,b] values in the same column represent statistically significant differences (ANOVA, P<0.05). 2PN: two pronuclei; PS: Polyspermic. Pronuclei assessment through Orcein staining.

Table 2. Embryo development of IVM-IVF prepubertal goat oocytes matured with different LA:ALA ratios.

Treatment (LA:ALA)	Total oocytes	Cleavage (%)	Blastocysts (%)	Blastocysts / cleavage (%)
Control FBS	187	123 (65.78)	24 (12.83)a	24 (19.51)a
Control BSA	184	104 (56.52)	21 (11.41)[a]	21 (20.19)[a]
200:50	190	102 (53.68)	5 (2.63)[b]	5 (4.90)[b]
100:50	195	127 (65.13)	26 (13.33)[a]	26 (20.47)[a]
50:50	212	142 (66.98)	28 (13.68)[a]	29 (20.42)[a]

[a,b] values in the same column represent statistically significant differences (ANOVA test, P<0.05).

In conclusion, LA:ALA at 200:50 ratio has a negative effect on IVF compared to control group, leading to a poor blastocyst production. Otherwise, supplementation during IVM has not shown any effect when 100:50 and 50:50 were used in comparison to the control group.

Notes

ANTI-MULLERIAN HORMONE (AMH) PROFILES AND OVARIAN RESERVE OR EMBRYO PRODUCTION IN HOLSTEIN COWS.

Rozner A, Verstegen J*

MOFA global, International Center for Biotechnology, 2633 State Rd 78, 53572 Mt Horeb, WI, USA

Anti-müllerian hormone (AMH) is a small peptide hormone that has been indicated as a marker of ovarian follicular reserve in bovine. AMH profiles, as well as the relationships between serum AMH to oocyte number and *in vivo* embryo production in Holstein animals were evaluated. Fifteen unstimulated cows were followed at monthly intervals to evaluate AMH over four months. AMH was evaluated for 394 male and 399 female developing Holstein animals from birth to adulthood. Forty one heifers were evaluated at OPU and 125 were evaluated at embryo-flushing. Superovulation was induced using a modified Ovsynch protocol with 4 days of decreasing FSH (Pluset H® MOFA). Blood samples were collected using serum tubes and spun within 2 hours. The samples were stored at -20C until evaluated for AMH using the MOFA AMH-Bovine specific immunoassay®. AMH levels in males and females peaked at 2 months of age and then fell as they reached adulthood. The average AMH level of adult cows was stable for each of the 4 monthly measurements with a high correlation between all values per animal (r^2 = 0.9077; P<0.01) suggesting AMH levels are consistent for at least 4 consecutive months. However, AMH levels were lowest during the summer months, suggesting a seasonal change in AMH secretion. Animals repeatedly ovarian stimulated showed decreasing AMH levels (509±295, 299±210; 211±119) and a significant decrease in embryo numbers recovered (5.7±4; 2.2±1.9; P=0.02) with subsequent stimulations. The number of oocytes was not altered by multiple stimulations (9.9±9.8; 8.1±6.2; P=0.57). Since AMH and embryo numbers decreased after multiple stimulations, the first AMH value and results of the first OPU or flush were used for the correlation of AMH to OPU/flushes. Animals were separated into three AMH categories, low (<100), normal (100-400), and high (>400 pg/mL). High AMH OPU animals had significantly higher numbers of oocytes than the normal or low AMH groups (13.8±9.2; 9.2±5.3; 5.6±3.9; P=0.001). High AMH flushed animals had significantly higher numbers of embryos than animals with low AMH (10.9±8.0; 5.7±5; P=0.002). In conclusion, AMH serum concentrations are consistent over multiple months however; blood should not be taken for animal selection by AMH after ovarian stimulations have begun and interpreted with caution during the summer months. AMH is highly associated with superovulation response, oocyte and embryo production and should improve efficiency of MOET.

*: *Corresponding author: jverstegen@mofaglobal.com*

Notes

OPU AND ITS IMPACT ON FERTILITY AFTER TIME FIXED ARTIFICIAL INSEMINATION IN A CONSERVATION PROGRAM OF MURCIANO-LEVANTINE BOVINE BREED

Ruiz S[1], Romero-Aguirregomezcorta J[1], Almela L[2], Peinado B[2], Poto A[2], Astiz S[3]

*[1]Dept. Physiology. University of Murcia. Murcia, Spain. [2]Animal Breeding. IMIDA. Murcia, Spain. [3]Animal Reproduction. INIA. Madrid, Spain. *E-mail: sruiz@um.es*

The Spanish Murciano-Levantine (M-L) bovine breed is considered at critical point of extinction. The follicular punction by *ovum* pick-up (OPU) allows the recovery of *in vivo* bovine oocytes from living donor females that can be used for *in vitro* embryo production. This increases the amount of offspring per cow. In order to consider OPU as an effective tool for obtaining oocytes, and therefore useful for the conservation of endangered breeds, it should be demonstrated that OPU does not adversely interfere with the fertility of the donor after its employment. Hence, the main objective of this study was to test whether the repeated use of OPU negatively affects subsequent fertility of these animals.

A total of eight M-L donor animals subjected to a session of OPU/week during seven weeks in average were studied. OPU involved removal of the dominant follicle by GnRH (Dalmarelin®, Fatro Iberica, Barcelona, Spain), followed 48 h later by 500 IU FSH-LH (Pluset®, Calier, Barcelona, Spain) and OPU 48 h later (Ruiz *et al.,* 2013). Donors were subjected to synchronization of ovulation with CO-Synch and CO-Synch7d+CIDR® (Zoetis, Paris, France) protocols (Geary and Whittier, 1998; Lamb *et al*., 2001) and timed fixed artificial insemination (TFAI) between 9 and 35 days after the last OPU and re-inseminated at observed estrus if not pregnant. Semen for AI was obtained from a M-L bull by electroeyaculation, packaged in 0.5 ml straws and frozen and stored in liquid nitrogen until use. Pregnancy diagnosis was performed by rectal palpation and ultrasonography with more than 30 days post-AI and reconfirmed within 2-3 months. Following reproductive data were analyzed: *a*. Pregnancy rate to first AI (PR1AI, i.e. percentage of females pregnant after first AI); *b*. Total pregnancy rate (TPR, percentage of pregnant cows in relation to total inseminated females); *c*. Mean days open (MDO, interval in days between the last OPU and gestation); *d.* Deliveries percentage (DP, number of calving cows divided by total number of pregnant cows). *e*. Sex ratio (SR, ratio between males and females of all calves born) and *f*. Number of AI/pregnancy (AI/P, number of inseminations performed between pregnancies obtained).

Two donor cows are still in the OPU program. Results including the six remaining animals were: PR1AI = 50% (3/6); one of the inseminated cows diagnosed as non pregnant was not inseminated again; the rest of the cows became pregnant after the 2nd AI after observed estrus. Therefore, TPR after OPU was 100%, excluding those donors not inseminated. MDO was 39 days (ranging 9-106 days). DP was 100% (5/5 pregnant) with 5 calves born (4 males and 1 female). SR was 4:1. PR1AI in CO-synch group was 25% (1/4) and 100% (2/2) for CO-Synch7d+CIDR® group. Finally, AI/P (excluding the donor discarded after 1st AI) was 1.4.

Reports of repeated applications of OPU and subsequent fertility to TFAI are scarce. Aller *et al.* (2010) found no difference in pregnancy rates between cows subjected to repeated OPU sessions and the control group, after double TFAI, 11 days after last OPU. Our results revealed a PR1AI after the last OPU session of 50%, after CO-synch protocol being 25% (1/4), while the PR1AI afterCO-synch7d+CIDR® was 100% (2/2). Although the number of animals and inseminations is very small and the objective of the study was not a comparison, it appears that CO-synch7d+CIDR® protocol has major advantages for synchronization, ovulation and pregnancy rates and MDO, than CO-synch protocol in M-L cattle breed.

We can conclude that the repeated use of OPU technique does not reduce the subsequent fertility of M-L cows used as donors in our study.

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Notes

THE ROLE OF SELECTED FACTORS FROM TGF-B SUPERFAMILY IN PORCINE OOCYTES MATURATION

Rybska M[1], Wozna M[1], Zuraw A[1], Kempisty B[2,3], Antosik P[1], Jozefiak A[1], Jaskowski JM[1]

[1]Institute of Veterinary Sciences, Poznan University of Life Sciences, Poland [2]Department of Histology and Embryology, Poznan University of Medical Sciences, Poland; [3]Department of Anatomy, Poznan University of Medical Sciences, Poland;

Members of the TGF-β are involved in several important cell functions including proliferation and differentiation. Moreover, the role of these cytokines in growth and development of theca and granulosa cells is well recognized. However, the relationship between the oocytes maturity stage or follicular size and expression of these genes in pigs is still not entirely known.

This study was designed to investigate the expression of few cytokines belonging to the TGF- β superfamily in oocytes: GDF-9, TGF-β2 and TGF-β3. The COCs were collected from small (<4mm), and large (7-10 mm) follicles from puberal gilts and sows after slaughter. Following selection COCs were cultured for 44h in NCSU-23 medium. The relative abundance of mRNA in oocytes was detected by RQ-PCR, before and after in vitro maturation (IVM).

We observed no differences in expression of GDF9, TGF-β2 in oocytes before IVM collected from large and small follicles. However after IVM significantly higher levels of GDF9 mRNA in oocytes collected from large follicles have been found in comparison to the cells isolated from small follicles ($P<0,05$). The TGF-β2 mRNA level also was higher in oocytes collected from large follicles in comparison to oocytes isolated from small follicles after IVM ($P<0.001$). The results obtained in this research could confirm the influence of TGF-β2, GDF-9 on in vitro gametes maturation. Also no differences in expression level of TGF-β3 factor was observed, which may suggest little impact on development of porcine oocytes.

Presented results suggest, that TGF-β factors are important in regulation of oocytes maturation potential as well as follicular growth in pigs ovaries.

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Notes

SCRIPTAID-MEDIATED EPIGENOMIC MODULATION OF *IN VITRO* CULTURED NUCLEAR RECIPIENT OOCYTES BIASES THE EFFICIENCIES OF BOTH THEIR MEIOTIC MATURATION AND SOMATIC CELL CLONING IN PIGS

Samiec M, Skrzyszowska M

National Research Institute of Animal Production, Department of Biotechnology of Animal Reproduction, 32-083 Balice n. Kraków, Poland

The present study was carried out to examine whether the inducible epigenetic modification of *in vitro* maturing oocytes that was triggered *via* new-generation inhibitor of histone deacetylases (designated as scriptaid; SCPT) impacts on their capability to acquire the nuclear maturity and to support the preimplantation development of cloned pig embryos reconstructed with their ooplasm. Meiotically immature cumulus-oocyte complexes (COCs) were cultured *in vitro* for 20 to 21 h in TC 199 medium supplemented with 1 mM L^{-1} dibutyryl cyclic adenosine monophosphate (db-cAMP), 5 mIU mL^{-1} porcine follicle-stimulating hormone (pFSH), 0.1 IU mL^{-1} human menopausal gonadotropin (hMG), 10% foetal bovine serum (FBS), 10% porcine follicular fluid (pFF), 10 ng mL^{-1} recombinant human epidermal growth factor (rhEGF), 5 ng mL^{-1} recombinant human basic fibroblast growth factor (rh-bFGF) and 0.6 mM L^{-1} *L*-cysteine. Subsequently, the COCs were incubated for a further 23 to 24 h in the db-cAMP- and pFSH+hMG-depleted medium enriched with 350 nM L^{-1} SCPT (i.e., 6-(1,3-dioxo-1*H*,3*H*-benzo[de]isoquinolin-2-yl)-hexanoic acid hydroxyamide). In the somatic cell cloning procedure, enucleated *in vitro*-matured oocytes provided the source of recipient cells for genomic DNA of serum-starved and trypsinised foetal fibroblasts. Nuclear transfer-derived oocytes were artificially stimulated using the protocol of simultaneous fusion and electrical activation (SF-EA). The complexes of ooplasts and fibroblast cells were subjected to plasmalemma electroporation by application of two successive DC pulses of 1.2 kV cm^{-1} for 60 μs. The electropermeabilisation of cell plasma membranes was performed in an isotonic dielectric solution with concentration of Ca^{2+} ions increased up to 1.0 mM L^{-1}. After SF-EA, nuclear-ooplasmic hybrids (clonal cybrids) were exposed to 5 μg mL^{-1} cytochalasin B for 2 h, followed by culture to morula and blastocyst stages in 0.4% bovine serum albumin (BSA)- and 10% FBS-supplemented NCSU-23 medium for 6 to 7 days. Sequential *in vitro* meiotic maturation (IVM) in the SCPT-deprived and SCPT-enriched medium contributed to reaching the metaphase II stage by 76/82 (92.7%^{a}) oocytes as compared to 69/83 (83.1%^{b}) oocytes in a control (i.e., SCPT-untreated) group [$^{a,b}P<0.05$; χ^2 test]. Moreover, the frequencies of cleaved embryos (61/70; 87.1%^{A}), morulae (45/70; 64.3%^{A}) and blastocysts (24/70; 34.3%^{C}) that developed from clonal cybrids originating from nuclear recipient oocytes exposed to SCPT during IVM were found to be significantly higher than in the SCPT-untreated group (44/63; 69.8%^{B}, 28/63; 44.4%^{B} and 14/63; 22.2%^{D}, respectively) [$^{A,B}P<0.001$; $^{C,D}P<0.01$; χ^2 test]. Collectively, increased competencies of porcine nuclear-transferred embryos to complete their *in vitro* development to the morula/blastocyst stages suggest the improved reprogrammability of epigenetic memory and thereby transcriptional activity for foetal fibroblast-inherited genomic DNA in an epigenomically-matured host ooplasm that has undergone exposure to SCPT.

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Notes

TRICHOSTATIN A AFFECTS THE COMPETENCIES OF ADULT MESENCHYMAL STEM CELL NUCLEI TO DIRECT EXTRACORPOREAL DEVELOPMENT OF CLONED PIG EMBRYOS

Samiec M, Opiela J

National Research Institute of Animal Production, Department of Biotechnology of Animal Reproduction, 32-083 Balice n. Kraków, Poland

The current research was conducted to explore the *in vitro* developmental outcome of porcine nuclear-transferred embryos reconstituted with adult bone marrow-derived mesenchymal stem cells (ABM-MSCs) that had been epigenetically transformed by treatment with non-specific inhibitor of histone deacetylases (HDACs), known as trichostatin A (TSA). Cumulus-oocyte complexes (COCs) were matured *in vitro* for 20 to 22 h in Tissue Culture Medium 199 (TCM 199). The maturation medium was enriched with 10% foetal bovine serum (FBS), 10% porcine follicular fluid (pFF), 5 ng mL^{-1} recombinant human basic fibroblast growth factor (rh-bFGF), 10 ng mL^{-1} recombinant human epidermal growth factor (rhEGF), 0.6 mM L^{-1} *L*-cysteine, 1 mM L^{-1} dibutyryl cyclic adenosine monophosphate (db-cAMP; bucladesine), 0.1 IU mL^{-1} human menopausal gonadotropin (hMG) and 5 mIU mL^{-1} porcine follicle-stimulating hormone (pFSH). Afterwards, the COCs were cultured for an additional 22 to 24 h in the bucladesine- and hMG+pFSH-deprived medium. Before their use for somatic cell cloning, the permanent ABM-MSC lines (between passages 1 and 2) that had been established from the primary cultures derived from bone marrow aspirates from the iliac crests of a prepubertal gilt were exposed to 50 nM L^{-1} TSA for 24 h during 24- to 48-h contact inhibition. Reconstruction of enucleated oocytes (ooplasts) was accomplished by their electrofusion with epigenomically modulated ABM-MSCs. Fusion of couplets comprised of ooplasts and ABM-MSCs was triggered using two consecutive DC pulses of 1.2 kV cm^{-1} for 60 μs. The same electric pulses that evoked the fusion of ooplast-nuclear donor cell complexes were simultaneously applied to initiate the activation of reconstituted oocytes (clonal cybrids). Immediately after electrofusion/electroactivation, clonal cybrids were incubated in North Carolina State University-23 (NCSU-23) medium supplemented with 5 μg mL^{-1} cytochalasin B for 2 h, followed by *in vitro* culture up to morula and blastocyst stages in NCSU-23/BSA/FBS medium for 144 to 168 h. The rates of dividing embryos (174/178; 97.8%), morulae (155/178; 87.1%^{A}) and blastocysts (116/178; 65.2%^{A}) that originated from nuclear-transferred oocytes derived from ABM-MSCs undergoing TSA treatment were significantly higher than in the TSA-unexposed group (152/161; 94.4%, 119/161; 73.9%^{B} and 71/161; 44.1%^{B}, respectively) [$^{A,B}P<0.001$; χ^2 test]. Altogether, the improvements in the morula and blastocyst yields of cloned pig embryos seem to arise from enhanced abilities for promotion of faithful and complete epigenetic reprogramming of TSA-treated ABM-MSC nuclei in a cytoplasm of reconstructed oocytes.

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Notes

EMBRYO PRODUCTION USING SEXED AND NON-SEXED SPERM FROM 5/8 GIROLANDO BULLS

Santos Filho AS, Guido, SI[1], Oliveira JVC[1], Nascimento PS[2], Chaves MS[2], Gomes ET[2], Bartolomeu CC[3]

[1] *Universidade Federal Rural de Pernambuco, Garanhuns- PE, Brasil.*
[2] *Instituto Agronômico de Pernambuco, Arcoverde- PE, Brasil.*
[3] *Universidade Federal Rural de Pernambuco, Recife- PE, Brasil.*

The use of in vitro fertilization (IVF) together with sexed semen is well accepted to enhance breeding progress. In turn, it is also known that sperm sorting weakens the sperm quality (JR SEIDEL, 2003). Many studies have been performed to identify factors influencing the fertilizing potential. However these studies have obtained contradictory results (Marques et al., 1995). This study aimed to compare the cleavage rate (CR), viable embryos (VE) and blastocysts (BP) produced by IVF with sexed (SS) and non-sexed sperm (NS) from three 5/8 Girolando Bulls.

Ovaries were collected at the slaughterhouse in the municipality of Arcoverde-PE, transported to the Laboratory of Animal Breeding and Reproduction of the Agronomic Institute of Pernambuco (IPA). Follicles between 3-8 mm were aspirated.

In groups 15/drop, grade 1 oocytes were matured for 24 hours in saturated humidity atmosphere with 5% CO2 at 38.5 °C. The sexed and non-sexed sperm were capacitated by a 90% Percol gradient. The insemination dose was 2 million for each treatment. Sperm and oocytes were incubated for 18 hours under the same conditions as already described. After 48 hours of culture the first "feeding" was done along with the cleavage evaluation, after 72 hours of culture the second "feeding" was done, and on the 7th day it was accomplished the evaluation of embryo viability and blastocysts. Data were analyzed by chi-square test at 5% of significance.

Statistical difference was found between sexed and non-sexed sperm when comparing the variables cleavage rate, viable embryos ($P < 0.01$), and blastocysts ($P < 0.05$). Cleavage rate for Bull 3 from sexed and non-sexed-sperm was 12.1 ±1.1 and 6.0 ±1.0, respectively; $P<0.01$. For Bull 2 viable embryos was 6.5 ± 2.3 and 9.5 ± 1.0, respectively; $P<0.01$ and for Blastocysts was 4.3 ± 1.7 and 6.0 ± 1.0, respectively $P<0.05$. Comparing the results of sexed-sperm from the three Bulls, Bull 1 presented the best result for viable embryos (13.0 ± 3.0) and Bull 2 the worst (6.5 ± 2.3) ($P<0.05$).

From the results it can be concluded that sexed-sperm produced a smaller number of structures. It has to be considered that the use of this technology provides an extra benefit for animal breeding, when producing sexed embryos. However, it was also shown that each individual bull exerted a main impact on fertilization capacity, embryo growth and development up to the blastocyst stage.

Notes

EFFECT OF COOLING AND FREEZING OF BLACK RACKA RAM SEMEN ON SEMINAL PLASMA ENZYME ACTIVITIES

Sarlós P, Huszár SZ, Rátky J, Páble T, Egerszegi I
istvan.egerszegi@atk.hu

National Agricultural Research and Innovation Center, Research Institute for Animal Breeding, Nutrition and Meat Science, 2053 Herceghalom Gesztenyés str.1. Hungary

Besides liquid preservation of ram semen there is an improved use of cryopreserved semen in breeding programs and conservation of rare breeds, however fertility rate after cervical insemination with frozen/thawed semen exceptionally is more than 50 %. One of the main reasons for decreased fertilizing ability is their membrane damages, which shorten lifespan. Biochemical analysis of seminal plasma could be an important indicator of altered membrane function during freezing process. The aim of the study was to determine changes in aspartate-aminotransferase (AAT) and lactate-dehydrogenase (LDH) enzyme activities during cryopreservation of semen from Black Racka ram.

Altogether 10 mature (2-4 years old, 50-65 kg) Black Racka rams were used. Ejaculates were collected via artificial vagina. Semen was diluted with Andromed for a final concentration of $3x10^8$ spermatozoa/ml after motility analysis and determination of concentration. Samples were divided to 3 parts; fresh semen (0h), equilibration at 5°C for 2 h (2h) and frozen one (F). In each group motility of semen was evaluated by computer assisted semen analyzer (CASA, SpermVision, Minitube), AAT and LDH activities were measured from seminal plasma and acrosome damages were determined on stained smears. One-way ANOVA was performed for statistical evaluation of data.

Changes of enzyme activities were found during the freezing process in each group. Significant increase was obtained in AAT level from 76.05±8.03 (0h) and 91.10±7.53 (2h) to 137.70±6.75 (F) U/$3x10^8$ spermatozoa/ml ($p<0.001$) and in LDH level 284.70±17.40 (0h) and 297.60±19.28 (2h) to 369.35±17.99 U/$3x10^8$ spermatozoa/ml ($p<0.001$), respectively. Percentage of acrosome damages was 2.5 fold higher in frozen samples (32.73±1.77) than in fresh one (12.10±1.44). Progressive motility of frozen thawed samples was less then 45% with more than 40% altered acrosome, when LDH level reached 400 U/$3x10^8$ spermatozoa/ml. Further experiment is needed to decrease membrane injuries during cryopreservation with possible use of antioxidants.

Notes

COMPARISON BETWEEN TWO VITRIFICATION METHODS FOR IN VITRO PRODUCED BOVINE EMBRYOS

Saucedo MN[1], Reichenbach M[2], Kurome M[1], Wolf E[1], Reichenbach H.-D[3]

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

In the present study we compared two different vitrification methods for *in vitro* produced bovine embryos: The CryoLogic Vitrifcation Method (CVM), CryoLogic® Australia and the Hollow Fiber Vitrification (HFV) Method (Matsunari et al. 2012). For IVP, ovaries from slaughtered animals were obtained from the abattoir. Only 2 to 8 mm follicles were aspirated and *in vitro* matured (IVM) for 22 h, followed by *in vitro* fertilization (IVF) for 18 h. After IVF presumptive zygotes were denuded for *in vitro* culture (IVC) in SOF supplemented with 5% OCS. Cleavage rate was recorded on day 3 (day 0 = IVF). *In vitro* cultured embryos were assessed on days 5 to 8. Only grade 1 morulae (day 5 or day 6), early blastocysts (day 6 or day 7) and blastocysts (day 7 or day 8) were vitrified either by the CVM or HFV method. Embryos were loaded in 0.7 – 1.0 µl of solution. Vitrification procedure was performed at room temperature (RT). Embryos were selected and handled in 20 mM HEPES TCM-199 supplemented with 20% fetal calf serum (FCS) (TCM) until they were transferred to the equilibration solution (ES) 1 (TCM + 7.5% EG + 7.5% DMSO) and equilibrated for 1 minute. Subsequently they were transferred to ES 2 (TCM+ 7.5% EG + 7.5% DMSO + 0.25M sucrose) for another minute, followed by ES 3 (TCM + EG 7.5% + DMSO 7.5% + 0.5M sucrose) for 1 minute, and finally transferred to the vitrification solution (VS) (TCM + 15.0% EG + 15.0% DMSO + 0.5 M sucrose) where they were held for 40 seconds. For thawing, embryos were immersed directly in thawing solution (TS) (TCM + 4% FCS + 1 M sucrose) at 38.5°C for 1 minute and subsequently transferred to the dilution solution (DS) 1 (TCM + 10% FCS + 0.5 M sucrose) at RT for 3 minutes and to ES 2 (TCM + 15% FCS + 0.25 M sucrose) at RT for another 3 minutes and finally washed two times in washing solution (WS) (TCM + 20% FCS) 1 and 2, five minutes each. After thawing procedure, embryos were *in vitro* cultured until day 12. Survival rate (judged by re-expansion rate) 24-48 h after thawing and hatching rate were recorded. Statistical difference was observed between the HFV method and CVM. Overall survival rates after thawing were higher when embryos (morulae: 62% (n=42) *vs* 76% (n=41); early blastocyst: 80% (n=60) *vs* 77% (n=35); blastocyst: 86% (n=81) *vs* 87% (n=70); HFV and CVM respectively) were vitrified by the CVM. An average re-expansion rate of 73.0 (HFV) vs. 78.6 (CVM) % ($p>0.05$) and hatching rate of 63.6 vs. 57.0 % ($p>0.35$) respectively, was observed. Hatching rate within the two groups equals the hatching rate of regular IVP (60.3%) observed in our laboratory. Early blastocysts and blastocysts seem to be the best embryo stages in order to achieve good hatching rates after thawing (morulae vs. blastocyst; $p>0.05$).

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Notes

EPIGENETICALLY MODULATED BLOOD-DERIVED FIBROBLAST-LIKE CELLS PROVIDE A NEW SOURCE OF NUCLEAR DONORS FOR SOMATIC CELL CLONING IN GOATS

Skrzyszowska M, Samiec M

National Research Institute of Animal Production, Department of Biotechnology of Animal Reproduction, 32-083 Balice n. Kraków, Poland

The purpose of our study was to evaluate the effect of valproic acid (VPA)-dependent epigenomic transformation of peripheral blood-derived fibroblast-like cells (PB-FLCs) on the *in vitro* developmental capacity of caprine cloned embryos generated using this new type of nuclear donor cells. The enucleated *in vitro*-matured oocytes were reconstructed with the cell nuclei of serum-starved PB-FLCs that had been either treated with 3 mM VPA (non-selective inhibitor of histone deacetylases) for 24 h (Group I) or had not been exposed to VPA (Group II). Ooplast-somatic cell complexes were simultaneously fused and electrically activated by a single DC pulse of 2.4 kV/cm for 15 μs. After a 1-h delay, nuclear-cytoplasmic hybrids (clonal cybrids) were additionally stimulated using 5 μM calcium ionomycin, followed by treatment with 2 mM 6-dimethylaminopurine for 2 h. Activated clonal cybrids were incubated in upgraded B2 INRA medium for 24 h. Afterwards, dividing embryos were cultured in the FBS-supplemented medium for an additional 144 to 168 h up to morula and blastocyst stages. Among 63 cultured cloned embryos assigned to Group I, 51 (81.0%^{A}) were cleaved. The percentages of embryos that reached the morula and blastocyst stages were 31/63 (49.2%) and 19/63 (30.2%^{A}), respectively. In Group II, out of 75 cultured embryos, 48 (64.0%^{B}) underwent cleavage divisions, but 24 (32.0%^{B}) and 13 (17.3%^{B}) developed to morula and blastocyst stages, respectively. Cumulatively, VPA-based inducible epigenomic modification of PB-FLCs resulted in the cleavage activity and morula/blastocyst formation rates of cloned goat embryos that increased significantly as compared to those for cloned embryos produced using VPA-unexposed nuclear donor cells ($^{A,B}P<0.001$; χ^2 test). This appears to be related to improved ability of donor cell nuclei to undergo transcriptional reprogramming in blastomeres of caprine cloned embryos.

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Notes

EFFECTS OF L-CARNITINE AND/OR RESVERATROL ON THE DISTRIBUTION OF CHROMOSOMES AND MICROTUBULES OF VITRIFIED BOVINE OOCYTES

Sprícigo JFW[1,2], Arcarons N[1], Morató R[3], Dode MAN[4], Mogas T[1]

[1]Universitat Autònoma de Barcelona, Spain [2] University of Brasília, Brazil. [3] University of Girona, Spain. [4] Embrapa - Genetic Resources and Biotechnology, Brasília-DF, Brazil.

The large intracellular lipid content and the wide cell volume increase sensitivity of bovine oocytes to chilling injury during cryopreservation. In addition, oocytes that survive cryopreservation accumulate reactive oxygen species. The aim of this work was to evaluate the spindle configuration of vitrified/warmed oocytes after IVM in a supplemented media supplemented with L-Carnitine and/or Resveratrol, a lipolytic and an antioxidant agent, respectively. For the IVM, viable COC's with at least three cumulus cells layers and homogenous cytoplasm were randomly distributed into four groups: 1) Control: conventional IVM media with TCM-199, EGF and 10% of FCS; 2) CAR: IVM media supplemented with 0.6 mg/ml of L-Carnitine; 3) RES: IVM media supplemented with 1µM/ml of Resveratrol and 4) L+ R: IVM media supplemented with 0.6 mg/ml of L-Carnitine and 1µM/ml of Resveratrol. After 22 h of IVM, half of the oocytes from each of the four groups were vitrified and warmed using the Cryotop method. After warming, the oocytes were allowed to recover to their respective media for two additional hours. After 24 h of IVM, oocytes from all treatments were completely denuded, fixed and microtubule and chromosome distribution was analyzed by immunocytochemistry under a fluorescent microscope. ANOVA was performed to analyze differences in meiotic spindle configuration after treatment. The level of statistical significance was set at $P<0.05$. Similar percentages of normal chromosome and microtubule configuration were observed for non-vitrified oocytes (Control: 76.9% ± 2.1; n=78; CAR: 88.1% ± 3.3; n=84; RES: 85.1% ± 5.6; n=67 and L+R: 82.9% ± 4.8; n=57), Similarly, no significant differences were observed among vitrification treatments (Control: 45.6% ± 4.4; n=57; CAR: 54.5% ± 2.6; n= 55; RES: 47.1% ± 6.1; n= 68 and L+R: 52.4% ± 6.6; n= 42). When comparing fresh with vitrified oocytes, those oocytes matured with CAR or with C+R before vitrification/warming triggered similar percentages of normal spindle and chromosome distribution than non-vitrified control oocytes. Despite vitrification and warming procedures cause a severe chromosome and spindle commitment, the supplementation of IVM media with L-carnitine alone or in association with resveratrol was able to reduce the injuries and may indicate a helpful alternative for improving bovine oocyte vitrification.

Notes

LONG TERM ELEVATED NEFA CONCENTRATIONS DURING *IN VITRO* MURINE FOLLICLE GROWTH REDUCE OOCYTE DEVELOPMENTAL COMPETENCE AND ALTER SUBSEQUENT EMBRYO METABOLISM

Valckx SDM[a], Simintiras C[b], Mckeegan P[b], Sturmey R[b], Cortvrindt R[c], Bols PEJ[a], Leroy JLMR[A]

[a]Gamete Research Center, University of Antwerp, Belgium; [b]Center for Cardiovascular and Metabolic Research, Hull York Medical School, UK; [c]EggCentris bvba, Belgium

Metabolic disorders, such as a negative energy balance in dairy cows or obesity and type 2 diabetes in human, are characterized by elevated serum and follicular fluid non-esterified fatty acid (NEFA) concentrations, due to an increased lipolysis. Such high NEFA concentrations during the final phase of oocyte maturation *in vitro* (24h) impair bovine oocyte developmental competence and subsequent embryo quality and metabolism. We recently showed that long-term elevated NEFA concentrations during murine whole follicle growth, more closely mimicking the *in vivo* situation, only moderately affect antrum formation, but substantially alter granulosa cell gene expression patterns and steroidogenesis. How this may affect the oocyte and subsequent embryo is unknown. Therefore, we hypothesized that long-term elevated NEFA concentrations may hamper oocyte developmental competence and subsequent embryo quality through an altered follicular physiology (indirect) or via direct effects at the oocyte level. The specific aim was to study the effect of elevated NEFA concentrations during murine *in vitro* follicle growth, on oocyte developmental competence and embryo metabolism, as a marker for embryo quality.

Murine early secondary follicles were cultured individually until the antral stage (12days), under the following conditions: BASAL NEFA [72µM palmitic acid (PA), stearic acid (SA) and oleic acid (OA) mix], HIGH SA (280µM SA) and HIGH NEFA (720µM NEFA mix). After a Day 12 ovulatory stimulus (hCG, EGF), oocytes from all antral follicles were isolated, fertilized and presumptive zygotes were cultured following standard laboratory procedures. Cleavage rate (Day 1 p.i.) and blastocyst formation (Day 5 p.i.) were quantified (4 replicates). Furthermore, on Day 2 p.i., 4- to 8-cell stage embryos were selected and cultured in groups of 10 in 5µl homemade ASSAY medium drops (specific supplementation of glucose and amino acids) under a mineral oil overlay. Embryos were allowed to grow for exactly 24h, after which the (mostly) morula stage embryos were removed from the culture drops. ASSAY medium droplets were then analyzed for glucose (ultrafluorometry) and amino acid composition (HPLC) (4 replicates). Data were analyzed with binary logistic regression (embryo development) or non-parametric Kruskal-Wallis tests (amino acids, glucose).

Cleavage rate was reduced for HIGH NEFA embryos (53%), compared to BASAL embryos (69%, $P<0.01$). Blastocyst formation was impaired in HIGH SA, HIGH OA and HIGH NEFA embryos (32%, 33% and 42% respectively), compared to the BASAL treatment (63%, $P<0.01$). Furthermore, HIGH SA embryos consumed significantly less glucose compared to BASAL and HIGH NEFA embryos ($P<0.01$). Amino acid analyses only showed a trend ($P=0.097$) for an increased overall amino acid production in HIGH NEFA embryos.

In conclusion, our results indicate that long-term elevated NEFA concentrations during follicular growth, alter follicular physiology, ultimately leading to an impaired oocyte developmental competence and embryos with an altered ('glucose intolerant') metabolism.

Notes

ANTI-MUELLERIAN-HORMONE (AMH) LEVELS IN PLASMA OF HOLSTEIN-FRIESIAN HEIFERS AS A PREDICTIVE PARAMETER FOR THEIR OVUM-PICK-UP OUTCOMES: PRELIMINARY RESULTS

Vernunft A[1], Schwerhoff M[2], Viergutz T[1], Kuwer A[2]

[1]*Institute of Reproductive Biology; Leibniz Institute for Farm Animal Biology, 18196 Dummerstorf, Germany*
[2]*Masterrind GmbH, ET-Station Nückel and Rodenkirchen, Germany*

Anti-Muellerian hormone (AMH) is expressed only in the gonads. In females it is produced only from granulosa cells, manly expressed from preantral, secondary follicles during follicle recruitment. As shown in mice and humans, the concentration of AMH in plasma reflects the total number of oocytes in the ovary and also the number of follicles, which are involved in antral follicular activity. Also in cattle it was found, that the antral follicle count (AFC) of small follicles is positively correlated (r=0.88) to the plasma levels of AMH (Rico et al., 2011). Moreover AMH plasma levels are mainly independent of the ovarian cycle. The aim of this study was to investigate whether AMH plasma levels of Holstein-Friesian heifers could be used to predict their ovum-pick-up outcomes.

Plasma samples were collected from 54 Holstein-Friesian heifers. To avoid possible impacts of the ovarian cycle on AMH plasma levels, samples were only taken when the animals were in heat. Plasma samples were analyzed with an ELISA-Kid (DSL-10-144400, Beckman Coulter, USA) for their AMH concentration. Afterwards, the heifers underwent repeated Ovum-Pick-Up (OPU) on two commercial ET stations for in vitro embryo production.

The heifers had mean plasma AMH levels of 0.37±0.03 ng/ml with a range from 0.09 to 1.39 ng/ml. Mean number of follicles, counted as be aspirated in an OPU session were 9.8±0.7 per animal and an average of 7.5±0.6 oocytes were recovered. Correlations between plasma AMH and number of aspirated follicles or recovered oocytes were low (r=0.46; r=0.38) but high between number of aspirated follicles and recovered oocytes (r=0.78). Dividing the animals into quartiles according to their AMH plasma levels it became apparent that animals of the upper quartile (AMH levels of 0.63±0.08 ng/ml) had significant more aspirated follicles (11.9±2.0 vs. 7.8±0.6; p=0.03) and recovered oocytes (9.0±1.2 vs. 6.1±0.4; p=0.04) than the lower quartile (AMH levels of 0.19±0.01 ng/ml).

Our preliminary results suggest the assumption that correlations between AMH plasma levels and outcomes of an OPU-IVF program are too low to use AMH as a good predictive parameter for the success of an OPU procedure in Holstein-Frisian heifers. But AMH can help to identify groups of very good or very poor oocyte donors.

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Notes

ULTRASOUND-GUIDED FOLLICLE INJECTION: A METHOD TO PROOF RESULTS OF BOVINE FOLLICLE CELL CULTURES IN AN IN-VIVO MODEL

Vernunft A, Viergutz T, Röttgen V, Weitzel JM

Institute of Reproductive Biology; Leibniz Institute for Farm Animal Biology, 18196 Dummerstorf, Germany

Basic research on cell functions is mostly done by using standardized in-vitro models like cell cultures. However, to test the relevance of cell culture findings for the whole organism, it is necessary to proof the results in in-vivo models, preferably in the investigated species. The use of the minimal invasive Ovum-Pick-Up (OPU) technique to inject substances in a follicle may provide an in-vivo model to test the relevance of findings from cell cultures for follicle development in cattle.

To inject follicles, we use an OPU system, which is constructed for the use of a 55cm long 17 G Ovum Pick-Up aspiration needle (Cook Australia) and equipped with a 6.5MHz fingertip ultrasound probe. Instead of the OPU-needle a 1.2 mm steel pipe is inserted into the needle duct of the OPU system, which carries a luer-lock connection on both sides. On one end a fine scaled 1-ml syringe is put on the pipe, and pipe and syringe are filled with sterile water. Then a 0.5 x 40 mm cannula (Sterican® 25G x 1.5", B. Braun, Germany) is set on the other side of the pipe and the substances, which should be injected, can be draw up by the syringe so that an air bubble separates substances and sterile water. Experimental animals are fixed and prepared by paradural anesthesia with 4 ml 2%-procain solution. If fixation of the animal is not possible, it is sedated with 1 ml of 2%-xylazine solution.

In a current study we investigated the relevance of blocking the LOX-1 receptor for the ovulation process, because in cell culture the LOX-1 receptor can affect the estrogen production of bovine granulosa cells. After heat induction, we injected 0.2 ml of different solutions in the dominant follicle of heifers and cows just before, two or sixteen hours after ovulation induction with a GnRH analogon. Injected follicles had diameters between 13 and 26 mm. After injection of 0.9% sodium chloride solution or solutions of 2.5µg unspecific mouse antibodies (negative controls) ovulations occurred and were observed between 28 and 32 hours after GnRH administration. However, injection of more than 5µg antibodies led to unspecific immune reactions in the follicle and disturbed ovulation. In contrast, injection of COX-2 inhibitors like flunixine or indomethacin (positive controls) could interrupt the ovulation process at each time point and ovulation was not observed unit 42 hours after GnRH administration. In an ongoing study we test specific LOX-1 receptor inhibitors and antibodies.

In conclusion, our current work demonstrates, that ultrasound guided follicle injection is a minimal invasive and economic model to investigate bovine follicle cell function in vivo.

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