AETE

Association Européenne de Transfert Embryonnaire

European Embryo Transfer Association

27ème COLLOQUE SCIENTIFIQUE

27th SCIENTIFIC MEETING

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Dr Maurice P Boland

Special Celebration

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Chester, England, 9th and 10th September 2011

AETE

Association Européenne de Transfert Embryonnaire

European Embryo Transfer Association

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Prof. Maurice P. BOLAND

A.E.T.E. Medalist 2011

Prof. Maurice P. BOLAND
A.E.T.E. Medalist 2011

Professor Maurice P. Boland
AETE Pioneer Award 2011

Maurice Boland was born on a small mixed farm at Kilanerin, near Gorey, County Wexford in what is known as 'the sunny south-east' of Ireland. The youngest of 4 children, his father died when Maurice was less than one year old; his mother, Claire, subsequently re-married and the family grew to a total of eight children over the following years.

Maurice graduated from University College Dublin with a BAgrSc Degree (1st Class) in 1970. He immediately began a postgraduate research career under the supervision of Professor Ian Gordon (himself a Pioneer Award recipient of the AETE in 1995 and IETS in 1998) obtaining a MAgrSc Degree in 1971 and a PhD in 1973. Much of his PhD was concerned with developing embryo transfer in sheep as a means of breed propagation. As a post-doc, Maurice worked extensively on embryo transfer in cattle and was one of the first to achieve success with non surgical embryo transfer techniques. At that time in Ireland there was a particular interest in establishing twin pregnancies in beef cows by transferring an embryo to the contralateral uterine horn of a bred recipient, thus increasing the efficiency of beef production. The establishment of a simple non-surgical technique for transferring embryos in cattle with relatively high levels of success led to the almost universal adoption of the technique by the embryo transfer industry as it was demonstrated that normal pregnancies and high twinning rates could be established using this simple transcervical procedures.

Two periods of sabbatical leave followed. The first of these was at the University of California at Davis from 1979-1980 working with Gary Anderson, Bob Bondurant and others on various aspects of embryo development including embryo survival following short term storage at 4 degrees. The second period of leave was at CSIRO at Prospect, Sydney from 1983-1984, working with Colin Nancarrow and Jim Murray. This period resulted in the publication of numerous papers ranging from studies on the ovarian response to PMSG and GnRH in ewes immunized against oestradiol 17-beta to the study of chromosomal abnormalities in embryos from superovulated Merino ewes. Maurice has acknowledged that these visits enhanced his view of the research questions he was interested in addressing and always encouraged staff to take such opportunities to see how other groups operated internationally.

Maurice returned invigorated from his stay in Sydney and embarked on significant research effort in the area of superovulation and embryo studies in cattle. Despite significant research in this area during the past four decades by many research groups and the transfer of up to 1 million embryos annually worldwide it is sobering to note that success rates in terms of viable embryos produced per donor treated have not improved much. During this time he worked extensively with Jim Roche, himself a pioneer of our understanding of follicle development and turnover in cattle. Over his career, Maurice's main areas of interest have included 1) production and transfer of embryos produced both in vivo and in vitro, 2) factors affecting follicular growth and atresia in cattle and sheep, 3) fertility in the high fertility dairy cow, and 4) the link between nutrition and reproduction.

Maurice's sizeable and impressive list of publications is testament to an extraordinary drive and work ethic. This has been acknowledged by the awarding of a DSc degree on Published Work in 1983, the most prestigious degree awarded by UCD and held by only a handful of people in the University. He supervised approximately 40 Masters students and 30 PhD students in animal reproduction. He served on the editorial boards of several international journals and has acted as an ad-hoc reviewer for most of the journals publishing research in his area. He has been an active member of both AETE and IETS, serving as a Board Member of both societies and as President of IETS in 1990-1991.

Within UCD, Maurice rose swiftly through the academic ranks from his appointment as College Lecturer from 1976-1986, Senior Lecturer 1986-1990, Associate Professor 1990-1994 to his appointment as Chair and Professor of Animal Husbandry in 1994. Maurice was appointed Head of what was the Department of Animal Science and Production in the Faculty of Agriculture in 1992 and retained this position until 2001. At the time, he was the youngest staff member in the Department, a measure of the President's confidence in him. On stepping down as Head of Department he took on the role of Associate Dean for Research for the next three years before being appointed Dean of the Faculty of Agri-Food and the Environment in 2004. Following a major re-structuring exercise in the University in 2005, which saw the disappearance of faculties and departments and their replacement with Schools and Colleges, he then became the Head of the new School of Agriculture, Food Science and Veterinary Medicine in that year. Under Maurice's guidance, the School, the largest School in UCD, became one of the best and most productive in the university. Finally (to date, at least) in January 2008, Maurice was appointed as Principal, College of Life Sciences. This was a significant achievement and a reflection of the esteem in which he is held within the university as the College Principals are also Vice-Presidents of the University and have a central role in the day-to-day running of the University determining University policy.

Whether in teaching, research or management Maurice has always worked hard and played hard. He leads by example and his work ethic has stayed with him as he made the move from research to management; his is typically the first car in the car-park in the morning and one of the last to leave in the evening. He has been, and continues to be, a strong voice for agriculture and for research in Ireland. He has a high national profile as evidenced most recently by his appointment to

the recently established Agri-Research Expert Advisory (AREA) Group by The Minister for Agriculture, Fisheries and Food, which is responsible for developing a strategic research agenda for agriculture research into the future.

Finally, behind every good man is an even better woman. Maurice is married to Geraldine ('Ger') and together they have 3 daughters, Lisa, Clara and the youngest, Emma, currently working in veterinary practice in New Zealand. Maurice and Ger now live in Kilteel, County Kildare, close to the Dublin border and are the proud grand-parents of five children.

Pat Lonergan
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TRANSITION FROM RESEARCH TO MANAGEMENT - LESSONS LEARNED

MAURICE P BOLAND
Vice President and College Principal

College of Life Sciences,
University College Dublin
Ireland

Introduction

This short paper attempts to indicate how lessons learned from research can be translated into management at University level. Having completed a BAgrSc degree I commenced a Master's research programme under the supervision of Professor Ian Gordon, I soon realised that I wished to remain active in research. I was fortunate to start my academic career under the supervision of such a gifted and visionary reproductive biologist. My research career was helped significantly by taking periods of sabbatical leave at the University of California at Davis (working with Gary Anderson et al.) and at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) at Prospect, Sydney (working with Colin Nancarrow and James Murray). Both of these invaluable experiences enabled me to become more independent, to become better at forming collaborative alliances and better at developing hypotheses and questions and becoming more efficient at scientific critique. In addition, at UCD I was fortunate to have the opportunity to work with Professor Jim Roche who provided real challenges and leadership to our reproductive biology group. His ability to understand and become familiar with many aspects of science was immense. Furthermore I was very lucky that we were able to hire a group of very bright and enthusiastic young scientists who have continued the tradition at UCD of having a very strong reproductive biology group in place. All of this indicates that one requires critical mass and being part of a team that is self critical and aware of international outputs to make some progress. Undertaking large animal experiments requires a large team of dedicated scientists, technicians and animal husbandry experts to ensure that the outcomes are reliable – this is particularly important when carrying out intensive blood sampling over many days and in some cases over many weeks.

I moved into management in the early nineties and this became my full-time job from early 2004. I was not trained for the administration/management role but applied the basic principles that I learned as a researcher to the job, such as focusing on the task at hand, ascertaining the facts before making a decision, not taking on too many commitments and making sure that the paperwork was completed, because no job is finished until the paperwork is done. Management can be a very lonely job, unlike animal research where one normally works as part of a team; team spirit plays a major role in the success of a research group and must be fostered at all costs. As with animal research, one must learn to delegate when in management, and when you delegate you must trust that person to deliver and be prepared to stand by the outcome, personally taking the responsibility for those actions.

When I commenced research for a Masters degree in 1970, I had no idea where my career would take me. However, having completed a PhD degree in 1973 I was hooked on science and was

determined to make a career in academia. I enjoyed research in animal reproduction and the idea of embryo transfer was fascinating, enhanced no doubt by the vision, enthusiasm and persistence of my mentor, supervisor and boss, Ian Gordon. His dedication to his research was unparalleled; his knowledge of the scientific literature was exceptional and therefore members of the team were always current on relevant literature.

Early research

My PhD was concerned with developing embryo transfer (ET) in sheep as a means of propagating particular breeds of animals; we were all either ignorant or unaware of the difficulties that we would face in trying to overcome the problems of superovulation. It is true to state that relatively little success has been achieved with superovulation during the past 40 years. While we understand considerably more about the whole area of follicular growth, dominance and atresia, we still face challenges of being able to harvest a consistent supply of good quality embryos from every donor selected to enter an ET programme. Working with cattle is in no way easier than sheep except that you can easily recover and transfer embryos non surgically. In the early 1970s one of the major challenges was to develop non surgical (NS) procedures for embryo recovery and transfer. Major progress was achieved using different procedures for embryo recovery; success with transfer was far more limited. One of the major breakthroughs involved the timing of transfer. Like many other groups we were interested in achieving pregnancies through NS transfer. Our second objective at that time was to establish twin pregnancies in beef cows. We concentrated our transfer attempts on modifying the approach used for artificial insemination (AI) but doing the transfers between days 5 and 7 of the oestrous cycle. We took the approach of inseminating beef recipients and then transferring a single embryo into the contralateral horn with the hope of seeking help from the native embryo to nurture the transferred embryo in the contralateral horn and help in the recognition of pregnancy. We had immediate success with the first contralateral transfer resulting in a twin pregnancy when the beef recipient was slaughtered at 33 days post AI. Expectations were raised and disappointment followed when we discovered only a single pregnancy resulting from AI in the ipsilateral horn in the next two recipients. After adding the appropriate numbers of recipients we discovered that we could establish about a 40% twinning rate in those animals that became pregnant following AI. Others were also working on N/S transfer and data revealed that one could achieve close to a 60% pregnancy rate using N/S transfer to the ipsilateral horn provided that a synchronized recipient was used. Taking early embryos and culturing those in the rabbit oviduct for a period of time seemed to enhance the selection pressure and result in higher embryo survival following transfer.

Training graduate students

One of the most privileged responsibilities that any scientist can have is to supervise and train graduate students; these are normally the brightest, most dedicated and totally committed individuals to the task at hand. One of the major limitations is that many of us started off supervising graduate students with little or no training for such a position. However, having the

opportunity to travel and collaborate internationally soon made the task easier. Learning to embrace rather than despise criticism was a major success story as far as providing guidance to graduate students. Interacting with collaborators from North America also honed our ability to enable graduate students to develop a workable and clear hypothesis; it also ensured that we were more likely to get our manuscripts published in higher ranking journals. This has become much more important in recent years as universities strive to be research intensive while also being internationally competitive. One also learned the importance of publishing research outcomes once they were verified rather than waiting to complete the PhD and then start the write up for publication. Inclusion of published papers as chapters of the thesis worked very well for the student during the viva voce. A key component in the training of graduate students includes development of their communication (oral and written) skills; we found that a series of seminars during the programme helped immensely in enabling each student hone their skills in developing their experimental hypothesis, completing the experimental design, analysing their data and ultimately presenting that data to their peers locally and at international conferences. Writing manuscripts for international journals ensured that they had to be concise and precise in the written word.

Management

Head of Department

My first serious interaction with major management was when the UCD President called me up and informed me that he was appointing me as Head of Department of Animal Science in the Faculty of Agriculture. This was a steep learning curve as I was the youngest academic staff member in the Department and thus had to earn the trust and respect of all staff within the Department. My mission for the Department was to develop it to be amongst the best in the Faculty and to ensure that we focused on research where we could deliver. I was aware that one needs critical mass to ensure that progress is made; we concentrated on three research areas – reproduction, nutrition and genetics and this helped the staff to work better as a group. A second major objective was to ensure that the quality of the undergraduate teaching was enhanced; this in turn led to more students wanting to pursue MSc or PhD programmes thereby increasing the research output from the department. Our Department was one of the first within the Faculty to undergo a Quality Assurance/Quality Improvement process and this was the catalyst to grow the department. Fortunately the review panel articulated the need for focus and critical mass and we could build on the report to ensure that new staff was employed to fulfil the mission of the Department. We grew the academic staff by close to 40% over the next 3-4 years and this in turn led to increased research funding, increased graduate student numbers; increased numbers of postdoctoral fellows and overall increased quality and quantity of output. Head of Department was a 3-year rotating post; I was reappointed on two occasions and had great difficulty persuading the President that he should appoint a different leader after 9 years. Appointing an alternative head was a real bonus as it ensured that new thinking and new ways of conducting business were employed – it is essential that change occurs on a frequent basis and in my case nine years was perhaps too long in that rotating post.

On stepping down as Head of Department I took on the role of Associate Dean for Faculty Research for 3 years. This enabled me to lead initiatives in Faculty research, to provide leadership in establishing some interdisciplinary research and to promoting research among staff who were not research active; it was a role that had little or no staff conflict – those who were research active were very supportive and some of those who were not research active came on board and others did not. The Dean provided a small budget and this helped to lead some initiatives.

Dean of the Faculty

UCD appointed a new President in January 2004 and this was the catalyst for major change. The newly appointed President invited me to take on the role of Acting Vice President for Research – I was chuffed and honoured and took on the role with gusto – but indicated that I was interested in the role of Dean of my Faculty which would be vacant in 3 months. I competed for and was successful in that competition and was appointed Dean of the Faculty of Agriculture in April 2004. I had significant plans to develop the Faculty in both teaching and research until the President announced that he wanted a complete overhaul of the University's Department and Faculty structure. Thus I held the post of Dean for a very short time because by September 2005 eleven faculties and over 90 Departments were abolished and were replaced by five Colleges containing thirty five Schools.

Head of School

I was appointed as Head of the School of Agriculture, Food Science and Veterinary Medicine – the most complex and largest school in the University with staff coming from 13 Departments within 3 faculties. No structures were put in place by the University and I was told to lead this with all staff supposedly reporting to me – over 200 including all grades. Again this was a very sharp learning curve and I immediately established a small management committee (group of 4 which I chaired) and a wider executive advisory committee that provided input to the management committee. It was essential that I maintained control of budgets – which I did, but against considerable resistance because there were many former Heads of Department that now had no budgetary control. This school was one of eight Schools within the College of Life Sciences. I indicated to all staff that I had a single agenda item – to make it the best and most competitive School within the College – and that referred to teaching, research and innovation. While many staff were uncomfortable with the new structures and new ethos, many also were very comfortable with the new arrangements especially as they had access to labs and equipment that might have been difficult under the former system. One challenge was to free up unused space in laboratories so that we could facilitate the increased funding opportunities that were available in Ireland at that time. One way of dealing with this was to identify each individual who required a lab space and work on the principle that one person could only be at one bench at any one time – this freed up considerable bench space and allowed labs to be used in a much more productive and effective manner for the benefit of all staff.

One of the major challenges in running such a large and diverse school is having the time and ability to communicate in an effective and productive manner with all staff such that they are invigorated to work in an optimum fashion and maintain the best output possible which is current and competitive in education and research.

College Principal

January 2008 saw my appointment as Principal, College of Life Sciences, which carried with it a role of Vice President of the University and member of the President's Senior Management Team. This role was again different in that responsibilities shifted; interaction was mainly with Heads of Schools rather than individual academic or support staff, but as College Principal one had a significant budget responsibility in that the complete budget for the College was under the control of the Principal, whose role it is to agree with the Bursar that it is distributed in a manner that will best suit the University. A major challenge during this period has been to return to a break-even budget for the College and to adjust to significant reduction in state support just after a period of unprecedented research funding opportunities. This role requires considerable effort in balancing, on the one hand, the desires of Schools to maintain/increase staffing levels and, on the other hand, to help the University maintain a balanced budget in a time of fiscal poverty, while trying to compete internationally in terms of publications, attracting and maintaining national and international staff and students. Collegiality was crucial to maintaining staff morale and output – otherwise many of the gains achieved by restructuring could be lost. Against this it is gratifying to note that UCD has secured its place in the top 100 universities (Times Higher Education World University Rankings).

Conclusions

Lessons learned from developing, leading and growing a competitive international research programme can be used very effectively in honing skills used in management. Working on the principle of establishing the facts, being aware of international standards, welcoming critique and seeking help when required will go a long way to ensuring that staff members work as part of a team. The major challenge is to be a conduit that will provide the best opportunity for staff to perform at an optimum level in an environment best suited to the individuals strength for the particular activity and where possible to have a good succession plan in place. Saying "no" can sometimes be the best thing that can happen. Major progress can be achieved by having critical mass while taking an interdisciplinary and multidisciplinary approach to the problem at hand. Being part of a team provides a stabilised structure to ensure delivery in a timely fashion; changing team members occasionally will help rejuvenate the members. Having a succession plan in place will enable a School/Unit build on past performance and avoid previous errors.

National Statistical Data of Bovine Embryo Transfer Activity in Europe (2010)

TABLE: 1 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: BELGIUM ***A.E.T.E 2011***

Data collected by Dr. Peter Vercauteren & Dr. Isabelle Donnay

Total number of approved E.T. teams in the country	8
Number of teams providing data	8

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	1174	B/A=
	Embryos collected	**B**		C/A= 5.2
	Embryos transferable	**C**	6052	C/B=
In vitro *(OPU)*	Nb of oocyte donors			
	Nb of OPU sessions			
	Nb of transferable embryos	**D**		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total i*n vitro* embryos		**F**	0	=(D+E)
Total number of transferable embryos		**G**	6052	=(C+F)
Number of sexed embryos			0	
EMBRYO TRANSFER				
In vivo	Fresh	**H**	3476	
In vivo	Frozen	**I**	3746	
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	7222	H+I+J+K=
Number of frozen stored embryos		**M**	3898	
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**	51.9%	(I+K)/L=

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	2046
Number of calves born from *in vitro* embryos	
Total	2046

TABLE: 2 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **CROATIA** ***A.E.T.E 2011***

Data collected by Dr. Martina Karadjole

Total number of approved E.T. teams in the country	1
Number of teams providing data	1

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**		B/A=
	Embryos collected	**B**		C/A=
	Embryos transferable	**C**		C/B=
In vitro *(OPU)*	Nb of oocyte donors			
	Nb of OPU sessions			
	Nb of transferable embryos	**D**		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total i*n vitro* embryos		**F**		=(D+E)
Total number of transferable embryos		**G**		=(C+F)
Number of sexed embryos			0	
EMBRYO TRANSFER				
In vivo	Fresh	**H**		
In vivo	Frozen	**I**	17	
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	17	H+I+J+K=
Number of frozen stored embryos		**M**		
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**	100%	(I+K)/L=

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	32
Number of calves born from *in vitro* embryos	
Total	32

TABLE: 3 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **CZECH REPUBLIC** ***A.E.T.E*** ***2011***

Data collected by
Dr. Jirina Peteliková

Total number of approved E.T. teams in the country	5
Number of teams providing data	5

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	229	B/A=	10.3
	Embryos collected	**B**	2365	C/A=	5.1
	Embryos transferable	**C**	1180	C/B=	49.9%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	1180	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	640		
In vivo	Frozen	**I**	573		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	1213	H+I+J+K=	
Number of frozen stored embryos		**M**	504		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	47.2%	(I+K)/L=	

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE: 4 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **DENMARK** ***A.E.T.E 2011***

Data collected by Dr. Henrik Callesen

Total number of approved E.T. teams in the country	14
Number of teams providing data	9

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	347	B/A=	8.9
	Embryos collected	**B**	3093	C/A=	7.2
	Embryos transferable	**C**	2497	C/B=	80.7%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	2497	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	1354		
In vivo	Frozen	**I**	700		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	2054	H+I+J+K=	
Number of frozen stored embryos		**M**	1056		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	34.1%	(I+K)/L=	

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	1534
Number of calves born from *in vitro* embryos	
Total	1534

TABLE: 5 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **ESTONIA** ***A.E.T.E 2011***

Data collected by Dr. Jevgeni Kurokin

Total number of approved E.T. teams in the country	1
Number of teams providing data	1

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	4	B/A=	3.0
	Embryos collected	**B**	12	C/A=	1.5
	Embryos transferable	**C**	6	C/B=	50%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	6	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	5		
In vivo	Frozen	**I**	6		
In vitro	Fresh	**J**	17		
In vitro	Frozen	**K**	0		
Total embryos transferred		**L**	28	H+I+J+K=	
Number of frozen stored embryos		**M**	1		
% of *in vitro* embryos transferred		**N**	60.7%	(J+K)/L=	
% of frozen embryos transferred		**O**	21.4%	(I+K)/L=	

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	9
Number of calves born from *in vitro* embryos	
Total	9

TABLE: 6 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **FINLAND** ***A.E.T.E 2011***

Data collected by Dr. Marja Mikkola

Total number of approved E.T. teams in the country	5
Number of teams providing data	5

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	486	B/A=	12.6
	Embryos collected	**B**	6135	C/A=	7.9
	Embryos transferable	**C**	3831	C/B=	62.4%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	3831	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	865		
In vivo	Frozen	**I**	2944		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	3809	H+I+J+K=	
Number of frozen stored embryos		**M**	2651		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	77.3%	(I+K)/L=	

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	1563
Number of calves born from *in vitro* embryos	
Total	1563

TABLE: 7 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **FRANCE** ***A.E.T.E 2011***

Data collected by Dr. Claire Ponsart

Total number of approved E.T. teams in the country	21
Number of teams providing data	19

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	5714	B/A=	8.9
	Embryos collected	**B**	50680	C/A=	5.2
	Embryos transferable	**C**	29585	C/B=	58.3%
In vitro *(OPU)*	Nb of oocyte donors		58		
	Nb of OPU sessions		74		
	Nb of transferable embryos	**D**	315		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	315	=(D+E)	
Total number of transferable embryos		**G**	29900	=(C+F)	
Number of sexed embryos			663		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	14252		
In vivo	Frozen	**I**	14592		
In vitro	Fresh	**J**	142		
In vitro	Frozen	**K**	169		
Total embryos transferred		**L**	29155	H+I+J+K=	
Number of frozen stored embryos		**M**	15174		
% of *in vitro* embryos transferred		**N**	1,1%	(J+K)/L=	
% of frozen embryos transferred		**O**	49.3%	(I+K)/L=	

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	15620
Number of calves born from *in vitro* embryos	
Total	15620

TABLE: 8 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **GERMANY** ***A.E.T.E 2011***

Data collected by Dr. Hubert Cramer

Total number of approved E.T. teams in the country	40
Number of teams providing data	19

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**		B/A=
	Embryos collected	**B**	23572	C/A=
	Embryos transferable	**C**	14596	C/B= 66.9%
In vitro *(OPU)*	Nb of oocyte donors			
	Nb of OPU sessions		1226	
	Nb of transferable embryos	**D**	2148	
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total i*n vitro* embryos		**F**	2148	=(D+E)
Total number of transferable embryos		**G**	16744	=(C+F)
Number of sexed embryos				
EMBRYO TRANSFER				
In vivo	Fresh	**H**	5010	
In vivo	Frozen	**I**	9062	
In vitro	Fresh	**J**	1104	
In vitro	Frozen	**K**	377	
Total embryos transferred		**L**	15553	H+I+J+K=
Number of frozen stored embryos		**M**		
% of *in vitro* embryos transferred		**N**	9.5%	(J+K)/L=
% of frozen embryos transferred		**O**	60.7%	(I+K)/L=

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE: 9 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **GREECE** ***A.E.T.E 2011***

Data collected by Dr. Samartzi Fonteini

Total number of approved E.T. teams in the country	2
Number of teams providing data	2

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	0	B/A=
	Embryos collected	**B**		C/A=
	Embryos transferable	**C**		C/B=
In vitro *(OPU)*	Nb of oocyte donors			
	Nb of OPU sessions			
	Nb of transferable embryos	**D**		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total i*n vitro* embryos		**F**	0	=(D+E)
Total number of transferable embryos		**G**	0	=(C+F)
Number of sexed embryos			0	
EMBRYO TRANSFER				
In vivo	Fresh	**H**		
In vivo	Frozen	**I**	5	
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	5	H+I+J+K=
Number of frozen stored embryos		**M**		
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**		(I+K)/L=

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE: 10 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **HUNGARY** ***A.E.T.E 2011***

Data collected by Dr. Ference Flink

Total number of approved E.T. teams in the country	3
Number of teams providing data	2

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	46	B/A=	12.7
	Embryos collected	**B**	592	C/A=	9.8
	Embryos transferable	**C**	453	C/B=	76.5%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	453	=(C+F)	
Number of sexed embryos			15		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	69		
In vivo	Frozen	**I**	180		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	249	H+I+J+K=	
Number of frozen stored embryos		**M**	204		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	72.2%	(I+K)/L=	

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	162
Number of calves born from *in vitro* embryos	
Total	162

TABLE: 11 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **IRELAND** ***A.E.T.E 2011***

Data collected by Dr. Pat Lonergan

Total number of approved E.T. teams in the country	5
Number of teams providing data	1

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	360	B/A=	9.2
	Embryos collected	**B**	3594	C/A=	5.3
	Embryos transferable	**C**	1910	C/B=	53.1%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	1910	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	559		
In vivo	Frozen	**I**	1053		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	1612	H+I+J+K=	
Number of frozen stored embryos		**M**	1351		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	65.3%	(I+K)/L=	

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE: 12 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **ITALY** ***A.E.T.E 2011***

Data collected by Dr. Giovanna Lazzari

Total number of approved E.T. teams in the country	
Number of teams providing data	

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	2039	B/A=	9.2
	Embryos collected	**B**	18856	C/A=	5.4
	Embryos transferable	**C**	11035	C/B=	58.5%
In vitro *(OPU)*	Nb of oocyte donors		61		
	Nb of OPU sessions		117		
	Nb of transferable embryos	**D**	373		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	308		
Total i*n vitro* embryos		**F**	681	=(D+E)	
Total number of transferable embryos		**G**	11716	=(C+F)	
Number of sexed embryos					
EMBRYO TRANSFER					
In vivo	Fresh	**H**	8500		
In vivo	Frozen	**I**	2000		
In vitro	Fresh	**J**	80		
In vitro	Frozen	**K**	1045		
Total embryos transferred		**L**	11625	H+I+J+K=	
Number of frozen stored embryos		**M**	2360		
% of *in vitro* embryos transferred		**N**	9.6%	(J+K)/L=	
% of frozen embryos transferred		**O**	10.7%	(I+K)/L=	

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE: 13 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **LUXEMBOURG** ***A.E.T.E 2011***

Data collected by Dr. Jacob Westphal

Total number of approved E.T. teams in the country	3
Number of teams providing data	3

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	86	B/A=	13.9
	Embryos collected	**B**	1196	C/A=	7.7
	Embryos transferable	**C**	665	C/B=	55.6%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	665	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	108		
In vivo	Frozen	**I**	313		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	421	H+I+J+K=	
Number of frozen stored embryos		**M**	715		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	74.3%	(I+K)/L=	

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE: 14 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **THE NETHERLANDS** ***A.E.T.E 2011***

Data collected by Drs. Tom Otter

Total number of approved E.T. teams in the country	
Number of teams providing data	

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	3499	B/A=	11.1
	Embryos collected	**B**	38938	C/A=	7.1
	Embryos transferable	**C**	24695	C/B=	63.4%
In vitro *(OPU)*	Nb of oocyte donors		254		
	Nb of OPU sessions		2649		
	Nb of transferable embryos	**D**	2686		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	177		
Total i*n vitro* embryos		**F**	2863	=(D+E)	
Total number of transferable embryos		**G**	27558	=(C+F)	
Number of sexed embryos			370		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	5240		
In vivo	Frozen	**I**	12975		
In vitro	Fresh	**J**	1995		
In vitro	Frozen	**K**	598		
Total embryos transferred		**L**	20808	H+I+J+K=	
Number of frozen stored embryos		**M**			
% of *in vitro* embryos transferred		**N**	12.5%	(J+K)/L=	
% of frozen embryos transferred		**O**	65.2%	(I+K)/L=	

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE : 15 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **NORWAY** ***A.E.T.E 2011***

Data collected by Dr. Eiliv Kummen

Total number of approved E.T. teams in the country	2
Number of teams providing data	2

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	19	B/A=	7.6
	Embryos collected	**B**	144	C/A=	6.6
	Embryos transferable	**C**	120	C/B=	83.3%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	120	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	36		
In vivo	Frozen	**I**	130		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	166	H+I+J+K=	
Number of frozen stored embryos		**M**	84		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	78.3%	(I+K)/L=	

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	75
Number of calves born from *in vitro* embryos	
Total	75

TABLE: 16 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **POLAND** ***A.E.T.E 2011***

Data collected by Dr. Jedrzej Jaskowski

Total number of approved E.T. teams in the country	4
Number of teams providing data	5

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	86	B/A=	8.5
	Embryos collected	**B**	729	C/A=	5.9
	Embryos transferable	**C**	510	C/B=	70.0%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	510	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	225		
In vivo	Frozen	**I**	285		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	510	H+I+J+K=	
Number of frozen stored embryos		**M**	247		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	55.9%	(I+K)/L=	

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

TABLE: 17 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **PORTUGAL** ***A.E.T.E 2011***

Data collected by Dr. Joao N Chagas e Silva

Total number of approved E.T. teams in the country	5
Number of teams providing data	1

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	34	B/A=	14.4
	Embryos collected	**B**	488	C/A=	9.1
	Embryos transferable	**C**	311	C/B=	63.1%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**	1007		
Total i*n vitro* embryos		**F**	1007	=(D+E)	
Total number of transferable embryos		**G**	1318	=(C+F)	
Number of sexed embryos			0		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	141		
In vivo	Frozen	**I**	49		
In vitro	Fresh	**J**	74		
In vitro	Frozen	**K**	55		
Total embryos transferred		**L**	319	H+I+J+K=	
Number of frozen stored embryos		**M**	250		
% of *in vitro* embryos transferred		**N**	40.4%	(J+K)/L=	
% of frozen embryos transferred		**O**	32.6%	(I+K)/L=	

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	107
Number of calves born from *in vitro* embryos	
Total	107

TABLE: 18 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **SPAIN** ***A.E.T.E 2011***

Data collected by Dr. Julio De la Fuente

Total number of approved E.T. teams in the country	10
Number of teams providing data	10

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	577	B/A=	10.2
	Embryos collected	**B**	5905	C/A=	4.4
	Embryos transferable	**C**	2528	C/B=	42.9%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**	0	=(D+E)	
Total number of transferable embryos		**G**	2528	=(C+F)	
Number of sexed embryos			118		
EMBRYO TRANSFER					
In vivo	Fresh	**H**	754		
In vivo	Frozen	**I**	1560		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**			
Total embryos transferred		**L**	2314	H+I+J+K=	
Number of frozen stored embryos		**M**	2687		
% of *in vitro* embryos transferred		**N**		(J+K)/L=	
% of frozen embryos transferred		**O**	67.4%	(I+K)/L=	

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	1180
Number of calves born from *in vitro* embryos	
Total	1180

TABLE: 19 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **SWITZERLAND** ***A.E.T.E 2011***

Data collected by Dr. Rainer Saner

Total number of approved E.T. teams in the country	6
Number of teams providing data	4

EMBRYO PRODUCTION					
In vivo	Flushed donors	**A**	467	B/A=	10.3
	Embryos collected	**B**	4832	C/A=	7.2
	Embryos transferable	**C**	3382	C/B=	70.0%
In vitro *(OPU)*	Nb of oocyte donors				
	Nb of OPU sessions				
	Nb of transferable embryos	**D**			
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**			
Total i*n vitro* embryos		**F**		=(D+E)	
Total number of transferable embryos		**G**	3382	=(C+F)	
Number of sexed embryos					
EMBRYO TRANSFER					
In vivo	Fresh	**H**	723		
In vivo	Frozen	**I**	2283		
In vitro	Fresh	**J**			
In vitro	Frozen	**K**	5		
Total embryos transferred		**L**	3011	H+I+J+K=	
Number of frozen stored embryos		**M**	2515		
% of *in vitro* embryos transferred		**N**	0.2%	(J+K)/L=	
% of frozen embryos transferred		**O**	76.0%	(I+K)/L=	

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	1550
Number of calves born from *in vitro* embryos	-
Total	1550

TABLE: 20 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **TURKEY** ***A.E.T.E 2011***

Data collected by Prof. Ebru Emsen

Total number of approved E.T. teams in the country	1
Number of teams providing data	1

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**		B/A=
	Embryos collected	**B**		C/A=
	Embryos transferable	**C**	0	C/B= %
In vitro *(OPU)*	Nb of oocyte donors			
	Nb of OPU sessions			
	Nb of transferable embryos	**D**		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total i*n vitro* embryos		**F**	0	=(D+E)
Total number of transferable embryos		**G**	0	=(C+F)
Number of sexed embryos			0	
EMBRYO TRANSFER				
In vivo	Fresh	**H**		
In vivo	Frozen	**I**	25	
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	25	H+I+J+K=
Number of frozen stored embryos		**M**	25	
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**	100%	(I+K)/L=

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	13
Number of calves born from *in vitro* embryos	
Total	13

TABLE: 21 EMBRYO TRANSFER ACTIVITY IN 2010

COUNTRY: **UK** ***A.E.T.E 2011***

Data collected by Dr. Ian Murphy

Total number of approved E.T. teams in the country	39
Number of teams providing data	36

EMBRYO PRODUCTION				
In vivo	Flushed donors	**A**	2527	B/A=
	Embryos collected	**B**		C/A= 5.9
	Embryos transferable	**C**	14903	C/B=
In vitro *(OPU)*	Nb of oocyte donors			
	Nb of OPU sessions			
	Nb of transferable embryos	**D**		
In Vitro *(Slaughtered donors)*	Nb of transferable embryos	**E**		
Total i*n vitro* embryos		**F**	0	=(D+E)
Total number of transferable embryos		**G**	14903	=(C+F)
Number of sexed embryos			0	
EMBRYO TRANSFER				
In vivo	Fresh	**H**	6598	
In vivo	Frozen	**I**	8361	
In vitro	Fresh	**J**		
In vitro	Frozen	**K**		
Total embryos transferred		**L**	14959	H+I+J+K=
Number of frozen stored embryos		**M**		
% of *in vitro* embryos transferred		**N**		(J+K)/L=
% of frozen embryos transferred		**O**	55.9%	(I+K)/L=

Number of E.T. calves born (2010)

Number of calves born from superovulated embryos	
Number of calves born from *in vitro* embryos	
Total	

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2010

I. EMBRYO PRODUCTION

(Data collected from 21 countries)

In vivo produced embryos (superovulation)*	
- number of flushed donors	17,694
- number of transferable embryos	117,813
- mean number per flushed donor	6,66
In vitro produced embryos:	
From OPU	
- number of OPU sessions	4,085
- number of transferable embryos	5,522
- mean number per session	1.35
From slaughterhouse collected ovaries	
- number of transferable embryos	1,633
Total *in vitro*	7,155
Total number of transferable embryos	109,646
Embryos sexed:	1,1664

* Specified data from two countries not available and not included.

(Hiemke Knijn, AETE, Chester, England 2011)

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY

IN EUROPE IN 2010

II. EMBRYO TRANSFERS

(Data collected from 21 countries)

	Number of embryos transferred
***In* vivo produced embryos**	109,414 (48,555 fresh / 60,859 frozen)
***In vitro* produced embryos**	5,661 (3,412 fresh / 2,249 frozen)
Total number of embryos transferred	114,976
Proportion of IVF embryos transferred	4.9%
Proportion of frozen embryos transferred	54.8%

(Hiemke Knijn, AETE, Chester, England 2011)

EMBRYO TRANSFER ACTIVITY IN OTHER SPECIES IN EUROPE IN 2010

Species	Embryo Production	Embryo Transfers	Countries
Sheep	273	446	Turkey Czech Republic Hungary
Swine			
Goat			
Horse	289	123	Czech Republic Hungary Italy Portugal

(Hiemke Knijn, AETE, Chester, England 2011)

INVITED LECTURES

GENOMIC ACTIVATION, FIRST STEPS OF EMBRYO DEVELOPMENT AND ENVIRONMENTAL EFFECTS

DURANTHON V
Biologie du Développement et Reproduction UMR1198

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Introduction

On a morphological point of view, mammalian early embryonic development's principal achievement is the formation of a blastocyst composed of an outer epithelial trophectoderm (TE) encircling a small group of cells, the inner cell mass(ICM) cells precursors of the fetus, and a fluid filled cavity the blastocoele. The TE is the first differentiated cell type of the embryo, it is a specialized tissue involved in initiation of feto-maternal interactions leading to implantation or attachment, and is the progenitor of the placenta. Although essential in function, the developmental period from fertilization to blastocyst formation seems thus relatively simple. It is characterized first by a series of cleavage divisions that give rise to smaller and smaller cells _the blastomeres_ while the whole embryo volume remains constant. During these divisions, the blastomeres first keep independent. The first important morphological event occurs with compaction; it is marked by an increase in cell to cell contacts between blastomeres driven by the establishment of adherens junctions. Whatever its timing that differs with species, it results in the formation of a morula with outer and inner cells that are the progenitors of TE and ICM respectively. Trophectoderm formation lies in compaction and establishment of cell polarity as reflected by the two main models of TE differentiation "inside-outside hypothesis" (Tarkowski and Wroblewska 1967) and "cell polarity model" (Johnson and Ziomek 1981) and molecular factors responsible for the decision to become ICM or trophectoderm are being identified. Very interestingly, it has been recently shown that these factors may differ between species and especially that the most studied mouse model may be particular regarding the regulatory circuitry determining ICM/TE identity (Berg et al. 2011). Blastocyst formation then relies on TE differentiation since ion and water transport through the trophectoderm are responsible for blastocoele formation (for review see Duranthon, Watson et al. 2008).

1.Embryonic genome transcriptional activation and reprogramming

Beside these relatively simple morphological events, the period of early mammalian development is in fact very complex on a molecular point of view. The challenge for the embryo is to manage the maternal-embryo transition. This involves of course the progressive transcriptional

activation of the embryonic genome together with the finely regulated elimination of the maternally encoded information (transcripts and proteins). Molecular complexity of early stages of development lies in the regulation of these two different kinds of genetic information: the maternal one and the embryonic one respectively, encoded by two different genomes. These two genetic information are regulated at two different levels: maternal information having been transcribed and stored as transcripts and proteins during oogenesis is regulated at post-transcriptional levels including regulation of maternal transcript translation and degradation and maternal protein storage and function, when embryonic information is mainly regulated at the transcriptional level. But embryonic genome transcriptional activation is not simply the reactivation of gametic genomes gene expression programs. Indeed one of the major functions of the oocyte cytoplasm while activating the transcription of the newly formed genome is to reprogram it. That is to epigenetically modify the two gamete-inherited genomes that are genomes from highly differentiated cells, into a unique totipotent (able to give rise to a whole individual) embryonic one. Such a function has consequences well beyond blastocyst formation, and concerns in fact full term development and adulthood. It supposes the involvement of maternal information into huge epigenetic modifications of the gamete-inherited and newly formed embryonic genome. These extensive epigenetic changes probably make the early embryo very acute to environmental conditions. Once reprogrammed, totipotency is a rather transient property : it is shared by the zygote and early blastomeres till the 16-32 cell stage in the mouse (Tarkowski et al. 2011). At the blastocyst stage, the embryo contains two different types of cells : the differentiated cells of the trophectoderm and the pluripotent cells (able to participate to each of the three embryonic lineages : ectoderm, mesoderm,endoderm,) of the inner cell mass.

Transcriptional activation of the embryonic genome is a very progressive process. In most species, the ability to transcribe is detected as soon as the one cell stage, but the stage when embryonic transcripts are necessary to the ongoing of development which corresponds to the major embryonic genome activation (EGA), varies with species. In the most studied model, the mouse one, this occurs as soon as the two cell stage (Flach et al. 1982). It however appears to be a very unique situation since in most species major EGA is delayed; in the bovine or the rabbit for example it only occurs at the 8-16 cell stage (Manes 1973) (Camous et al. 1986). This makes the period for embryonic genome modifications by maternal factors longer and more progressive. Some maternal transcripts synthesized during oogenesis are oocyte specific which means that the corresponding genes are no more transcribed in embryonic or adult tissues. In this case, mutations in the maternal genes but not the embryonic one induce a phenotype in the oocyte or embryo. The corresponding genes are thus true “maternal effect genes”. But other maternal transcripts are also expressed after the activation of the embryonic genome, a relay is thus ensured between maternally

and embryonically encoded transcripts for the same gene. While very few natural "maternal effects genes" mutants are available in mammals, targeted mutagenesis experiments mainly carried out in the mouse, have made it possible to specifically affect the maternally inherited transcripts. In this species, oocyte specific knock-out of candidate genes may be obtained using the transgenic mouse strain expressing the cre recombinase under control of an oocyte specific promoter (ZP3 promoter for example) (Lewandoski et al. 1997). The main functions encoded by maternal transcripts in the mouse have been recently reviewed by Li (Li et al. 2010). In non murine mammals however evidencing the function of maternal transcripts mainly rely on RNA interference experiments that provoke the down regulation of maternal transcripts but not their total inhibition, making the phenotype observed less obvious. According to data obtained in the mouse, the most common phenotype among mouse embryos with such mutations is an arrest or delay in cleavage period (Li et al. 2010). But, as soon as maternally encoded transcripts are involved in epigenetic modifications of the newly formed embryonic genome, they may affect longer term development. Although still scarce in the literature, there are now few evidences for long term effects of maternal information defaults. Such defaults have consequences far beyond the onset of embryonic genome transcription, although being directly related to alterations in the oocyte "quality" or developmental competence (Duranthon and Renard 2001). Probably the most demonstrative example for this has been reported several years ago by Erhardt et al. (Erhardt et al. 2003). These authors produced mouse oocytes devoid of maternal Ezh2 (Enhancer of zest 2) transcript. When fertilized by normal males, such oocytes produced heterozygous embryos, with one single active paternally inherited allele that is expressed as soon as the four cell stage. While hemizygous embryos with only one functional allele are viable and normal, experimental embryos devoid of the only maternal transcript were born with a reduced (about one third) size. This evidenced that maternal Ezh2 is involved in epigenetic modifications of the embryonic genome that have to take place before the four cell stage, but have long term consequences, interfering with fetal growth and resulting in new born reduced size. Such an experimental proof remains rare, it demonstrates however the potential long term effects of defaults in maternal transcript regulation. On the other hand, lot of data accumulated describing the effects of modifications of the oocyte environment on some candidate maternal transcripts. Despite the molecular basis for such effects of the environment on maternal transcripts content has not been elucidated yet, and especially whether it relies on alteration of transcript synthesis or stability, it may appear that such alterations have functional effects on embryo development.

2.Long term effects of oocyte and early embryo environment.

Interestingly, while early development has been obtained in rather diverse conditions - especially in vitro- that remained compatible with full term development, recent data have

accumulated pointing to the sensitivity of the early embryo to its environment and to long term effects of environmental modifications. In fact the DOHaD hypothesis (for Developmental Origin of Health and Diseases) (Barker et al. 2002) has initially emphasized the effects of maternal under nutrition during fetal development on long term adult diseases in the offspring. According to Barker's hypothesis, maternal malnutrition or placental deficiency led to fetal growth restriction, which disturbs organogenesis and results at adulthood into metabolic syndrome induced by external factors linked to the way of life such as obesity, aging and sedentarity. This is described as fetal or metabolic programming. DOHaD together with the Adaptative Predictive Response (Gluckman and Hanson 2006) implies that the fetus adapts to a suboptimal intrauterine environment, but at adulthood is no more able to face new environment it has not been programmed for, so that environmental changes increase the risk for cardiovascular and metabolic diseases in the offspring. More recent experimental data have now evidenced that pre and peri conceptional maternal nutrition and environment also may program the adult phenotype. Especially early preimplantation embryo appears as a target for environmental alterations. Long term effects of periconceptional environment have been demonstrated both in ruminants and in laboratory models (Chavatte-Palmer et al. 2008). In vivo alterations of this environment mainly result from alterations of maternal nutrition but also may result from maternal physiopathologies such as diabetes. In vitro studies provide opportunities to develop complementary mechanistic analyses of the molecular basis sustaining these long term effects. Different modifications of maternal environment have been analyzed.

Modifications often concern a still relatively wide window including the pre and periconceptional periods but more precise periods have also been targeted. First, it has been shown that maternal nutrition may affect gonad development and oogenesis of the fetus. In sheep for example, a nutritional restriction (50% decrease regarding the nutritional requirements) during the first 30 days after fertilization, resulted in a delayed ovarian development at 110 days of gestation (Rae et al. 2001). Maternal undernutrition affects cell proliferation and apoptosis gene expression in fetal ovary (Lea et al. 2006). Such a restriction thus affects progeny's gonad function.

But maternal environment may also have effects on oocyte and embryo quality. In the bovine for example, increasing the lipid supply during the periconceptional period reduces the number of small and medium follicles in the ovary. It does not affect the cleavage rate after fertilization, but increased the blastocyst rate and quality assessed by the increased number of cells both in the inner cell mass and the trophectoderm (Fouladi-Nashta et al. 2007). In the mouse, exposure of the oocytes in the ovary to an environment with high omega-3 polyunsaturated fatty acids by introducing them in the mother's diet during four weeks before fertilization, resulted in

altered mitochondrial distribution, calcium levels and increased reactive oxygen species in the oocyte. After *in vivo* fertilization, embryos from such oocytes have a decreased ability to develop to the blastocyst stage. Very interestingly such effects on embryo development are not observed after *in vitro* fertilization which points to fertilization as a period sensitive to oocyte/embryo early environment modifications (Wakefield et al. 2008).

In the rabbit, we have recently shown that a hyperlipidemic, hypercholesterolemic diet, when started before conception, induced gene expression alterations in the early embryos and intra-uterine growth retardation observed as soon as day 9 of gestation. At birth, the offspring are smaller than controls, but become heavier after weaning (Picone et al. 2011).

Another example of periconceptional programming is provided by the restriction of the supply of vitamin B12, folate and methionin in physiological ranges from mature female sheep's diet starting 8 weeks before conception and until 6 days post conception. Such a restriction had no effect on pregnancy rates and birth weights were normal. However, adult offspring were both heavier and fatter, insulin-resistant and had elevated blood pressure and altered immune responses especially in males (Sinclair et al. 2007). Alterations in DNA methylation status of the adult offspring were evidenced. Common phenotypic effects were observed in male rates in parallel studies (Maloney et al. 2011). Gametes and/or preimplantation embryos are thus programmable by maternal diet.

Oocyte maturation has been more precisely shown to be sensitive to programming. In the mouse, a hypoprotein diet administered to the females during 3.5 days before mating, does not affect gestation length, litter size, nor postnatal growth but induces anxious behavior, alterations of cardiovascular development and reduction in the number of nephrons in the kidney of offspring (Watkins et al. 2008a).

The preimplantation period of development has also been precisely targeted for programming in the studies performed by T. Fleming's team. Mice females were fed with a hypoprotein diet during the 3.5 days following fertilization. Such a diet leads to offspring with increased weight from birth, sustained hypertension, and abnormal anxiety-related behavior, especially in females. Transfer of blastocysts from such females to control recipient females does not prevent the phenotype of the offspring thus showing that preimplantation embryo is already determined to develop the phenotype (Watkins et al. 2008b). The functioning of extra embryonic tissues and especially the yolk sac has been shown to be affected by this treatment. In the rat, hypoprotein diet fed during the first 4.5 days of preimplantation development results in reduced insulin and amino acid levels and associated mild increase in glucose level in the maternal environment. This affects cell proliferation and results in modifications of the size of stem cell

lineages (Kwong et al. 2000). The long term consequences of such early environmental effects are mediated by epigenetic modifications of the embryonic genome.

3. Epigenetic modifications during early development make the embryo very sensitive to its environment:

Environmental modifications affect early gene expression in the embryo. In the mouse, diet induced obesity decreased IGF1R expression in the preimplantation embryo (Jungheim, Schoeller et al. 2010). In our rabbit model of hyperlipidemic hypercholesterolemic maternal diet, Adipophilin a protein involved in lipid droplets formation is overexpressed at the stage of embryonic genome activation in (Picone et al. 2011). Such short term environmental effects on gene expression imply epigenetic modifications of these genes. But more importantly these epigenetic modifications are responsible for long term environmental effects. In Human for example it has been recently shown that glycemic memory is associated with epigenetic changes of genes involved in vascular function. Very interestingly transient hyperglycemia results in alterations of the epigenetic status of these genes that remain even five years after the hyperglycemic period (Siebel et al. 2010). Whether environmental modifications occuring during oogenesis directly affect oocyte genome, or are mediated by post-transcriptional modifications of the stored maternal information that subsequently affect embryonic genome remains largely unknown. Whatever the case, and this is especially evidenced in the case of preimplantation embryo programming, embryonic genome is the end-target for epigenetic modifications of numerous genes involved in the regulation of the various physiological functions and responsible for long term effects of environmental modifications.

Early embryo appears especially sensitive to such environmental induced epigenetic alterations probably because preimplantation period is one of the two periods of huge epigenetic reprogramming during the life of an individual (the other one takes place in primordial germ cells with the erasure of inherited DNA imprinting before its replacement during gametogenesis). During the preimplantation development period, reprogramming differentiated gametic genomes into a totipotent embryonic one relies on extensive epigenetic modifications. DNA methylation represents the most studied one and the only known modification of DNA occurring in vertebrates. After fertilization the paternal pronucleus is rapidly demethylated in an active way that is before DNA replication. This active demethylation first evidenced in the mouse (Mayer et al. 2000) (Santos et al. 2002) and the bovine (Dean et al. 2001), appears well conserved between species, despite variations in its extent and timing (Park et al. 2007) (Reis e Silva, 2011). Cleavage period is then characterized by a progressive and passive demethylation of the embryonic genome. Both of these phases of demethylation are sensitive to environmental conditions. This has been evidenced by comparing demethylation kinetics in *in vivo* and *in vitro* developed embryos (Zaitseva et al.

2007) (Reis e Silva et al., 2011), (Reis e Silva et al. in preparation). Besides these very global analyses of the embryonic genome methylation level, modifications of the DNA methylation status of specific gene loci by embryo environment have also been reported: *in vitro* culture of mouse embryos resulted in the modifications of DNA methylation at specific epiallele loci affecting their expression during further development and resulting in adult phenotype modifications (Morgan et al. 2008).

Interestingly, a recent paper (Wakefield et al. 2011) evidenced the long term effects of mitochondrial function perturbations during preimplantation development. Reducing mitochondrial activity during this period reduced blastocyst development, and the ICM and trophectoderm cell numbers. Moreover it may affect placental and fetal development. In a mouse model, maternal diet induced obesity has been reported to alter mitochondria function and DNA content in the oocyte and early embryo (Igosheva et al. 2010). Since both mitochondria DNA content and mitochondrial metabolism can interfere with nuclear gene DNA methylation (Naviaux 2008) it may be that mitochondrial functions in the early embryo play a key role linking metabolic and environmental perturbations to epigenetic alterations and their long term effects.

Conclusion

Preimplantation period of development despite morphologically simple and apparently highly tolerant to environmental perturbations, is in fact very complex on a molecular point of view and particularly sensitive to subtle environmental changes that have long term effects on new born and adult phenotype. The molecular bases for such long term effects are being elucidated. Their analysis in the oocyte and at early stages has been yet largely impaired by the scarcity of the biological material available and progress in large scale epigenetic analyses of scarce material would be of great interest both for the understanding of such effects and for the control of the quality of embryo environment either *in vivo* or *in vitro*.

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THE ROLE OF ENDOMETRIUM IN BOVINE PREGNANCY ESTABLISHMENT

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Introduction

Assisted reproductive technologies (ART) have been the major tool towards increasing pregnancies in livestock industry. The contribution of embryo transfer has paved the way in increasing the bomber of calves borne alive in dairy and beef cattle. Despite the advancement in ART protocols and embryo transfer procedures, in some cases the pregnancy rates are still relatively low and higher rate of early embryonic mortality is reported after embryo transfer. From this, 40 % of total embryonic losses are estimated to occur between day 8 and day17 of pregnancy (Thatcher *et al.* 1994). The majority of this blame is mainly falls on the quality of the embryo and the endometrial receptivity of the recipient animal that is sheltering the fetus for 275-290 days. This can suggest the molecular genetic pathways of the recipients endometrium and the embryo that are crucial to implantation process and the mechanism of their interaction are barely understood (Wang & Dey 2006). Thus understanding of the molecular signals that are associated with endometrial receptivity could lead to the establishment of strategies to correct implantation failure and improve pregnancy rates (Dey *et al.* 2004). In addition, the methodologies to assess cattle endometrial receptivity is lacking although the qualities of embryo are mainly evaluated based on morphological parameter. To address this issue, more recently, we have investigated the possibility of using transcriptome profile of pre-transfer endometrial and/or embryo biopsies as predictors of recipients with receptive endometrium or embryo that could develop to term (El-Sayed *et al.* 2006, Salilew-Wondim *et al.* 2010). This strategy may be particularly relevant in the field of fertility as it gives non-invasive option for selecting cows of better endometrial receptivity and embryos of having higher developing potential. This review highlights the significance of endometrium for successful pregnancy establishment, the molecular and genetic markers of endometrial receptivity and the embryo-uterine dialogue.

Endometrial receptivity and embryo implantation

The endometrium is the inner layer of the uterus consisting of the luminal epithelium, uterine glands and connective tissue and it plays a crucial role in early embryo-maternal communication and pregnancy establishment (Tabibzadeh & Babaknia 1995, Tabibzadeh 1998,

Wolf *et al.* 2003, Spencer *et al.* 2004, Bauersachs *et al.* 2005). Endometrium receptivity describes a period in which the endometrial epithelium is functionally and structurally ready in terms of ovarian steroid to allow the embryo to attach, adhere, penetrate, and form placenta that will provide an interface between the conceptus and the maternal circulation (Aplin 2000, Ghosh & Sengupta 2004). In fact endometrial receptivity is not a single event but it consists of a set of sequential and complex events that can allow the embryo to adapt to the new environment (Bergh & Navot 1992, Martin *et al.* 2002). For optimal results in assisted reproductive technology, it is critical to recognize the time of embryo transfer that would best corresponds with the implantation time. Therefore, assessment of endometrial receptivity is required to identify endometrium that will be suitable for assisted reproduction technology. However, the mechanism of uterine receptivity is a biological mystery that remains to be explored (Makker & Singh 2006).

Pretransfer endometrium gene expression and pregnancy success

The intrinsic factors associated with the aberrant gene expression in the uterine endometrium could be one of the major causes of pregnancy failure in cattle. However, selection of those cows of adequate endometrial receptivity based on the gene expression pattern has been a greater challenge. Therefore, selecting recipients having adequate endometrial receptivity based on the gene expression pattern may increase the number of calves borne following embryo transfer. In an effort to gain detailed insights whether the endometrial and embryo gene expression pattern during the time embryo transfer has a direct effect on the upcoming pregnancy success, we compared the pre-transfer endometrial gene expression of heifers eventually resulted in calf delivery and those resulted in no pregnancy using endometrial samples collected during days 7 and 14 of the oestrous cycle (Salilew-Wondim *et al.* 2010). The most noticeable findings of that study were, heifers resulted in successful pregnancy and calf delivery were molecularly distinguishable from those resulted in no pregnancy at day 7 of the oestrous cycle (Figure 1). This may suggest that the occurrence of different molecular events in the endometrium whose expression is either induced or turned off in the expectation of blastocysts arrival into the uterus. Among those, the expression levels of solute carrier cluster, cell division cycle clusters, adaptor-related protein complex, kinesin clusters, leucine-rich repeats, coiled domains, ATPases, protein kinases and phosphatase clusters, transmembrane proteins, junctional adhesion molecules, and integrins were higher in endometrium of heifers resulted in successful pregnancy and calf delivery. However, the expression levels of cluster of differentiation molecules, chemokine molecules, homeobox genes, collagen families, S100 calcium binding protein, and zinc finger proteins were found to be lower in those heifers resulted in successful pregnancy and calf delivery. Here the fundamental question to be raised is what contributes for higher transcriptome abundance differences and transcriptomic function

alteration in heifers that resulted in successful pregnancy and calf delivery group versus no pregnancy group at day 7 than day 14 of the estrous cycle. Although further experimentation is necessary to answer this question, the higher gene expression differences between those two groups of heifers at day 7 than day 14 of the estrous cycle may be attributed to the degree of endometrial response to the circulating progesterone.

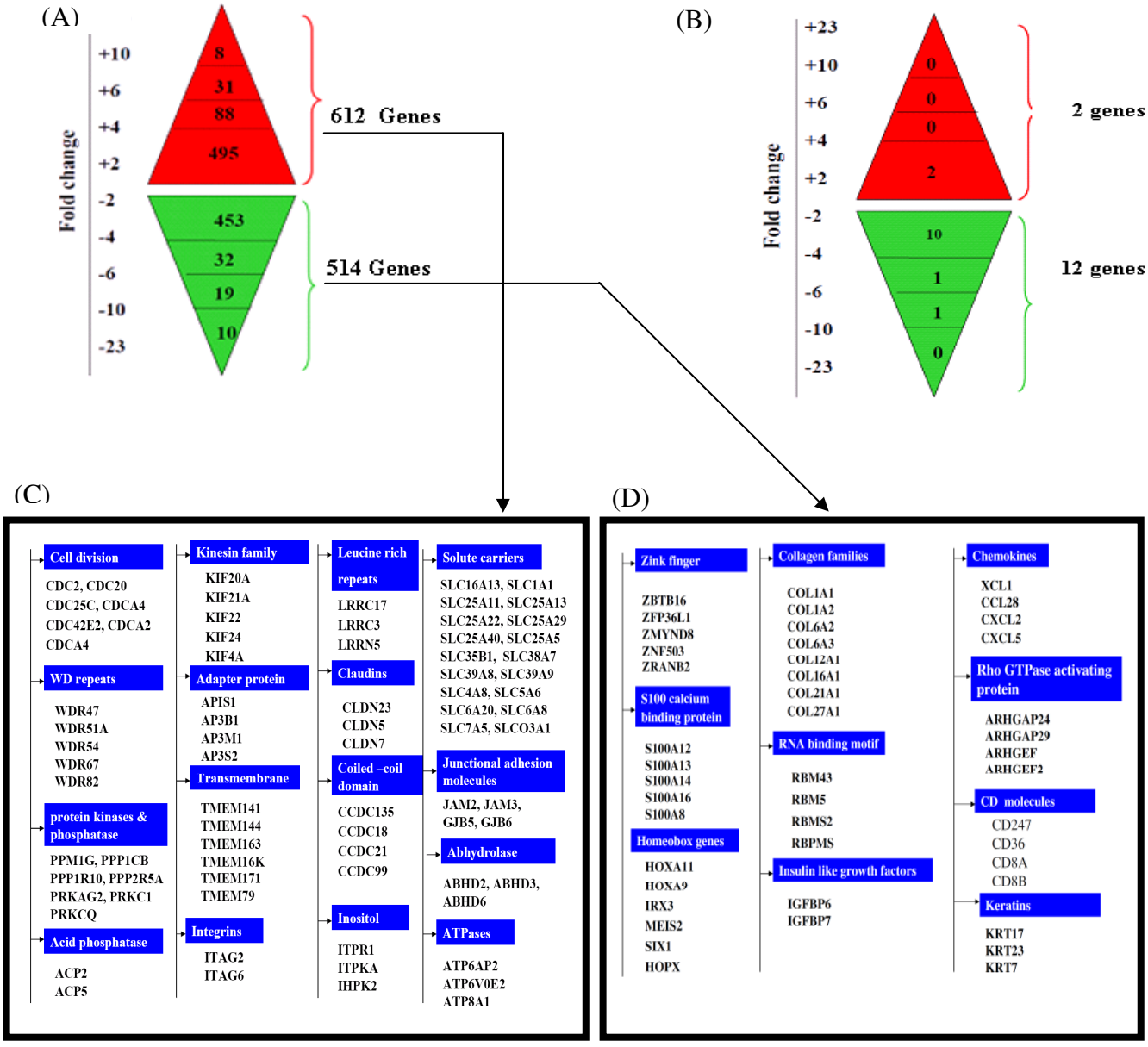

Figure 1:
Pre-transfer endometrial transcriptome alteration between heifers that resulted in calf delivery and those resulted in no pregnancy at day 7 (A) and day 14 (B) of the estrous cycle. The number and fold change distribution of genes elevated (red colors) downregulated (green) in heifers that resulted in calf delivery compared to and those resulted in no pregnancy. (C) Gene clusters exhibited increased expression level or decreased expression level (D) in heifers that resulted in calf delivery compared to those resulted in no pregnancy at day 7 of the estrous cycle.

In the absence of the embryo, the endometrium undergoes dynamic biomolecular alteration as it proceeds from one phase of the oestrus cycle to others. These changes can be correlated to different functions of the endometrium at specific phases of the oestrous cycle. For instance, at later stages of oestrus, the bovine endometrium is endowed with genes believed to be involved in cell-to-cell adhesion, cell motility and extracellular matrix but, 12 days post oestrous, the same endometrium is enriched by genes required for transport of proteins and ion (Bauersachs et al. 2005). Similarly, it has shown that the human endometrium at the secretory phase is enriched by genes required for cholesterol trafficking and signal transduction (Kao et al. 2002). Studies on Rhesus monkey endometrium also indicated that the expression level of certain genes including PLK, SAT2, SLPI and MT1G to be higher in proliferative phase compared to the secretory phase (Ace & Okulicz 2004). Similarly, the diestrous endometrial transcriptome dynamics of heifers that resulted in calf delivery or no pregnancy was investigated between day 7 and 14 post oestrus (Salilew-Wondim *et al.* 2010). Thus, the temporal transcriptome dynamics revealed a total of ~1800 genes to be differentially expressed between day 7 and 14 of the oestrous in heifers resulted in the calf delivery group. Those abundantly expressed at day 7 were found to be involved in various biological processes including, cellular material transport, metabolic process, phosphorylation, post-translational protein modification. On the other hand, immune responses and apoptotic process were among the main biological processes overrepresented in those genes whose transcript level was increased in at day 14 of the estrous cycle. In the same study, the endometrial transcriptome alteration in those heifers that resulted in no pregnancy has shown ~200 transcripts to be differentially expressed between day 7 and 14 of the estrous cycle. In spite of the endometrial response to the pregnancy, 121 genes were commonly differentially expressed between day 7 and14 in both heifer groups (Figure 2). This may suggest there are certain endometrial genes which can change their expression irrespective of the receptivity of the endometrium. Those genes that were elevated at day 7 were found to be involved in regulation of transferase activity, protein kinase activity, phosphorylation, protein metabolic process and phosphate metabolic process. However, those increased in day 14 of the oestrous cycle were found to be involved mainly in immune response, cytoskeleton organization and biological adhesion.

Endometrium as embryo sensor: molecular and biochemical changes of the endometrium during and post embryo implantation

The endometrium provides an optimum and favorable environmental condition for the incoming embryo during and after implantation by maintaining the hormonal control. Thus, the ovarian hormone induces the changes that could result endometrial maturity and receptivity (De Ziegler *et al.* 1993, De Ziegler *et al.* 1994, De Ziegler 1995, De Ziegler *et al.* 1998). This suggests

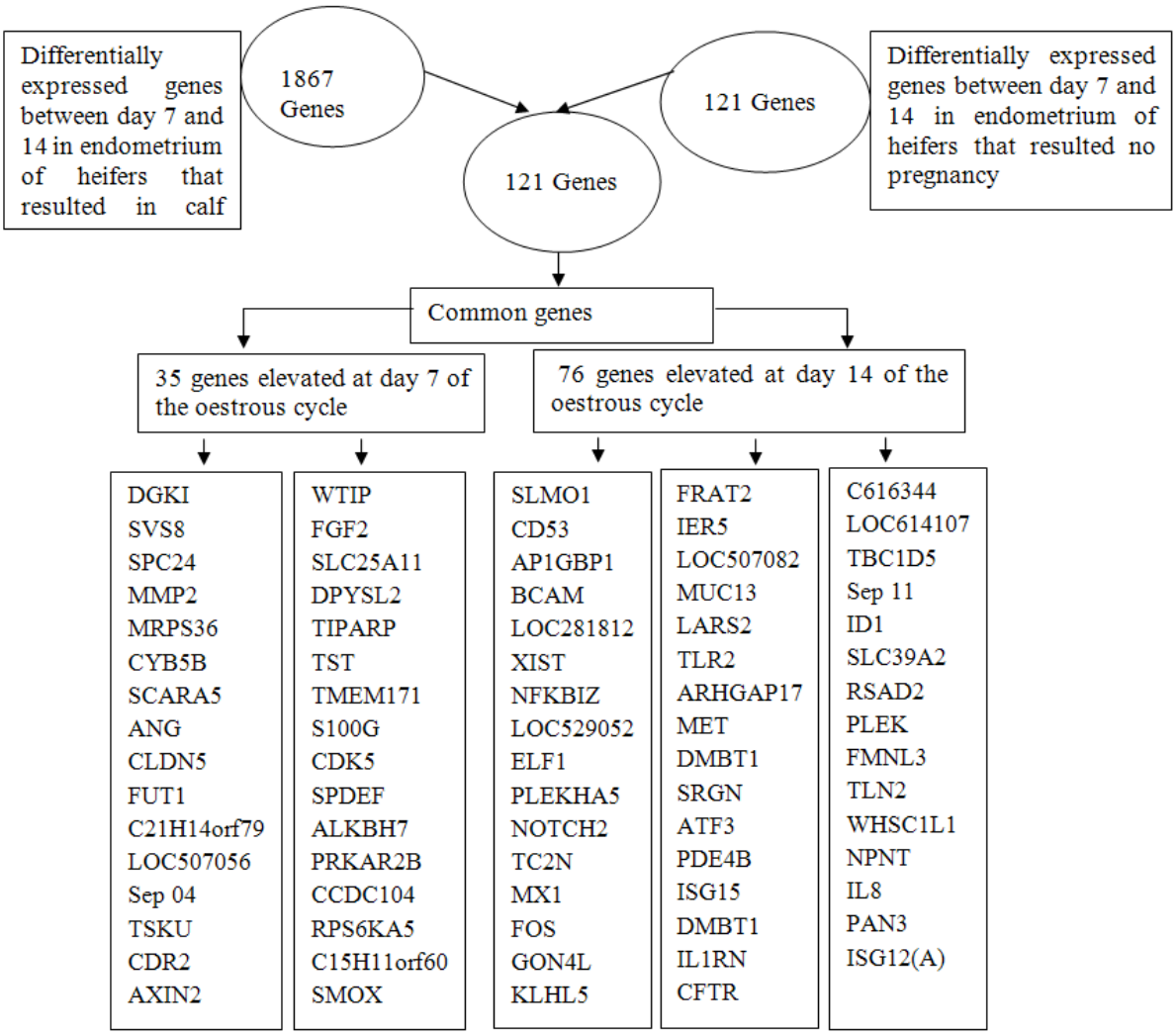

Figure 2:
Genes elevated at day 7 or 14 of the estrous cycle both in heifers that resulted in calf delivery and in no pregnancy.

that, successful embryo implantation is mainly depend on embryonic and maternal endocrine, paracrine and autocrine modulators and a failure in one of these could affect the embryo and uterine dialogue consequently resulted in embryonic losses (Diedrich *et al.* 2007). Furthermore, evidences have shown that several molecules of endometrial or/and embryo origin are required to establish the dialogue between the conceptus and the maternal environment (Glencross *et al.* 1973, Tabibzadeh & Babaknia 1995, Benson *et al.* 1996, Spencer *et al.* 1996, Tabibzadeh 1998, Paria *et al.* 2000, Paria *et al.* 2001, Paria *et al.* 2002, Riesewijk *et al.* 2003, Dey *et al.* 2004, Giudice 2004, Bauersachs *et al.* 2006, Wang & Dey 2006, Spencer *et al.* 2007b, Spencer *et al.* 2008, Bauersachs *et al.* 2009, Boomsma *et al.* 2009). Here the principal question is what is the mechanism that endometrium senses the incoming embryo? To what extent the endometrium is hospitable or hostile to the incoming embryo? Whether hospitality or hostility of the endometrium towards embryo dependant on the source of embryo? In fact to clearly address those questions and to reach at logical

conclusion, it inevitable to conduct series of experiments to gather quantitative and qualitative data. Recently, Bauersachs *et al.* (2009) have evaluated whether the endometrium behaves differently to the different source of embryos. In that study, comparison of transcriptome abundance in endometrium after transfer of somatic nuclear transfer (SCNT) and in vitro fertilized embryos showed differences in mRNA profiles to be greater in the SCNT group than in the IVF embryo transfer endometrium group. This may give a clue that endometrium reacts differently depending on the origin of the embryo.

Embryo triggers the endometrium biochemical and physiological changes

As the embryo enters in the uterus, it undergoes functional and morphological changes and after 14-17 days, the bovine conceptus is enlarging and produces Interferon-τ (IFNT) (Naivar *et al.* 1995, Bazer *et al.* 1997, Spencer *et al.* 2008) which binds to the endometrial type-I IFN receptors (IFNAR1 and IFNAR2) in limunal and superficial glandular epithelial cells of the endometrium (Li & Roberts 1994). IFNT initiates antiluteolytic and is believed to reduce the prostaglandin F2α in the luminal and glandular epithelial cells of the uterus by stimulating consistent production of progesterone by the ovarian corpus luteum (Naivar *et al.* 1995, Spencer *et al.* 1995, Rosenfeld *et al.* 2002, Spencer *et al.* 2007a, Spencer *et al.* 2008). Thus along with progesterone IFNT stimulate a several array of genes including many type I IFN-stimulated genes in endometrial stroma and glandular epithelium using signal transducer and activator of transcription 1 (STAT1)-dependent pathways. In addition, Spencer *et al.* (2008) proposed that in early pregnancy period of ewe, IFNτ activates CST3, CTSL, LGALSIS and WNT7A in luminal epithelium and superficial glands however, in the later stages of pregnancy, IFNτ activates several genes including STAT1, STAT2, ISG15, B2M, MIC, OAS, RSAD2 and INFIH.1. Similarly, Naivar *et al.* (1995) identified endometrial proteins which have an approximately 8, 16, and 28 kDa sizes and the 16 kDa was found to be sharing epitopes with human ubiquitin cross-reactive protein and this protein was called bovine UCRR (Austin *et al.* 1996). In humans the embryonic chorionic gonadotropin believed to modify the endometrium for embryo implantation (Ghosh & Sengupta 2004). Similarly, infusion of hCG that mimics blastocyst transit in the baboon, has proven to modulate the uterine environment prior to implantation (Fazleabas *et al.* 1999). In addition, the embryonic IL-1 believed to increase the expression of integrins in the endometrial epithelial cell (Simon *et al.* 1997). Apart from these, the embryonic IL-1, PAF, PGE, spermine–spermidine, proteases can alter the state of endometrium towards blastocyst implantation (reviewed by Ghosh & Sengupta 1998) . Thus, these and other data can indicate that the blastocyst stage embryos can directly or indirectly influence endometrial receptivity and implantation.

Blastcyst gene expression as predictors of pregnancy success

The genetic constitution of the blastocyst is one of the major components for successful implantation. Thus, the blastocyst needs to be enriched with transcripts and molecular signals required for controlling cell differentiation and maternal recognition of pregnancy (Spencer et al. 2008). In addition, in an attempt to investigate the gene expression pattern of in vitro produced blastocyst that develop to terms, El-Sayed et al. (2006), identified the relative abundance of COX2, CDX2, ALOX15, BMP15, PLAU and PLAC8 to be associated with embryos that resulted in calf delivery. Further investigations of the gene expression difference between in vivo derived blastocysts that resulted in calf delivery and no pregnancy evidenced the direct association of transcript abundance with developmental competence (Salilew-Wondim *et al.* 2010). Accordingly the transcript level of *SPAG17 (PF6), UBE2D3P, DFNB31, AMD1, DTNBP1NUP35, GDE1 (MIR16), ARL6IP, NUDT2, FAM161A, LGMN, LEO1, CCL16AP2B1, PTDSS1* and HSPA8 was higher but the transcript level of *SGK1, GBF1, FADS1, KRT8, RIF1, GART, DTX2, WDR26, RNF34*, *KPNA4, ARL8B, RYBP* and *WDR13* was lower in embryos that resulted in calf delivery compared to no pregnancy groups. Further, bioinformatics analysis on those differentially expressed genes indicated that those genes are involved in different biological and molecular functions, namely, protein binding, transferase activity, chemokine activity and cell to cell signaling, signal transduction, physiological response to stimulus, protein/mRNA transport, and regulation of transcription, adenosylmethionine decarboxylase activity and apoptosis. Similar investigation has been conducted in human showing the relative abundance of transcripts involved in cell adhesion and cell communication to be different between implantative and non implantative embryos (Jones *et al.* 2008).

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OOCYTE COMPETENCE: AN IMPORTANT FACTOR FOR PREPARING EMBRYO DEVELOPMENT

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Introduction

In modern animal agriculture, with increasing milk production there is a continuous decline in the fertility of dairy cows leading to higher economic loss (Macmillan et al. 1996). This decline in fertility can be explained by management changes within the dairy industry and also negative genetic correlations between milk production and reproduction. One of the primary mechanisms that depresses fertility in lactating cows is abnormal preimplantation embryo development, which that may be a result of poor oocyte quality (Snijders et al. 2000, Lucy 2007). Oocyte developmental competence is defined as the ability of an oocyte to resume meiosis, to cleave following fertilisation, to develop to the blastocyst stage, to induce a pregnancy and bring offspring to term in a good health (Krisher 2004, Sirard et al. 2006). This competency is acquired gradually during the course of folliculogenesis as the oocyte grows and its companion somatic cells differentiate (Eppig et al. 1994). Many factors have been shown to affect the oocyte's developmental potential, including follicle size (Lonergan et al. 1994), health of the follicle (Blondin & Sirard 1995, Vassena et al. 2003), phase of follicular wave (Hagemann 1999, Machatkova´ et al. 2004), hormonal stimulation (Blondin et al. 2002; for review Sirard et al. 2006), maturation environment (Warzych et al. 2007; for review Sutton et al. 2003), season (Al-Katanani et al. 2002, Sartori et al. 2002), nutrition (Fouladi-Nashta et al. 2007) and age (Rizos et al. 2005). Although previous studies support the notion that oocyte competence depends on multiple factors, it remains difficult to draw clear and reliable criteria for oocyte selection.

Parameters of oocyte competence

Morphological assessment of oocytes based on thickness, compactness of the cumulus investment and the homogeneity of the ooplasm (Gordon 2003) is a relatively popular and convenient way of evaluating oocyte quality in practice. However, results derived from this non-invasive approach are often conflicting, largely due to subjectivity and inaccuracy. Morphological evaluation alone is insufficient to distinguish competent oocytes that have the ability to bring about full-term pregnancy (Lonergan et al. 2003, Coticchio et al. 2004, Krisher 2004). With the urgent need for establishing non-invasive and non-perturbing means for oocyte selection, the brilliant cresyl blue (BCB) staining test has been successfully used to differentiate oocytes with different developmental capacity in various species, including pig (Ericsson et al. 1993, Roca et al.1998, Wongsrikeao et al. 2006), goat (Rodriguez-Gonzalez et al. 2002) and cattle (Alm et al. 2005,

Bhojwani et al. 2007). During the course of their growth, immature oocytes are known to synthesise a variety of proteins, including glucose-6-phosphate dehydrogenase (G6PDH; Mangia & Epstein 1975, Wassarman 1988). The activity of this protein is decreased once this phase has been completed and oocytes are then likely to have achieved developmental competence (Wassarman 1988, Tian et al. 1998). BCB is a dye that can be degraded by G6PDH (Ericsson et al. 1993, Tian et al. 1998); thus, oocytes that have finished their growth phase show decreased G6PDH activity and exhibit cytoplasm with a blue colouration (BCB^+), while growing oocytes are expected to have a high level of active G6PDH, which results in colourless cytoplasm (BCB^-). In our previous studies, it has been shown that oocytes screened based on BCB staining differ in their developmental potential to reach blastocyst stage (Alm et al. 2005) and efficiency in utilisation for somatic cell nuclear transfer (Bhojwani et al. 2007). Moreover, oocytes screened with BCB staining were reported to differ in various oocyte quality markers like cytoplasmic volume and mitochondria DNA copy number (El-Shourbagy et al. 2006). However, little is known about the molecular and the subcellular characteristics of these oocytes.

Investigation on gene expression in oocytes before maturation

The success of in vitro production of bovine transferable blastocysts using oocytes aspirated from slaughterhouse ovaries does not exceed 40–50%. Various studies have shown the quality of the oocyte to be the main determinant of blastocyst rate, while the culture environment affects their quality (Rizos et al. 2002, Lonergan et al. 2003). Therefore, selection and further use of good quality or developmentally competent oocytes is vital for the success of various embryo technologies. The use of BCB staining based on the presence of active G6PDH in immature oocytes has proven to be efficient tool to screen developmentally competent or incompetent oocytes for various species including cattle (Alm et al. 2005, Bhojwani et al. 2007). A previous study further evidenced differences in subcellular organisations and transcript abundance between the two oocyte groups. In terms of biological processes, the expression profiles of BCB^+ oocytes were markedly different from those of BCB^- ones. The majority of expressed genes in BCB^+ oocytes are associated with regulation of the cell cycle (NASP, MLH1, PRC1, UHRF2, UBE2D3, CCNB1, MPHOSPH9, CETN3, ASPM, NUSAP1 and AURKA), transcription (SMARCA5, ZFP91, ZNF519, ZNF85, HMGN2, PA2G4, STAT3, DNMT1 and FANK1) and translation (EEF1A1, RPS27A, RPS14, RPS15, RPS29, RPL18A, RPL9 and RPL24); while BCB^- oocytes encoded genes controlling ATP synthesis (ATP5A1), mitochondrial electron transport (FL405) and calcium ion binding (S100A10). Numerous factors involved in cell cycle regulation have been more recognised in BCB^+ than BCB^- oocytes. Among these cell cycle regulators, a NASP was first identified as a nuclear-associated protein in rabbit testis (Welch & O'Rand 1990, Welch et al. 1990). This gene has high homology with Xenopus histone-binding protein, N1/N2, which is expressed in oocytes (Kleinschmidt et al.

1986, Kleinschmidt&Seiter 1988). NASP is an H1 histone-binding protein that is cell cycle regulated and occurs in two major forms: tNASP, found in gametes, embryonic cells and transformed cells; and sNASP, found in all rapidly dividing somatic cells (Richardson et al. 2000). Moreover, it was strongly expressed in mouse embryos developed under non-blocking culture conditions in which embryos do not exhibit developmental arrest at the two-cell stage; however, the function of this transcript in early embryonic development remains unknown (Minami et al. 2001). NASP was one of the genes with increased expression in very fast moving bovine oocytes, which showed higher blastocyst rate compared with the slow groups after dielectrophoretic separation (Dessie et al. 2007). It is not surprising that cell cycle regulator genes category is the one of the largest highly expressed transcripts in BCB^+ oocytes. The embryo has to divide thrice to reach maternal zygotic transition (MZT) in conditions of very low transcription (Barnes & First 1991). Therefore, the competent oocyte must store enough mRNA coding for cell cycle proteins like CCNB1 (Tremblay et al. 2005) to ensure that these proteins will not be limiting the embryo progression. Both the assembly of transcriptional machinery and organisation of appropriate chromatin structure are critical for establishing the programme of early mouse development shortly after fertilisation (Sun et al. 2007). Changes in chromatin structure are thought to play an important role in reprogramming gene expression during zygotic genome activation (ZGA) (Schultz & Worrad 1995, Kanka 2003). For example, an apparent increase in histone acetylation accompanies the one- to two-cell transition in the mouse (Sarmento et al. 2004). Chromatin remodelling enzymes belong to the SNF2 family of DNA-dependent ATPases, all of which have a helicase-like ATPase domain (Henikoff 1993). The SWI/SNF ATP-dependent chromatin remodelling complexes are example of these families and SMARCA5 represents one of its members. Mammalian SWI/SNF-related chromatin remodelling complexes regulate transcription and are good candidates for being involved in ZGA in mice (Bultman et al. 2006). The expression of SMARCAL1 as another member of this family was increased in eight-cell embryos compared with MII oocytes, which suggest a potential role in regulation of embryonic genome activation (Misirlioglu et al. 2006). In addition, the early cleavage stages can dramatically affect embryo development (Magnani & Cabot 2007). Homozygous SMARCA4 knockout mouse embryos arrest during pre-implantation development (Bultman et al. 2000). Several other subunits of SWI/SNF-related complexes, often referred to as BRG1-associated factors, have also been knocked out and confer periimplantation lethality as well (Klochendler-Yeivin et al. 2000, Guidi et al. 2001). Consistent with this, greater mRNA abundance (6.7-fold change) of the SMARCA5 transcript was detected in BCB^+ (with higher developmental competence) when compared with BCB^- oocytes. Alterations in the expression of some of genes encoded chromatin regulatory factors in rhesus monkey oocytes of different developmental

potentials suggest that the expression of such transcripts could provide useful markers of oocyte quality (Zheng et al. 2004).

Studies on cytoplasmic maturation in competent oocytes

The bovine oocyte, zygote and embryo have a profound need for protein synthesis. However, the mRNA transcripts for these proteins are not synthesised throughout development, but rather during specific phases (Hyttel et al. 2001). In mammals, synthesis of RNA, up to 60–65% of which is ribosomal (rRNA), increases during oocyte growth and reaches a peak at the beginning of follicular antrum formation (Wassarman & Kinloch 1992). This is in accordance with our investigation concerning meiotic configuration in BCB^- oocytes. These oocytes with insufficient cytoplasmic maturation, under the control of high G6PDH activity, and in the end of oocyte growth showed a proportion of 21.4% with morphological features for rRNA synthesis – nucleoli. In contrast, in BCB^+ oocytes only a small proportion (1.8%) showed germinal vesicles with nucleoli. This process of nucleolus remodelling in GV-containing oocytes is a marker for the finished r-RNA synthesis for the establishment of sufficient ribosomes for the following protein synthesis during the final oocyte maturation after GVBD. In our previous studies, we found an increased level in protein synthesis during final oocyte maturation after GVBD, not before (Tomek et al. 2002a, 2002b). Elongation factor 1a is a component of the eukaryotic translational apparatus and it is also a GTP-binding protein that catalyses the binding of aminoacyl tRNAs to the ribosome (Tatsuka et al. 1992). The tRNA carries the amino acid to the ribosome, which is then used in protein synthesis, thereby inferring a crucial role for this factor in the translation process in protein biosynthesis. Acquisition of high developmental capacity in mammalian oocytes is dependent on high rates of RNA and protein synthesis, imprinting processes and biogenesis of organelles such as mitochondria (Eichenlaub-Ritter & Peschke 2002). Consistent with this, oocytes with greater developmental potential (BCB^+) showed higher mRNA transcript abundance for RPS27A and EEF1A1 that represent members of ribosomal and translation related genes respectively. Collectively, it is possible to conclude that BCB^+ oocytes have greater stores of cell cycle, transcription and protein biosynthesis transcripts that could be used for resuming meiosis (Tatemoto & Horiuchi 1995) and supporting maternal to zygotic transition (Hyttel et al. 2001). This is in accordance with the results obtained with respect to the developmental competence of BCB^+ and BCB^- oocytes. Concerning the activity of cell cycle proteins in oocytes, it has been shown previously that maturing bovine oocytes posses the highest phosphorylation of MAPKs in MII and of Akt in MI stage (Tomek & Smiljakovic 2005, Bhojwani et al. 2006). Furthermore, it has been shown that these phosphorylations are tightly correlated with the activities of the kinases. Therefore, from our observations, it can be concluded that BCB^+ GV stage oocytes have a higher basal activity regarding MAPK and Akt, which probably positively influences their developmental competence

and which is well reflected by corresponding gene expression. The reduced developmental capacity of early embryonic development has been associated with mitochondrial dysfunction and low ATP in mammalian oocytes and embryos (Keefe et al. 1995, Barnett et al. 1997, Van Blerkom et al. 1998, Van Blerkom 2004). Recently, the amount of mitochondrial DNA and transcripts has been quantified in bovine oocytes and embryos (May-Panloup et al. 2005) showing that bovine oocytes that failed to cleave contained significantly lower transcripts implicated in mitochondrial biogenesis. A global down-regulation of mitochondrial transcripts has been reported in human compromised oocytes and embryos (Hsieh et al. 2004). In the pig, competent BCB^+ oocytes contain more copies of mtDNA and are more likely to be fertilised than incompetent BCB^- oocytes (El-Shourbagy et al. 2006). However, supplementation of BCB^- oocytes with mitochondria from BCB^+ oocytes, and subsequent improved fertilisation outcome, again demonstrates the association between mitochondrial number and fertilisation outcome. Mouse BCB^+ oocytes gained better cytoplasmic maturity than BCB^- oocytes as determined by a higher intracellular glutathione (peroxidase 1) level, fully polarised mitochondrial distribution (most of mitochondria aggregated in the oocyte hemisphere around the MII spindle). In this study, it is remarkable that oocytes with high G6PDH activity (BCB^-) had an increased level of mitochondrial fluorescence intensity and up-regulation of mitochondrial transcripts (ATP5A1 and FL405) compared with BCB^+ oocytes. One can speculate that the reason for the higher fluorescence intensity of labelled mitochondria in BCB^- oocytes is likely the increased respiratory activity to provide ATP for still unfinished processes in cytoplasmic maturation. In a recent study, incompetent (BCB^-) oocytes exhibited a delay in mtDNA replication due to the delayed onset of expression of their nuclear-encoded replication factors and the oocyte attempts to rescue this during the final stages of maturation. Consequently, oocyte competence in terms of mtDNA replication and composition is not fully synchronised and will result in either failed fertilisation or developmental arrest (Spikings et al. 2007). In addition, it could be possible that the higher level of mitochondrial fluorescence intensity in BCB^- oocytes may be due to increased oxidative stress in these oocytes.ATP5A1 is a nuclear-encoded gene whose protein contributes to the overall function of the ATP synthase and it is the universal enzyme for cellular ATP synthesis (Pedersen 1994). It has been reported that null mutations in 3-subunit of mitochondrial ATP synthase gene in Drosophila lead to embryonic death (Kidd et al. 2005). ATP6V1E1 transcript was up-regulated at two-cell block mouse embryos (Jeong et al. 2006). From the above-mentioned facts, it is clear that alterations in mitochondrial distribution, DNA replication, copy number and transcripts may lead to overall dysfunction for the mitochondria and influence the ability of embryos to scavenge free radicals and also induce an oxidative stress response, which contributes to impaired development. It seems also that the competency of oocytes is highly

dependent on distinct set of genes mainly regulating transcription, translation, cell cycle, chromatin remodelling and mitochondrial machineries which may interact to fulfil this task.

Function of cumulus and granulosa cells during oocyte maturation

The oocyte and their surrounding cumulus and granulosa cells stay in close contacts to each other via gap junctions. These direct contacts serve for bidirectional exchange of regulatory molecules and metabolites between the oocytes and their surrounding cells which is required for the development of both cell types (Lingenfelter et al. 2008, Brower & Schultz 1982, Larsen et al. 1986, Driancourt & Thuel 1998, Vozzi et al. 2001). Therefore, the dynamic partnership between the oocyte and its cumulus and granulosa cells leads the oocyte to complete its maturation.

It has been suggested that the oocyte with completion of growth and RNA synthesis has to pass a stage of the so-called prematuration before final maturation. In vivo, this prematuration takes place during the preovulatory development. One of the major factors influencing oocyte developmental competence seems to be the grade of atresia in the surrounding follicle cells (Opiela et al. 2008). However, visible signs of early atresia in the follicle cells (e.g. slightly expanded cumulus cells) may not be necessarily detrimental to the oocytes, but it may promote the oocytes to acquire developmental competence (Hazeleger & Stubbings 1992; Blondin & Sirard 1995; De Wit & Kruip 2001; Bilodeau-Goeseels & Panich 2002). This suggests that a slight degree of atresia in the follicle acts as a prematuration-like effect on the oocyte development and growth (Hyttel et al. 1986; Sirard et al. 1999; Hendriksen et al. 2000). The molecular mechanism of apoptosis process is considered as the underlying causes of follicular atresia (Hsueh et al. 1994). Although several studies investigated the relationship between apoptosis of follicular cells and its use as a marker for the oocyte quality, they are still incomplete and contradictory. To understand the occurrence of apoptosis related to the oocyte developmental competence it is important to detect different stages of apoptotic changes in follicular cells using a combination of different techniques including caspase-3 activity, Annexin-V and TUNEL assay.

We showed that the developmental competence of bovine oocytes was influenced positively by a slight increase of apoptotic granulosa cells (BCB+:17.0% vs. BCB-: 10.9%) in the follicle and a higher activity of the enzyme caspase-3 in granulosa cells. Cumulus cells of BCB+ oocytes were also characterized by an increased caspase-3 activity, but not by an increased manifested apoptosis detected by TUNEL-staining (BCB+: 4.9% vs. BCB-:4.4%). Therefore, moderate apoptotic changes in the follicle seem to support the prematuration-like changes of the oocytes, while oocytes of non atretic follicles are blocked in their maturation (Hendriksen et al. 2000; Zeuner et al. 2003). It was also reported that there is always present a certain degree of apoptotic cells in the follicle (Zeuner et al. 2003). Thus, apoptosis of granulosa cells and cumulus cells in cattle appeared even in healthy follicles (Blondin & Sirad 1995; Yang & Rajamahendran 2000; Zeuner et al. 2003). However, if a

certain threshold of apoptotic cells is exceeded, the viability of the oocyte is influenced (Zeuner et al. 2003). For example, an apoptosis grade of 5-15% in granulosa cells of goats had a positive impact on the developmental rate, while a higher or lower rate of apoptosis had a negative effect on the developmental competence of the oocyte (Han et al. in 2006). In cattle, it was also shown that a proportion of apoptosis in granulosa cells of 10-40% influences the blastocyst rate positively (Feng et al. 2007). In cumulus cells a grade of atresia with more than 10% affected the blastocyst rate negatively (goat: Anguita et al. 2009). In other investigations was observed that an increased rate of apoptosis in cumulus cells have a negative influence on the developmental potential of the oocyte (Host et al. 2000; Alisch et al. 2003; Zeuner et al. 2003; Yuan et al. 2005; Feng et al. 2007). However, in our investigations no differences appeared in the apoptosis rate of cumulus cells from different developmental competent oocytes.

Gene expression pattern in follicular cells

We have identified, 34 and 37 genes were differentially expressed in BCB+ vs. BCB- cumulus and BCB+ vs. BCB- granulosa cells derived from follicles size of 3-5 mm size whereas 2062 and 1672 genes were differentially expressed in BCB+ vs. BCB- cumulus and BCB+ vs. BCB- granulosa cells derived from follicles size of 6-8 mm size. This may suggest that the cumulus or granulosa cells that surround the developmentally competent oocytes exhibited a significant difference in gene expression compared to those cumulus or granulosa cells surrounding non-competent oocytes. However, the degree of transcriptome abundance difference was largely dependent on follicular size. The finding of Antosik et al. (2009) who reported that the level of transcripts and proteins accumulation contributes to the fertilizing ability of the porcine oocytes was associated with follicular size.

For instance, two genes controlling the rate of steroid hormone synthesis, namely CYP19A and FSHR in granulosa cells isolated from large follicle size (6-8 mm) enclosing BCB+ oocytes. This data are similar to the findings of Xu et al. (2010) who stated that changes in mRNA levels for gonadotropin receptors (LHCGR, FSHR) and steroidogenic enzymes (CYP19A, CYP17A1) are the key processes resulting in the conversion of the estrogen-secreting mature follicle into the primarily progesterone-secreting corpus luteum. In general, expression of mRNA for the gonadotropin receptors, steroidogenic enzymes, and steroidogenic acute regulatory protein (STAR) increase with progressive follicular development and is highest when dominant follicles approach maximum size (Bao & Garverick 1998). Thus, it seems that it is fundamental for these two genes to be over-expressed in granulosa cells isolated from large follicle size (6-8 mm) enclosing BCB+ oocytes. In addition, FSHR transcript abundance was also up-regulated in cumulus cells of enclosing BCB+ oocytes from large follicle size. This confirms that the large follicle enclosing competent oocytes is more advanced in its cellular differentiation.

Our result is in accordance with that of Calder et al. (2003) who clearly indicated the higher abundance of FSHR mRNA in better quality porcine COCs. Changes in mRNA level for gonadotropin receptors (FSHR) were also consistent with reported processes resulting in follicular maturation and preovulatory cascade (Xu et al. 2010). FSH is an important pituitary hormone which controls granulosa cell steroidogenesis in the mammalian ovary by interacting with specific receptors located on granulosa cells (Richards 1994). FSH receptors are located exclusively on granulosa cells to drive its proliferation, growth and differentiation.

In support to this idea CYP19A (converting androgen to estrogen) was also up-regulated in granulosa cells of 6-8 mm follicles. Cyp19 plays an important role in the development, function, and regulation of the female reproduction cycle and it was considered to be a potential candidate gene affecting fertility performance in cattle (Fürbass et al. 1997).

For the cumulus cells to cope with the changes occurred in granulosa cells to prepare competent oocytes, BMP-2 transcript abundance was up-regulated in BCB+ derived cumulus of large follicles. BMP-2 induced follicular stimulating hormone (FSH) receptor and aromatase expression, while decreasing luteinizing hormone (LH) receptor and steroidogenic acute regulatory protein expression in human granulosa cells (Shi et al. 2010). BMPs may play an important role in follicular growth and differentiation, cumulus expansion and ovulation.

Follicular cells of developmental competent oocytes were also characterized by an increased transcript abundance of STAR, ADAM and AREG. In the follicle these genes appear during ovulation und support the maturation in oocytes (Hernandez-Gonzales et al. 2006; Feuerstein et al. 2007; Yamashita et al. 2009).

In accordance with previous studies (Richards 2007; Assidi et al. 2008) BCB+ follicle cells show higher abundance of genes responsible for immune cell-related processes (CD9, TLR). It has been suggested that inflammatory factors exert an effect on cumulus expansion, ovulation, transport and fertilization (Liu et al. 2008).

Concluding remarks

Overall, the prevailing data provides a genome-wide expression profiling of genes that could be associated with functional relevance for the establishment of developmental competence in oocytes. However, further functional investigations based on these data could help to define the exact key regulatory genes controlling oocyte quality, which could be considered as good biomarkers for oocytes with high or low developmental competence.

Furthermore, the recent results support the idea that follicular cells enclosing developmental competent bovine oocytes categorically differ in the degree of apoptosis incidence and the accumulation of gene transcripts compared to those follicular cells enclosing non-competent oocytes.

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SHORT COMMUNICATIONS

THE EFFECTS OF SEMI-DEFINED DILUENTS AND DURATION OF SPERM-OOCYTE INCUBATION ON THE NUMBER AND QUALITY OF IVP EMBRYOS

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The duration of sperm-oocyte co-incubation is known to affect rates of early cleavage and blastocyst formation of *in vitro produced* (IVP) embryos in various mammalian species. Reducing co-incubation length results in faster embryonic development with superior morphological quality, better implantation rates and increased male sex ratio than the generally accepted overnight protocol. The objective of this study was to determine the combined effect of semi- defined diluents prepared from different concentrations of the cryoprotectant bovine serum albumin (BSA) and sperm- oocytes co-incubation length on fertilization, subsequent development and embryo quality.

Thirteen hundred and forty two matured sheep oocytes were divided randomly into three main groups and fertilized with frozen- thawed spermatozoa at a concentration 1×10^6 spermatozoa/ ml prepared with either 10, 15 or 20 % BSA (lacking EY) [Ali and Naitana, A.E.T.E. 2009 Poznan/ Poland. pp. 132]. The oocytes and spermatozoa were co- incubated briefly for 1, 2, 3 or 20 hrs as standard co-incubation length, and then transferred directly into culture wells. The numbers of cleaved and expanded blastocysts per group were evaluated at 46 hours post-insemination (hpi) and at 6- 8 days post-insemination, respectively. The quality of embryos in each group was evaluated accordingly to Sifer C et al. [Hum Reprod. 20 (10): 2769- 75, 2005].

The mean cleavage and blastocysts rates were increased linearly with co- incubation length in all BSA groups. However, three hours co-incubation length resulted in a significantly higher cleavage rate compared with 1, 2 and 20 hpi (260/ 311, 83.60 %; 184/ 316, 58.22 %; 248/324, 76.54 % and 227/ 381, 59.58 %), respectively. Subsequently, this group (3 hpi) had a significantly higher mean blastocyst rate compared with 1 and 20 hpi (152/ 311, 48.87 %; 98/ 316, 31.01% and 150/ 381, 39.37%), respectively. In addition, the cleavage rate was significantly increased ($P < 0.001$) after 1 and 2 hpi in oocytes fertilized with semen prepared with 20 % *versus* 10 or 15 % BSA (83/107, 77.57 %; 42/100, 42.0 %; 59/109, 54.12 %) and (96/109, 88.07 %; 61/101, 60.39 %; 91/114, 79.82 %), respectively. However, prolonged co-incubation length over 3h had no additional effects on fertilization, efficiency of subsequent embryo development, or embryo quality in all BSA groups. In conclusion, (1) both, cleavage and blastocysts rate increased linearly with sperm-oocytes co-incubation length, and better fertilization results were obtained when sperm-oocytes co-incubated $\geq$ 2h with semen prepared with 20 % BSA. (2) Prolonging co-incubation time over 3 h had no additional effects on fertilization, efficiency of subsequent embryo development, or embryo quality.

Notes

THE ROLE OF OVIDUCTAL SECRETION ON PORCINE AND EQUINE FERTILIZATION: IS DMBT1 INVOLVED?

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Oviductal environment contains important molecules that affect the sperm–oocyte interaction and the subsequent developmental competence of zygotes. The molecular mechanisms underlying gamete interactions are not fully understood. Deleted in Malignant Brain Tumours 1 (DMBT1) is a scavenger receptor cysteine-rich protein with functions in innate immunity and epithelial differentiation. No role in reproduction mechanisms has been shown at the moment, but the presence of CUB and ZP domains in DMBT1 leads us to hypothesize such a role. The aim of this study is to evaluate the effect of oviductal fluid (OF) and oviductal cells (OC) and the possible involvement of DMBT1 on *in vitro* fertilization (IVF) in porcine and equine species. These two species represent divergent models, as generally porcine IVF is very efficient with high rates of polyspermy, while equine IVF rates are low and polyspermy is scarce.

Porcine oviducts were collected from slaughtered sows 6 h after ovulation and used to recover OF and OC. After in vitro maturation, porcine and equine oocytes were co-incubated for 30 minutes with: 1) TBM culture medium or OF with or without anti DMBT1 antibody (Ab; 1mg/ml; anti gp340 gift from U. Holmskov); 2) oviductal cells culture medium with or without OC. In porcine oocytes co-incubated with OC, the effect of anti DMBT1 Ab was also tested. Then, oocytes were used for IVF. After 24 hours they were fixed and stained with Hoechst 33258 to label nuclear chromatin.

PORCINE SPECIES: Oocytes co-incubation with OF significantly increased the IVF rates compared to the control group (64%, 71/111 vs 50%, 79/157 for OF and control; $P<0.05$). Moreover, polyspermic fertilization rates were lower in OF group compared to control (18%, 13/71 vs 59%, 47/79 for OF and control; $P<0.001$). The addition of anti DMBT1 Ab during oocyte co-incubation with OF, showed a significant increase of polyspermic fertilization (49%, 17/35) compared to OF (18%, 13/71; $P<0.001$), cancelling the positive effect of OF. No effect of anti DMBT1 Ab was shown on IVF rates compared to OF (57%, 38/67 vs 64%, 71/111 for OF + Ab and OF; NS). Oocytes co-incubated with OC showed fertilization rates (62%, 99/160) similar to control group (61%, 57/93; NS). Polyspermic fertilization rates were also not affected (25/57, 44% vs 41/99, 41%, for OC and control). No effect on IVF and polyspermy was shown after the addition of anti DMBT1 Ab (69%, 58/84 for fertilization and 53%, 31/58 for polyspermy).

EQUINE SPECIES: Oocytes co-incubated with OF showed a significant increase of IVF rates compared to the control group (65%, 20/31 vs 32%, 13/41 for OF and control; $P<0.05$). The positive effect of OF on fertilization was not affected by the addition of anti DMBT1 Ab (65%, 20/31 vs 52%, 11/21 for OF and OF + Ab; NS), even if a decreasing tendency could be noted. Oocytes co-incubation with OC showed fertilization rates (46%, 11/24) similar to control groups (30%, 10/33; NS).

Our study shows a beneficial effect of OF on IVF in porcine and equine species and suggests an involvement of DMBT1 in fertilization. Co-incubation with OC does not seem to affect IVF. To our knowledge, this is the first study analyzing the role of DMBT1 on fertilization, further studies are in process to confirm this role.

Notes

BOVINE OOCYTE QUALITY: FROM PHENOTYPE TO GENES

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In the bovine, most gestation failures which occur in the first few days post conception are influenced by genetic effects. These links represent a major component to explain the decreased fertility of dairy cows. Due to the critical role of maternal factors in sustaining early embryo development, such early failures point out a suboptimal oocyte quality as a major determinant in this phenomenon. We have developed a program aiming at producing a gene expression footprint of the bovine oocyte associated with its embryonic development potential.

Montbeliard cows have been phenotyped on oocyte quality, based on their ability to produce viable embryos after seven days of in vitro culture (Guyader-Joly et al., IETS 2008). Among them, two sets of three extreme animals were isolated, who showed in vitro development rate comprised between 0-17% and 36-55% respectively. These animals have been genotyped on a 54K SNPchip, in order to reveal putative phenotype-associated polymorphisms.

Comparative gene expression studies have been performed between oocytes collected from these two groups of animals, both at the immature and at the mature stage (i.e. one day or a few hours before ovulation). Transcriptomic profiles were characterized using a 22K oligochip, and differential transcripts were revealed between the two animal groups, at both stages. Overall, the difference in oocyte developmental potential was associated with a moderate alteration of a large number of genes (1455/85 genes in immature oocytes, 2212/196 genes in mature oocytes after Benjamini-Hochberg 5% / Bonferroni 5% correction respectively). These genes have been mapped onto the bovine genome and several are located in previously identified fertility QTL.

Following the identification of these potential oocyte quality markers, functional studies will be performed for the most pertinent genes. Further sequencing and search for polymorphisms will pave the way for implementing their use in genomic selection.

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Notes

DETECTION OF *ZONA PELLUCIDA* GLYCOPROTEIN 3 (PZP3) AND INTEGRIN-BETA-2 (ITGB2) IN PORCINE OOCYTES *

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It was clearly demonstrated in several studies that *zona pellucida* is modified during oogenesis and folliculogenesis, which are important stages of mammalian oocyte maturation. However, still little is known about differential expression and various distributions of proteins involved in fertilization, eg. *zona pellucida* glycoprotein 3 (pZP3), integrin-beta-2 (ITGB2) within the porcine oocytes. Since the morphology of female gamete has a significant impact on the ability of oocytes to successfully undergo maturation and fertilization, this study aimed at investigating the distribution of pZP3 and ITGB2 in four morphologically different porcine oocytes using confocal microscopic observations.

The porcine COC's were morphologically evaluated in four graded scales with special relation to colorization of cytoplasm and cumulus cells layers, cultured in culture medium NCSU-23 for 44 h at 38°C, and then subsequently fixed with anti-pZP3 and anti-ITGB2 antibodies, and analyzed using confocal microscopic observations.

As results, we found that pZP3 protein was localized in oocytes graded as I and II in *zona pellucida* and cytoplasm. In oocytes graded as III and IV, pZP3 was distributed in cytoplasm. Regarding the ITGB2, in oocytes graded as I the *zona pellucida* localization, was observed. In the other grades of oocytes the strong cytoplasmic expression of ITGB2 was detected.

In conclusion, the expression of both pZP3 and ITGB2 proteins as well as the differential distribution of these proteins within the female gamete are associated with the morphological type of porcine oocytes.

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Notes

EMBRYO MORTALITY RATE IN TRANSFER PROGRAMS DURING SUMMER SEASON IN SUB-TROPICAL REGION

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Data was registered to determine the effect of the donor breed on embryo mortality in periods of high ambient temperature (higher than 35°C). The work corresponds to commercial programs of 1,055 embryo transfers carried out in six cattle ranches under extensive and semi-intensive conditions. These enterprises were located in the sub-tropical region of Argentina and Paraguay. It includes donors of the Bos indicus (Brahman), Synthetic (Brangus, Santa Gertrudis and Simbrah) and European (Angus and Fleckvieh) breeds. The females ranged between 14 to 180 months of age and treatments were performed during the months of November and February of 1995 to 2004.

The superovulatory treatments began between days 8 and 14 of the estrous cycle with the placement of progesterone containing intravaginal devices with injectable estradiol and progesterone and decreasing doses of Foltropin – V (Vetrepharm – Canada) during 4 days, with a total dose of 200mg NIH – FSH-P1 of Foltropin in donors of Bos indicus and synthetic breeds, and 280 mg NIH – FSH – P1 Foltropin on European breeds. On the third day of the treatment 2 doses of 15 mg of luprostiol (Intervet – Holland) with a 12 hour interval were administered. The AI was performed at 12 and 24 hours after observed estrus.

The embryos were collected by non-surgical means, flushing with PBS between the 6th and 8 th day, post-AI and classified by IETS standards. The recipients of Bos indicus breed or Bos indicus crosses were synchronized with 2 doses of 15 mg of luprostiol (Intervet – Holland) with an interval of 11 days. The transfers were performed with fresh embryos (n=1055). The pregnancy diagnosis was carried out at 28-33 days of gestation by transrectal ultrasound (Aloka SDD500V) and confirmed by rectal examination between 60 and 80 days of gestation. The results are presented in Table 1.

All embryos of different breeds transferred produced similar pregnancy rates ultrasound diagnosis. Pregnancy rates determined by rectal examination were also similar. However, higher embryo mortality rates were observed in synthetic and European breeds (Table 1). Of the total loss, 52% corresponded to Bos indicus breeds and 38% (n=221) to synthetic breeds. These results show that the embryos and recipients of Bos indicus origin suffer less embryonic losses after 28-33 days of gestation compared to European and synthetic breeds challenged with high ambient temperatures during summer in the sub-Tropical region.

Table 1: Pregnancy and Embryo Mortality as related to donor breed

Donor Breed	Total TE	Pregnancy 28-33 days	Pregnancy 60-80 days	Embryo Mortality
Brahman	280	146(52%)	135(48%)	11(8%)
Synthetics	542	298(55%)	249(46%)	49(16%)
European	233	136(58%)	109(47%)	32(24%)
TOTAL	**1055**	**580(55%)***	**493(47%)****	**92(16%)*****

*P>0.2 **P>08 ***P>0.06

Notes

SUPEROVULATION TREATMENTS IN THE BRANGUS BREED RETROSPECTIVE EVALUATION OF DIFFERENT COMMERCIAL PRODUCTS

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The objective of this work was to carry out a retrospective analysis of superovulatory treatments (ST) to compare the results of three hormonal compounds of seven manufacturers. A total of 1024 ST were carried out on Brangus females between 16 and 72 months of age with protocols used in commercial embryo-transfer programs. Females belonged to 34 ranches in Argentina and Paraguay and ST were performed between the years 1991 to 2006. Ninety six percent of these ST were collected (n=983). Collections were performed on day 7 after artificial insemination (AI). The commercial products used were: porcine FSH (FSH-P, Shering-Plough USA; Super OV, Ausa USA; Pluset, Serono Italy; Foltropin, Vethepharm USA; Antrin, Denka Japan), ovine FSH (Ovagen, ICP New Zeland) and eCG (Novormon, Syntex Argentina). The number of ST and the total embryos recovered (ETR) were 101 and 1273; 136 and 1496; 342 and 1496; 219 and 2147; 17 and 164; 153 and 1187; 56 and 631; respectively. Data were analyzed by ANOVA and Turkey´s test was to distinguish significance among means.

The average of ETR by ST and the fertilized percentage were of 12.6±0.2 and 37.3; 11.±0.4 and 49.; 11±0.4 and 49; 9.8±0.4 and 55; 9.6±0.4 and 65; 7.8±0.5 and 59.3; 11.3±0.3 and 42.4% for the commercial products as described above. Transferred embryos (TE) and TE x ST were 475 and 4.7; 734 and 5.4; 734 and 5.4; 1173 and 5.4; 106 and 6.2; 704 and 4.6; 268 and 4.8, respectively.

The results obtained by means of the Anova analytical system, demonstrate superior and significant differences ($p<0.05$) with the Antrin (Denka, Japan) by which a greater percentage of efficiency was obtained in relation to the other products.

Nevertheless, since the number of ST performed with Antrin was substantially lower than those of the other products, further ST with this formulation need to be carried out to confirm these results. We can also conclude that all the commercial products utilized have been successful for ST treatments in Brangus donor cows.

Notes

INVESTIGATION ON FIRST LUTEAL ACTIVITY IN HIGH PRODUCING DAIRY COWS IN RELATION TO THEIR METABOLIC AND ENERGETIC STATUS

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Energy demands increase after calving in high producing dairy cows and their metabolism is affected intensely by elevated glucose output due to milk production. It is known this interferes with reproductive mechanisms. As cows differ in fat mobilization, dry matter intake and milk performance around calving, the aim of the study was to investigate the energy balance in relation to reproductive traits. The first luteal activity is known as an indicator for reproductive fitness of post partum cows. Therefore cows were grouped according to different means of liver fat content (LFC), dry matter intake (DMI) and energy balance (EB) in relation to the detection of their first luteal activities.

Cows (n=27; milk yield >11500kg in 2nd lactation) were studied from dry off up to 12 weeks in their 3rd lactation and were fed TMR *ad libitum*. DMI and milk yield were recorded daily, body weight (BW), back fat thickness (BFT), BCS, and milk composition were measured weekly. Plasma concentrations of NEFA, BHBA, glucose, and insulin were measured in blood, taken at 56, 28, 15, 5 d before expected calving and once weekly up to slaughtering. Liver biopsies were taken at day 1, 14, 28 to measure LFC. Clinical and ultrasound guided examinations of the reproductive organs as well as estimation of progesterone concentrations in plasma and milk were carried out twice weekly. Embryo recovery rate after timed insemination and developmental competence of Cumulus-Oocyte-Complexes (COC) aspirated from follicles (diameter 3-5 mm) on day 0, 4 and 12 were analysed. Data were analyzed by the Mixed Model of SAS.

Mean hepatic fat concentrations were different ($P < 0.05$) among groups: 351 ± 14, 250 ± 10 and 159 ± 9 mg/g liver. DMI was lowest ($P < 0.05$) before calving in the group with the highest mean LFC and increased ($P < 0.01$) after calving in all groups. Milk yield was not affected by LFC, but energy balance was least negative ($P < 0.01$) in the group with the lowest mean LFC. An influence of the energy balance on the occurrence of the first luteal activity was observed according to time of the energy nadir (2nd vs. 4th week after parturition) however independently from the amount of the energy nadir. Ovulations were detected from the 2nd to 12th week. There is an individual variation in postnatal fat mobilization among cows indicated by a wide range of postnatal NEFA changes in blood plasma and different reductions of BW and BFT correlated to the occurrence of the first luteal activity.

Developmental rate (morulae and blastocysts) after IVM/IVF and IVC varied according to the day of slaughtering between 0 % and 32 %. The quality of the COC aspirated from preovulatory follicles (n=9) of high producing dairy cows is similar to the quality of COC from cattle with positive energy balance. These data indicate that these cows are able to develop competent oocytes however different embryo recovery rates on day 4 and day 12 (66% vs. 33%) implicate that embryonic mortality could play a role in these cows.

Notes

LARGE SCALE GENE EXPRESSION ANALYSIS OF ELONGATED EMBRYOS DERIVED FROM NUCLEAR TRANSFER, ARTIFICIAL INSEMINATION AND IN VITRO PREGNANCIES

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Nearly two decades evidence showed that the efficiency of cloned embryos is lower as compared to sperm fertilized control groups either vivo or vitro. The latest consensus directs the reason to be an error in reprogramming of a genome of a differentiated donor cell. Most embryo losses are pronounced at about day 16 and/or prior to day 21. During this period embryos are in the process of changes to establish a better dialogue between embryo and the uterus. Our hypothesis is abnormal embryo-maternal communication develops during this period and may be associated with transcriptom differences between cloned and sperm fertilized day 16 embryos.

Bovine genome affymetrix array (with 24128 probes) was used to investigate gene expression differences between elongated embryos derived from somatic cell nuclear transfer (SCNT), artificial inseminated (AI) and in vitro fertilized (IVF) pregnancies and donor fibroblast cell. Following total RNA isolation from 3 replicates of each group, biotin labelled cRNA was hybridized on 12 bovine chips. Normalization of the data was done by using Guanine Cytosine Robust Multi-Array Analysis (GCRMA) and data analysis was performed using LIMMA written on R package which maintained the Bioconductor.

The result of the present experiment revealed that the NT elongated embryos' gene expression profiles were different from those of their donor cells and closely resembled those of in vitro fertilized groups. Only 10 genes were found to be differentially regulated between NT and IVF embryos ($p \leq 0.05$, fold change ≥ 2 and FDR 20%). On the other hand 303 and 336 gens were differentially expressed in AI vs IVF and AI vs NT embryos comparisons, respectively. Majority of the transcripts were found to be up regulated on IVF embryos while only 21 transcripts were down regulated. Moreover 158 and 178 genes were up and down regulated, respectively in NT embryos as compared to AI. NT embryos were enriched with transcripts responsible for biological regulation (DLC1, F2RL2, EFNA1 and TGFB3) and system development (CGA, ATP1B1, ALAD, A1F1) as compare to AI embryos. Whereas transcripts involved in cell process (WARS, ATP1B1, MTCH2, PIGW, DLD and TXNDC9) were more abundant on NT embryos as compared to IVF embryos. IVF embryos were found to be enriched with genes responsible for establishment of localization (TF1, SLC44A2, ATP1B3 and SLC 15A1). In conclusion, day 16 embryos from NT and IVP pregnancies show differences in expression of genes involved in various biological processes compared to their AI counterparts. Moreover, highly pronounced differences in gene expression were observed between NT embryos and their donor fibroblast cells.

Notes

ASSESSMENT OF DIFFERENT CONCENTRATION OF PROGESTERONE IN CANINE COC'S CULTURED *IN VITRO*[*]

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The role of progesterone in oestrus cycle as well as in IVM efficiency and reaching of oocytes the MII stage in the canine species is well recognized, although no reports have been published as yet in the field of its influence on expression of genes encoding proteins involved in fertilization, such as *zona* glycoproteins.

In this study canine COCs recovered from anoestrus mongrel bitches (after ovariohisterectomy) cultured in serum-free TCM 199 medium, was used. The expression of ZP2 and ZP3 mRNAs using quantitative real-time PCR (RQ-PCR) as well as ZP3 and ZP4 proteins using western-blot analyzes in canine oocytes before IVM (control, C1), after IVM (control, C2) as well as after 0.5 µg/ml, 1 µg/ml and 2 µg/ml, was performed.

After using RQ-PCR we found an increased expression of both ZP2 and ZP3 mRNAs after supplementation of 1 µg/ml of progesterone as compared to 0.5 µg/ml and 2 µg/ml, ($P<0.01$, $P<0.05$, and $P<0.001$, $P<0.001$, respectively). Regarding expression of ZP2 in both controls, we showed higher expression of this gene in C1 oocytes as compared to C2. Contrary to these results, the expression level of ZP3 in C1 was lower than in C2 oocytes. After using the western-blot analysis, we found stimulatory effect of progesterone used in the dose of 1 µg/ml, as compared to 0.5 µg/ml and 2 µg/ml for both ZP3 and ZP4 proteins, ($P<0.001$). Similarly, to the ZP2 and ZP3 mRNAs expression pattern, differences in ZP3 and ZP4 protein level between C1 and C2, ($P<0.001$, $P<0.01$, respectively), was also found.

Our results first demonstrate that; (i) progesterone may up- or down-regulate expression of *zona* glycoproteins in a dose dependent manner, (ii) the dose of 1 µg/ml of progesterone stimulate expression of these genes and proteins as compared to 0.5 µg/ml and 2 µg/ml, and (iii) that differences in expression of *zona* glycoproteins between oocytes before or after IVM may reflect the stimulatory or inhibitory effect of IVC conditions, which could be explained by a gene dependent modification.

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Notes

ICSI, IVF AND PARTHENOGENETIC ACTIVATION PROCEDURES IN PREPUBERTAL SHEEP BCB SELECTED OOCYTES

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Brilliant Cresyl Blue (BCB) test determines the activity of glucose-6-phosphate dehydrogenase, an enzyme which activity decreases when oocytes reach their growth phase. In buffalo (*Theriog.*2007 **68**:1299), cattle (*Theriog*.2004 **61:**735), goat (*Theriog*.2002 **57:**1397), mouse (*Cell Res*.2007 **17**:722), pig (*Rep.Fert& Dev.*2010 **22**:830) and sheep (*Rep.Fert&Dev.*2011 **23**:223) BCB test was considered a useful technique in order to select the most competent oocytes to develop up to the blastocyst stage. Actually, intracytoplasmatic sperm injection (ICSI), *in vitro* fertilization (IVF) and parthenogenetic activation (PA), are the most common techniques to produce embryos in *in vitro* conditions. This abstract reports on our progress using selected BCB oocytes to produce *in vitro* embryos by ICSI, IVF and PA.

Cumulus oocyte complexes (COC) were exposed to 26 µM BCB during 1 h and classified: oocytes with blue cytoplasm or grown oocytes (BCB+) and oocytes not coloured or growing oocytes (BCB-). COCs were then matured in conventional TCM199 medium with hormones and fetal bovine serum (FBS) during 24 h and then ICSI, IVF or PA was performed. ICSI was done by immobilizing thawed spermatozoa by breaking its tail in medium containing 10% PVP. The spermatozoon was aspirated tail-first into an 8 µm inner diameter injection pipette and injected into a metaphase II oocyte. Subsequent oocytes were activated placing them into PBS containing 5 µM ionomycin during 4 min. IVF was performed during 20 h in SOF medium supplemented with 20% of oestrous sheep serum with $1x10^6$sp/mL spermatozoa selected by Ovipure density gradient (Nidacon EVB S.L.) and 15 oocytes/ 50µL drop. PA was done in PBS containing 5 µM ionomycin for 4 min, then carefully washed and placed during 3 h in TCM199 media with 1.9 mM DMAP. After ICSI, IVF and PA presumptive zygotes were cultured for 7 days in SOF with 10% of FBS at 38.5 ºC, 5% CO_2 and 90% N_2. Results are shown in table 1

Table1. Blastocyst production using BCB+ and BCB- selected prepubertal sheep oocytes after ICSI, IVF and PA at day 7 of IVC.

	BCB+				BCB-			
	n	Cleavage n, (%)	Blastocyst n, /total (%)	Blastocyst n, /cleavage (%)	n	Cleavage n, (%)	Blastocyst n, /total (%)	Blastocyst n, /cleavage (%)
ICSI+Io	77	59 (77)^{a,A}	11(14)^{a,A}	(19)^{a,b,A}	68	54 (76)^{a,A}	7(10)^{a,A}	(13)^{a,A}
IVF (10% OSS)	137	104(76)^{a,A}	33(24)^{a,A}	(32)^{a,A}	149	90(60)^{b,B}	6(4)^{a,B}	(7)^{a,B}
PA (IO+DMAP)	182	161(88)^{b,A}	33(18)^{a,A}	(20)^{b,A}	173	138(80)^{a,B}	7(4)^{a,B}	(5)^{a,B}

Different letters in the same column (^{a,b,c}) or road (^{A,B}) differ significantly (Fisher Test, P<0.05).

In conclusion, BCB + oocytes led to a higher number of blastocyst produced by IVF and PA than with BCB- oocytes. Whereas in ICSI-oocytes no differences were found between BCB+ and BCB- oocytes and this could be due to the previous MII selected oocytes before ICSI in both BCB categories.

Notes

INFLUENCE OF SHORT TIME UREA INTAKE ON THE ULTRASTRUCTURE OF 16-CELL STAGE EMBRYOS IN DAIRY HEIFERS

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The aim of the study was to evaluate an influence of short time urea intake on the ultrastructure of 16-cell stage embryos in dairy heifers. Holstein heifers (age 16 months, body weight 450 kg) were fed by 270 g of urea (divided into 3 doses) daily for 7 days (n=8, mean urea concentration 10.65 mmol/l) or not (n=8, mean urea concentration 3.41 mmol/l). Heifers were synchronized by prostaglandin and GnRH treatment twice within 11 days. Dominant follicles were aspirated on day 7 of oestrous cycle after synchronization (D -2). Superovulation was induced by 8 decreasing doses of FSH/LH (Pluset, Calier SA, Spain)(D0 – D3). Prostaglandin treatment was performed 60 and 72 hours after the initial FSH treatment, induction of ovulation was performed by injection of 2000 IU of hCG i.v. 48 h after the first prostaglandin treatment together with insemination.

Embryos at 16-cell stage were recovered by oviductal flushing using transvaginal endoscopic approach on D9. The developmental stage as well as the morphological integrity of the recovered embryos was assessed according to IETS guidelines. Mean number of CLs, all recovered ova/embryos, unfertilized ova, degenerated embryos, embryos <16-cells, 16-cell stage embryos and embryos >16-cells was 18, 14.1, 1.8, 1.8, 3.5, 5.1, 2 and 14.1, 10.1, 2.3, 1.6, 2.9, 2, 1.4 in urea group and control group, respectively.

Five 16-cell bovine embryos exposed to urea and five 16-cell control embryos were standardly processed for transmission electron microscopy. The following parameters were assessed in embryos: occurrence of multinucleated blastomeres, structure of nuclei and nucleoli, structure and distribution of organelles (mitochondria, endoplasmic reticulum and ribosomes, Golgi apparatus, vacuoles), formation of intercellular junctions. Generally, no remarkable differences were found between two experimental groups. Some insignificant differences were found out in mitochondrial morphology (reduced number of mitochondrial cristae, higher density of mitochondrial matrix in urea group) and cytoplasmic vacuoles (more variable content of vacuoles in urea group).

In conclusion, short time urea intake does not affect the ultrastructure of 16-cell stage embryos in dairy heifers.

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Notes

EFFECT OF E-64 INHIBITION CATHEPSIN B DURING *IN VITRO* MATURATION OF PREPUBERTAL CALF OOCYTES ON EMBRYONIC DEVELOPMENT: LEVELS OF CATHEPSIN B PROTEIN AND APOPTOSIS OF CUMULUS CELLS

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Cathepsin B belongs to a family of lysosomal cysteine proteinases which participe in a variety of proteolytic processes. Recently, the quantity of cathepsin transcripts in cumulus cells, especially cathepsin B, was found to be associated with low-developmental competence of adult bovine oocytes. In the present study, we investigated the effect of a cathepsin B inhibitor (E-64) during *in vitro* maturation of prepubertal calf oocytes on embryo development and cathepsin B protein activity. Cumulus-oocyte complexes (COCs) were matured in medium TCM 199 containing FCS and EGF (control), and supplemented with 1 (E1), 10 (E10) and 100 (E100) μM of E-64. After *in vitro* maturation, the oocytes were fertilized and cultured *in vitro*. Cleavage and blastocyst rates were determined on day 2 and 8 after fertilization. In order to evaluate cathepsin B protein levels in COCs matured *in vitro* for 24 h, oocytes and cumulus cells were stored at -80°C separately and analyzed using the western blotting technique. Cleavage rate was significantly lower for E100 (67.3%) group, when compared to the control (77.7%), but not for E1 (74.3 %) and E10 (72.9%) groups. At day 8 pi, blastocyst rate was significantly lower for E100 (3.9%) when compared to the other groups (12.5%, 11.5% and 8.7% for control, E1 and E10, respectively). Results obtained from the Western blotting analysis clearly indicate an increase of protein levels of the cathepsin B protein in oocytes matured in presence of 100 μM of E-64, when compared to other groups. Oocytes matured at the lowest concentration of E-64 (1 μM) contained similar amounts of cathepsin B protein when compared to oocytes matured in the absence of inhibitor. No differences in the intensity of signal were observed among groups when cumulus cells were analyzed. These data suggest that only the presence of higher concentrations of E-64 during *in vitro* maturation of prepubertal calf oocytes had a significant effect on embryo development. Probably, this buffering effect would be related to a compensatory action induced by the inhibitor on the oocyte total cathepsin B protein content.

Notes

FFECTS OF DIFFERENT DOSES OF GAMMA RAYS IN *IN VITRO* PRODUCED EMBRYOS AFTER TRANSFER IN SHEEP

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Cryopreservation of cells, tissues, and embryos offers the most secure form of conservation. Nevertheless, frozen biological material is exposed to a natural background of ionizing radiations (IR) which might induce untoward effects. The purpose of this study was to evaluate the effects of IR, estimated of about 1 mGray per year (mGy), on vitrified sheep embryos irradiated in LN_2 with different doses of gamma-rays (^{137}Cs γ-rays, 50 mGy/sec).

Sixty embryos were cultured in synthetic oviduct fluid with 8 mg/ml BSA until blastocyst stage and were vitrified and exposed to different radiation doses (0, 0.3, 2.4, 19.2 Gy) in LN_2. After warming, embryos were transferred in pairs into synchronized ewes. Pregnancy was confirmed by ultrasonography on day 40 and was carried out until term. Lambs born were monitored by cytofluorimetric analysis for seven months to evaluate blood values and were also monitored for body and health parameters until the first pregnancy and lambing. Lambs derived from transferred irradiated embryos were mated to each other to verify fertility parameters.

Pregnancy rates were 55% (5/9), 42% (5/12), 0% (0/5), 75% (3/4) and lambing rates were 28% (5/18), 21% (5/24), 0% (0/10), 50% (4/8) for 0.3, 2.4, 19.2 Gy and control group respectively. Gy 0.3 group had low mortality and almost normal pregnancy and survival rate, while Gy 2.4 group had higher mortality when compared to the control group. One lamb out of 5 born showed abnormal limbs and 2 died within two hours from lambing due to several organ defects (emphysema, pneumonia, hydrapericardium, fill bladder, hepatosis). As expected, no embryo survived in 19.2 Gy group. Furthermore, survived lambs (5 males and 3 females) were monitored for body and health conditions. Males and females were allowed to mate each other to test fertility parameters and the pregnancy of the three females was brought to term.

Results suggest that a low dose of Gamma rays (0.3 Gy) does not produce effects, a middle dose (2.4 Gy) produces differences in the pregnancy and survival rate and a high dose (19.2 Gy) induces embryo death.

Notes

ASSESSMENT OF DIFFERENT CAPACITATION TREATMENTS TO EVALUATE THE ABILITY OF NON-FILTERED AND FILTERED FROZEN-THAWED STALLION SPERM TO PENETRATE ZONA PELLUCIDA-FREE BOVINE OOCYTES MATURED *IN VITRO*

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The fertilizing capacity of cryopreserved spermatozoa from many stallions is lower than those from fresh or cooled spermatozoa. Filtration through a density gradient has been used in recent years to select the best sperm cells from stallion ejaculates, including those best suited to withstand preservation procedures. Although laboratory assays, such as sperm motility, can be used to rapidly evaluate a semen sample, motility evaluations are not highly correlated with the fertilizing potential of frozen-thawed sperm. The aim of this study was to compare different capacitation treatments to evaluate the ability of non-filtered or filtered frozen-thawed stallion sperm to penetrate zona pellucida-free bovine oocytes matured *in vitro.*

In the first experiment, zona-free i*n vitro* matured cow oocytes were inseminated with frozen-thawed stallion sperm previously treated with five different capacitation protocols: ionomcin (1.0µM; 0.1µM; 0.05µM or 0.01µM) or caffeine (200mg/mL) for 15 min. In the second experiment, frozen-thawed semen was divided into four treatments: non-filtered and filtered through EquiPure^{TM} in order to assess the ability to penetrate zona pellucida-free bovine oocytes using as a capacitation protocols 0.1µM and 0.05µM ionomicin. After 18 h of co-incubation, oocytes were fixed, stained with DAPI and examined for sperm penetration. In the first experiment, no sperm penetration was observed after treatments with 1.0µM ionomicin, 0.01µM ionomicin and 200mg/mL caffeine whereas semen treated with 0.1µM and 0.05µM of ionomicin triggered sperm penetration rates of 19.1% and 17.1%, respectively. In the second experiment, significantly higher rates of sperm penetration using filtered semen (41.3% and 6.6%) were observed when compared to non-filtered (2.3% and 0.0%) using 0.1µM and 0.05µM of ionomicin, respectively. These findings suggest that treatment with ionomicin, specially the 0.1µM concentration, is a good alternative to induce the sperm stallion capacitation. Heterologous IVF assays with zona-free IVM bovine oocyte would seem to be a good way to evaluate the quality of frozen-thawed stallion semen although further studies are needed to correlate sperm variables and heterologous penetration with *in vivo* fertility.

Notes

FOUR YEARS PRACTICAL EXPERIENCE WITH SEXED BULL SEMEN IN NORTH-WEST GERMANY

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There is a big increase in the use of sexed semen of Holstein bulls in AI all over the world. More heifer calves and easy calving are the main advantages for the farmers.

In the present study, data on the fertility of cows after insemination with sexed semen and on the sex of their calves are presented. Furthermore, preliminary results on the verification of the sex ratio in sexed sperm samples using a modified commercially available kit for sexing bovine embryos (SEX-Y^{TM}; Minitube) are reported.

11,897 first inseminations with flowcytometrically sorted semen (n = 28 Holstein bulls; certified purity = 90 % X-bearing sperm) were performed in the area of the East Friesian Cattle Breeding Association (VOST) from July 2007 to February 2011. 56-day nonreturn rates (NRR-56), number of inseminations (NI), and the calving rate (CR) were compared to 108,325 first inseminations with unsorted semen of the same bulls.

NRR-56 and CR proved to be significantly lower ($P < 0.001$) after AI with sex sorted semen (difference of -10.1 % and -9.3 %, resp.), while NI (+0.16) and FIC (+5.5 days) increased significantly ($P < 0.001$).

5,668 calves born after AI with sexed semen and 103,504 calves after AI with unsorted semen were analyzed. 85.5 % female calves resulted from inseminations with sorted semen. In comparison, only 47.3 % female calves were born after AI with unsorted semen. There were significant differences in the sex ratio of progeny after insemination with sexed semen between the bulls.

The modified SEX-Y™ proved to be a reproducible and effective method for measuring the sex ratio of spermatozoa in selected sperm samples, and for controlling the quality of sperm sexing procedures.

Notes

GHRELIN AFFECTS IN VITRO MATURATION OF BOVINE OOCYTES IN A TIME DEPENDED MANNER

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Ghrelin a stomach derived endogenous ligand for the growth hormone secretagogue receptor, is primarily involved in energy homeostasis in many species. Ghrelin also suppresses LH and promotes prolactin secretion, while its expression has been shown in many tissues, the ovary included. The aim of this study was to investigate the effects of ghrelin on the in vitro bovine oocyte maturation that would be evaluated on the basis of subsequent blastocysts formation rate.

Immature cumulus oocyte complexes (COCs) were obtained by aspirating small-medium size follicles (2-6mm) from slaughtered cows' ovaries. COCs were matured in TCM-199 supplemented with 10% (v/v) FCS and 10 ng/ml EGF at 39^{o}C under an atmosphere of 5% CO_2 in air with maximum humidity. Matured oocytes were inseminated using frozen-thawed swim-up separated bull sperm at a concentration of 1 x 10^6 spermatozoa/ml. Gametes were co-incubated at 39^{o}C under an atmosphere of 5% CO_2 in air with maximum humidity. At approximately 20 h post insemination (hpi), presumptive zygotes were denuded and transferred to 25 μl culture droplets under mineral oil and cultured in SOF + 5% (v/v) FCS at 39^{o}C in 5% CO_2, 5% O_2 and max. humidity. Cleavage and blastocyst formation rate were evaluated at 48 hpi and on days 7, 8 and 9 respectively. Comparisons on cleavage and blastocysts formation rates were carried out by χ^2, significance was set at 0.05 level.

In the first experiment 1780 grade 1 and 2 COCs were used (10 replicates). Maturation medium was modified by the addition of 3 different concentrations of bovine acylated ghrelin: 200pg/ml n=414; 800pg/ml, n=524; 2000pg/ml, n=404; 438 oocytes served as untreated controls. Maturation lasted for 24 hours.

Based on the results obtained from the 1st experiment the concentration of 800pg/ml was selected and tested for the 18hour maturation. In 5 replicates 892 COCs were divided in three groups; G800 (n=481) and two control groups, control 1 (n=168) for the 18 hour and control 2 (n=243), for the 24 hour maturation.

Among groups no difference was recorded in cleavage rate (73.3%, 68.6% 67.8%, 70.3%, for Control, G200,G800, G2000 respectively). All ghrelin concentrations used suppressed ($P<0.003$) blastocysts formation rate on days 7 and 8 in comparison with the control (day 7: 22.4%, 16.2%, 13.0%, and 14.1%; day 8: 25.3%, 19.1%, 17.9% and 18.6%, for Control, G200, G800 and G2000 respectively). On day 9 significant difference ($P<0.05$) was only detected between G800 (18.7%) and the control (24.7%).

In exp 2, blastocyst formation rate did not differ between groups, while at all days G800 yielded more blastocysts compared to that of the respective group in exp 1 (23.5%, 27.4% and 25.2% for days 7, 8, and 9 respectively, $P<0.02$).

Our results imply that ghrelin has a direct effect on maturation of bovine oocytes. We infer that irrespectively of the concentration used ghrelin accelerated maturation process that resulted to "aging" of the oocyte at the time of fertilization. This although did not preclude fertilization from occurring, it hampered developmental capacity of the zygotes.

Notes

SEPARATION AND PURIFICATION OF RAM SEMEN: EFFECTS ON FERTILITY RATES IN SARDA EWES: PRELIMINARY RESULTS

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The quality of semen is one of the mayor limiting factors affecting the success of artificial insemination in mammalians. The presence of dead spermatozoa, bacteria and cell debris in the semen has been suggested to have a detrimental effect on the fertilizing ability of normal sperm cells. A density gradient system that separates and purifies semen, selecting a sub-population of motile spermatozoa might improve fertility. The aim of the study was to test the efficiency of a density gradient system, Ovipure™ (Nidacon, Sweden) to select and purify ram semen and test its fertility in Sarda ewes.

Twenty Sarda ewes were synchronised with progestagen sponges for 14 days. At sponges removal the ewes were injected with 400IU of PMSG and inseminated 55 hours later with fresh semen (n=10; control group) and fresh semen purified with Ovipure™(n=10; Ovipure group). The ejaculates of 10 Sarda rams of proved fertility were pooled and diluted in skim milk extender to obtain an insemination dose of $1.6x10^9$ spermatozoa/ ml. The diluted semen was then divided into two parts: one was put into 0.25 ml straws and left at 15°C until the time of insemination (control group); the remaining part was processed with Ovipure™. Briefly, each ml of diluted semen was layered on the top of 4ml of a colloidal solution (Ovipure™) and centrifuged at 300g x 20 minutes. Afterwards, the supernatant was gently removed leaving 2ml of sperm suspension. A washing medium (4ml, Oviwash™) was added and the suspension was centrifuged at 500g x 10 minutes. The supernatant was removed leaving the sperm pellet (~1 ml) that was re-suspended in skim milk extender (1:2) and centrifuged at 500g x 10 minutes. The upper part of the suspension (~1 ml) was gently removed and the pellet checked for sperm concentration. The sperm suspension was then put into 0.25 ml straws and kept at 15°C until the time of insemination. After insemination the fertility rates were evaluated through oestrus detection on day 16 to 20 using vasectomised rams and through trans-abdominal scan on day 28.

The final concentration of the sperm pellet processed with Ovipure was $600x10^6$ spermatozoa/ml ($150x10^6$ spermatozoa/0,25 ml straw). The fertility rates were 50% (5/10) for the control group and 60% (6/10) for the Ovipure group.

Although further investigations are needed, the results show that inseminating ewes with a lower number of selected and purified spermatozoa (150 million vs 400 million) gave satisfactory fertility rates.

Notes

EFFECT OF A MODIFIED WOW-SYSTEM AND EMBRYO DENSITY DURING IN VITRO CULTURE ON DEVELOPMENTAL RATE OF CAPRINE EMBRYOS

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The most common and efficient systems used in IVP of embryos in small ruminants consist in large number of oocytes (20-70) matured, fertilized and cultured together in the same medium, thus preventing traceability of the origin of the oocytes. Individual oocyte in vitro fertilization and embryo culture has been proven to be unsuccessful. Therefore, our objective was to trace the donor animal by handling small groups of oocytes (n=5) instead of the oocyte individually.

Oocyte-cumulus complexes collected from slaughterhouse derived goat ovaries were matured, fertilized and zygotes were cultured in SOF-BSA in presence of serum to the blastocyst stage as previously described (Cognie *et al.*, 1995, AETE, 146). To improve the developmental rates, we tried to use a modified Well-of-the-Well system (WOW) consisting in culturing groups of 5 presumptive zygotes (PZ) in microwells within a conventional four well dish. We compared the following 4 experimental culture conditions: 5 PZ in a 15µL drop (5/15d; n=118), 5 PZ in a single WOW in a 15µL drop (5/15wow; n=93), 15 PZ in a 50µL drop (15/50d; n=160); 15 PZ in 3 WOW (3x5 PZ) under the same 50µL drop (15/50wow; n=339). These groups allowed us to separately assess the effect of the culture system (CS ; Drop *vs* WOW) and the effect of the number of PZ per group (NPZ ; 5 *vs* 15) on the developmental rate, while the standard in vitro culture (IVC) system consisting in 25 PZ in a 25µL drop (25/25d; n=423) was also performed as a control group. The effects of the CS and the NPZ, as well as their interaction on cleavage and blastocyst rates were analyzed by generalized linear models for categorical variables. Comparisons of groups vs. the standard IVC system were performed by the Dunnett's test for proportions. Both, CS and NPZ had a very significant effect ($p<0.002$ and $p<0.0001$, respectively) on the developmental rate in favor of the WOW system and the group of 15, with no significant interaction. Cleavage rate did not differ between all groups. Developmental rate at day 8 was higher in the 25/25d control and 15/50wow groups (58% and 59% respectively). The 15/50d group yielded an intermediate rate of development of 49%, and the 5/15d and 5/15wow groups showed significantly lower developmental rates than the control group (22% and 41%, $P < 0.001$ and $P < 0.05$, respectively).

These results demonstrate the beneficial effects of culturing large number of PZ together and the suitability of a modified-WOW system, which allows small groups of PZ to share the same medium, to achieve traceability. The promising results of the modified WOW-system make it interesting to use for in-vivo derived oocytes from OPU to handle oocytes by donor and assure their filiations.

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Notes

SMALL HYALURONAN FRAGMENTS MEDIATE OOCYTE NUCLEAR MATURATION AND IMPROVE PREIMPLANTATION EMBRYO DEVELOPMENT IN RUMINANTS

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Hyaluronan (HA) is an important of component extracellular matrix and is present in the follicular oviductal and uterine fluids. HA and its main receptor CD44 are expressed in the cumulus oocyte complexes and all stages of embryo development. It is important for cumulus cells expansion during oocyte maturation and is a marker of oocyte quality. HA cleaves into small fragments by hyaluronidase (HYAL) which is a membrane bound enzyme present in the oviduct and uterus. HYAL-2 disrupts HA-CD44 interaction leading to initiation of cell signaling. The effects of HA is dependent on its molecular/fragment size.

Experiment 1. Bovine oocytes were collected from abattoir and cultured in the presence or absence of HYAL-2 (300U/ml). The rates of cumulus cells expansion, oocytes nuclear maturation to metaphase II stage, cleavage after IVF and development to blastocyst stages were analyzed. Presence of HYAL-2 in the maturation medium resulted in total inhibition of cumulus cells expansion but did not affect oocyte maturation. These oocytes had similar fertilization and blastocyst rates. Combination of HA and HYAL-2 resulted in significant increase in the number of hatched blastocysts.

Experiment 2: In vitro produced bovine cleaved embryos were cultured in the absence or presence of increasing concentrations of HYAL-2. Presence of HYAL-2 increased blastocyst rate in a dose dependent manner with the highest rate at 300U/ml. These embryos also had better quality assessed based on the total and apoptotic cell numbers and the number of hatched blastocysts.

Experiment 3. Sheep was used as an experimental model. Ewes were synchronized and supper-ovulated and mated naturally. On day 2 after mating, the animals received infusion of PBS or HA alone in the presence or absence of HYAL-2 (300U/ml) into the ligated oviducts after laparoscopy. Two sizes of large and small HA were applied. Presence of HYAL in the infused media increased the number and quality of recovered blastocysts regardless of the HA treatment.

These studies provide some evidence for the critical roles of HYAL-2 induced small HA fragments during oocyte maturation and pre-implantation embryo development.

Notes

FLOCK-REPROD: HORMONE-FREE NON-SEASONAL OR SEASONAL GOAT REPRODUCTION FOR A SUSTAINABLE EUROPEAN GOAT-MILK MARKET

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FLOCK-REPROD is a European project co-financed by the Seventh Framework Programme (GA n°243520, Capacities, Research for SME Associations). It began in December 2009 and brings together 15 partners including research institutes and SMEs: CAPGENES (France, coordinator of the project), ACRIMUR, KPRA and CABRAMA (Spain), ANCRAS (Portugal), ARAL (Italy), CAPRIROM (Romania), OLYMPOS (Greece) and OPG Moravec (Croatia). In the European context of increased restrictions in the use of exogenous hormones (currently used by the majority of dairy goat breeders using artificial insemination (AI)) in breeding systems (96/22/EC), this project aims at providing the dairy goat industry with an innovative and sustainable technology to ensure the hormone-free production of goats' milk and related products, in full conformity with EC regulation. The work plan has been structured in 6 work packages (WP). The first three ones are dedicated to "Research and Technological Development": experiments based on the use of the male effect and light treatments are carried out to obtain a high-level of oestrus synchronisation and thus to optimize AI conditions, in and out of the breeding season. Seasonal variations of sexual activity and ovulatory response to the male are studied in breeds for which those physiological data are lacking (WP1). In other breeds, photoperiodic treatments without melatonin (WP2) and progestagen-free (prostaglandin-based) / hormone-free AI protocols (WP3) are already being tested. Moreover, protocols will be delivered according to breeders' technical and geographical situations. The different breeds concerned are: Alpine goats in France; Saanen goats in Croatia; Murciano-Granadina goats in Spain; Sarda goats in Sardinia; Serrana goats in Portugal; Capra-Prisca, Damascus and Skopelos goats in Greece; White of Banat and Carpathian goats in Romania. Once standardized "male effect + AI" protocols developed, they will be tested and validated in large-scale field conditions by SMEs (WP4) before dissemination and technological transfer (WP5). Finally, technical and practical guides will be produced (for technicians and breeders), as well as a training DVD and a trademark. The last work package (WP6) is dedicated to the management of the project. The first results show that the male effect is a very promising non-pharmacological alternative to the use of hormones for inducing and synchronising oestrus in some breeds of goats. Depending on the breed and/or the time of the year, a photoperiodic treatment of the females and/or the males might be necessary to optimize the response to the male effect.(*http://www.flock-reprod.eu*)

Notes

DIFFERENCES IN TRANSCRIPTOME PROFILE OF BOVINE BLASTOCYSTS DERIVED FROM ALTERNATIVE IN VIVO AND IN VITRO CULTURE CONDITIONS

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Understanding of gene expression patterns due to altered environmental conditions during specific phases of early embryonic development would yield insights into the molecular pathways controlling early development, to improve our knowledge in regulation of embryonic development and to improve success of embryo culture. In the current study, we aimed to understand the influence of alternative culture conditions (in vivo or in vitro) during embryonic genome activation (EGA) stage of development on gene expression pattern and subsequent influence on pathways and biological functions of bovine blastocysts. Six different blastocyst groups were produced under alternative in vivo and in vitro culture conditions. The first two groups (Vitro_4-cell and Vitro_16-cell) were matured, fertilized and cultured in vitro until 4- and 16-cell stage, representing the developmental stages before and after EGA, respectively. Then, each group was endoscopically transferred to synchronized recipients oviduct and blastocysts were collected at day 7 by uterine flushing. The second two groups (Vivo_4-cell and Vivo_16-cell) were matured, fertilized and cultured in vivo until 4- and 16-cell stage, respectively then flushed out and cultured in vitro until day 7 in which blastocysts were harvested. Totally in vitro (IVP) and in vivo blastocysts were produced and used as control groups. A unique custom microarray (Agilent) containing 42,242 oligo probes (60-mers) involved 30,461 genes/pseudo genes expressed in bovine (including 9,322 embryo specific novel transcripts) was used over six replicates of each blastocyst group vs. in vivo control group (with dye swap hybridizations).

Blastocyst groups which spent EGA stage under in vitro culture conditions (Vivo_4-cell and Vitro_16-cell), either started under in vivo or in vitro conditions, showed higher number of DEG's than their counterparts (Vivo_16-cell and Virtro_4-cell) which spent EGA stage under in vivo culture conditions compared to complete in vivo control group. Ontological classification of DEG's showed that metabolic processes including lipid, carbohydrate, nucleic acid, and amino acid, as well as, cell signalling, cellular development and cell death were the most significant functions in all groups, with different trends, compared with in vivo control group ($P \leq 0.05$). Interestingly, genes involved in lipid metabolism were found to be differentially expressed in Vitro_16-cell and Vivo_4-cell groups. Both groups spent the time of EGA in vitro but showed opposite pattern, in which lipid metabolism genes were down-regulated in Vitro_16-cell and up-regulated in Vivo_4-cell group. Pathway analysis revealed that signalling pathways and retinoic acid receptors activation pathways were the dominant pathways in Vitro_4-cell, Vitro_16-cell and IVP groups. However, NRF2-mediated oxidative stress pathway was the dominant pathway in Vivo_4-cell and Vivo_16-cell groups compared to in vivo control group. This study provides the transcriptome dynamics and gene expression patterns of bovine blastocysts produced under alternative in vivo and in vitro culture conditions with respect of EGA developmental stage and will serve for improving our knowledge in embryonic development regulations.

Notes

PRELIMINARY RESULTS: COMPARISON OF TWO PROTOCOLS FOR IN VIVO EMBRYO PRODUCTION IN DAIRY CATTLE AT HIGH ALTITUDE CONDITIONS

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The objective was to compare the efficiency, in terms of total number of transferable embryos produced, of two protocols for superovulation and embryo production in dairy cattle at high altitude conditions in the Peruvian highlands (15°, 51′, 39″S latitude, 70°, 0′, 57″W longitude and 3926 meters elevation). Three Brown Swiss adult cows (7 years old) were used repeatedly (6 times each) for embryo production using two different protocols. Protocol I was used in the first 3 superovulation and embryo collection runs and Protocol II was used in the last 3 runs. The interval between runs was of 2 months. The cows were not lactating when subjected to the superovulatory treatments and embryo collection. For superovulation, cows in both treatments and all runs received on Day 0 a CIDR® vaginal device (1,9 g progesterone, InterAg, Hamilton, New Zealand) plus 1,5 mg estradiol benzoate (IM, Estrovet®, Montana, Lima, Peru) following from Day 4 to Day 7 with twice a day IM injections of FSH (Folltropin –V, Bioniche Animal Health Inc., Belleville, Ontario, Canada) in decreasing doses (Day 4: 70 mg, 70 mg; Day 5: 60mg, 60 mg; Day 6: 40 mg, 40 mg and Day 7: 30 mg, 30 mg) with a total dose of 400 mg of FSH. All cows received 526 ug of cloprostenol (IM, PGF Veyx forte®, Veyx Pharma, GmbH, Deutschland) on day 6 AM, CIDR devices were removed 12 hours after PGF2∝ injection. Donors under Protocol I (T1) received 2500 UI HCG (IM; Pregnyl®, Organon USA Inc., Roseland, NJ) on Day 8 AM and were fix time artificially inseminated (FTAI) 12 and 24 hours after the HCG injection. Protocol II donors (T2) received 2,5 ml of GnRH (IM, Conceptal®; Intervet International) on Day 8 AM and were FTAI at 18, 24 and 36 hours after GnRH treatment. Ova/embryos were collected nonsurgically and classified according to IETS recommendations on Day 15 AM. Data were analyzed by non parametric Kruskal Wallis test and means compared using Dunn's test. There was no effect on ova/embryos and total transferable embryos produced between T1 and T2 ($p>0.05$), however treatment 1 with 2 inseminations 12 and 24 hours after HCG resulted in a significantly lower number of fertilized ova than treatment 2 with 3 inseminations 18, 24 and 36 hours after GnRH ($p<0.05$). These results suggest that it is possible to obtain transferable embryos with FTAI in highlands conditions and the higher fertilization rate can be achieved with 3 FTAI 18, 24 and 36 hours after GnRH injection. More research is required to optimize and standardized bovine superstimulation and embryo production in high altitude conditions.

Table 1. Ova/embryo production in Brown donors maintained in altitudes conditions treated with two or three FTAI after GnRH or HCG injection.

Treatment	FTAI	N° collections	N° ova/embryos collected ± SD	N° fertilized ova ± SD	N° transferable embryos ± SD
T1+HCG	12 and 24 h	9	13.22 ± 13.67[a]	4.33±7.18[a]	3.33 ± 7.48[a]
T2+GnRH	18, 24 and 36h	9	20.11±18,06[a]	13.00±13.80[b]	9.0 ± 10.38[a]

[a,b] Values differ between T1 and T2 ($p<0.05$)

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Notes

BOVINE IVP EMBRYO SPEED DEVELOPMENT IN SOF MEDIUM DO NOT INFLUENCE SEX RATIO : PRELIMINARY RESULTS

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Faster developing bovine blastocysts within an in vitro production system are generally recognized as more viable (i.e., higher survival after transfer) than those developing more slowly. Some studies demonstrated that after IVF, faster developing embryos were in some culture systems preferentially males and thus increased percentage of male offspring making this system economically less viable for the dairy industry (Gutierrez-Adan et al., 2001 Theriogenology 55, 1117 – 1126). Since October 2009 our OPU-IVP unit is producing embryos from high genetic merit donor Holstein females. This study was designed to investigate a possible relationship between IVP embryo speed development and their sex ratio in our culture conditions and compare it with their in vivo produced counterparts.

Three hundred oocytes from slaughterhouse ovaries were *in vitro* matured in M199 supplemented with FCS, FSH/LH, estradiol and EGF for 22 hours. They were then fertilized in TALP medium and cultured in vitro in SOF medium (Minitüb, Gamarra *et al*, AETE 2010) for 8 days at 38.5 °C in 5% CO2, 5%, O2, 90% N2 atmosphere with maximum humidity. On days 6.5 and 7.5 development stages and quality of the embryos were recorded. Grade 1 Blastocysts (B) and expanded blastocysts (EB) were selected for biopsy and sexing. Biopsy and sexing embryos were performed as previously described by Lacaze *et al*. (AETE 2007 p188). During the same period, 77 *in vivo* derived embryos were sexed as control. Percentages between groups were compared by chi-square test.

From 300 inseminated oocytes, 87.7% (264/300) cleaved and 35.7% (107/300) develop to the blastocyst stage from which 77.6% (83/107) were grade 1 embryos. Sex determination was achieved in 73 embryos out of the 77 selected for biopsy (95 %).

Table 1: Sex ratio of *in vitro* produced D 6.5 and D 7.5 embryos

	Total biopsied	Female n	%	Male n	%
D 6.5	35	16	46.0	19	54.3
D 7.5	38	19	50.0	19	50.0
Total	73	35	47.9%	38	52.1%

Results indicated that sex ratios on D 6.5 and 7.5 were not different. This was also not different from the one observed on *in vivo* produced embryos: 42.9 % females (33/77) and 57.1 % male (44/77).

In conclusion, these preliminary results show that using a SOF culture system 25.7 % of the inseminated oocytes developed in grade 1 blastocysts selected for biopsy and sexing. Moreover no influence of IVP embryo speed development was observed on sex ratio but this result needs to be confirmed by further experiments.

Notes

EVALUATION OF EPROCHECK® ELISA TEST MACHINE FOR MILK PROGESTERONE ASSAY IN CATTLE

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Progesterone (P4) assay is a useful tool for reproduction management in cattle, mainly used to check resumption of ovarian activity following calving and embryonic survival after artificial insemination. Moreover, it could be used in donor cows before starting the superovulation treatment and/or in recipients before embryo transfer, in order to evaluate the corpus luteum functionality. eProCheck® (Minitüb) is an easy to use and portable ELISA test machine allowing automatic, quantitative and immediate (20 minutes duration) P4 assays in milk or serum samples.

The objective of this study was to evaluate the automated eProCheck® analyzer for determination of progesterone concentrations in bovine milk samples, compared to a classical ELISA method under laboratory conditions.

P4 concentrations were measured from 30 milk samples in UNCEIA laboratory with the routinely used ELISA method (Ovucheck® kit, Biovet) considered as the reference method and compared to the eProCheck® analyzer (Minitüb) in order to evaluate its intrinsic characteristics. In each sample, P4 concentration was measured once using the reference method and compared to 9 replicates using the eProCheck® protocol with the following experimental design: each sample was measured 3 times within the same eProCheck® cycle (repeatability assessed by the intra-assay coefficient of variation) and then repeated in 3 different eProCheck® cycles (reproducibility assessed by the inter-assay coefficient of variation).

Considering quantitative results, a high Pearson correlation coefficient (R^2=0.85) was observed when progesterone assays obtained with eProCheck® were compared with the reference method. Regarding qualitative results (negative/doubtful/positive), different thresholds were tested and concordance rates were calculated. When UNCEIA thresholds were applied for both methods (< 2.5 ng/ml: negative / [2.5-3.5] ng/ml: doubtful / > 3.5 ng/ml: positive), the concordance rate between eProCheck® and UNCEIA methods averaged 76.7 % from the 30 milk samples. When using the threshold values initially recommended by Minitüb to interpret eProCheck® results (< 3 ng/ml: negative / [3-4.5] ng/ml: doubtful / > 4.5 ng/ml: positive), the concordance rate between both methods averaged 70% ($p<0.0001$).

The intra-assay and inter-assay coefficients of variation averaged 17.8 ± 7.4 and 18.2 ± 11.0 respectively for the eProCheck® test machine, which is quite high in comparison to classical ELISA tests.

In conclusion, concordance rates obtained in this study indicate that the eProCheck® test machine could be used in recipient or donor cows under field conditions in order to obtain a quick progesterone assay from milk samples. However, as results showed a high intra- and inter-assay variability, it still should be recommended to perform 2 replicates of each sample or to repeat doubtful samples. Moreover, these preliminary results suggest to work further on optimal threshold values to interpret eProCheck® results from milk samples.

Notes

SHEEP BREED CATHEGORIES AS A SOURCE OF VARIATION IN EMBRYO PRODUCTION

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The results of embryo transfers from 28 donor from different breeds are presented, and the data analyzed to investigate the influence of breed cathegories on embryo production. The breed of sheep was cathegorized as prolific (Romanov and its F1, G1 crosses), mutton (Charollais and its F1 crosses) and fur (Karakul). A total 28 donors (Prolific breed: 16; Mutton breed: 7; Fur breed: 5) were superovulated using FSH-p with 200 mg NIH-FSH-P1 (total of 20 ml) (Folltropin-V; Vetrepharm, Canada) applied in 8 decreasing doses of 1.5, 1.5, 1.5, 1.25, 1.25, 1, 1, 1 ml i.m. at 12 h intervals, starting 60 h before sponge withdrawal. Donors recieved 1ml esturumate and 100 I.U. PSMG 36h prior sponge removal and an additional 200 I.U. PMSG were injected at sponge removal. Donors underwent intrauterine insemination with fresh diluted semen (10 x 107) 40 h after sponge removal. Ewes were tested for estrus and 6 or 7 d later were laparotomized and surgically flushed to recover embryos. The number of corpora lutea (CL), the total number of embryos and of viable embryos were recorded. Embryo recovery was performed according to a procedure previously described (Naqvi et al., 2000). The donor ewe breed was a significant source of variation in the results of embryo transfer. Percentage of superovulation response ranged 60% to 85%, number of CL per collection ranged between breeds from 9.7 to 15.3, percentage of embryo recovery rates from 41% to 53% and the avarage number of transferrable emrbyos from 4.0 to 8.0. Prolific breed was recorded with higher 15.25 ± 2.06 CL than mutton ewes (11.1±2.70) and fur breeds (9.67±4.13). The most transferable embryos were collected from prolific breed (8.75±2.00), and followed by mutton (4.7±2.62) and fur (4.0±4.01). Superovulatory response to superovulation varied between breeds (prolific: 75%, mutton: 85%, fur: 60%) but this did not account for mutton and fur breed differences in total CL number and embryo production. Fertilization failure and/or degeneration of embryos and recovery rates of viable embryos in prolific breed of donors with higher ovulation rates were similar with mutton and fur breeds with lower ovulation rates.

Notes

A SIMPLE METHOD FOR OESTRUS SYNCHRONIZATION OF GUINEA PIG (*CAVIA PORCELLUS*)

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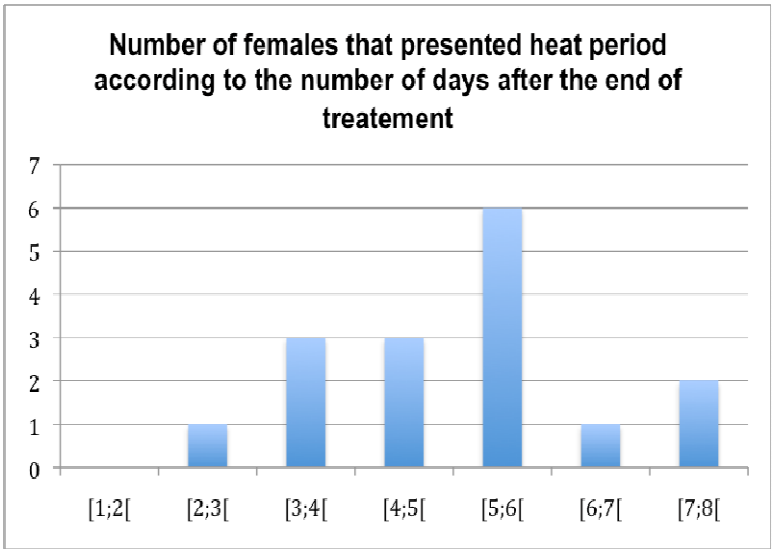
Guinea pigs (*Cavia porcellus*) play an important role in the Andes societies as a source of protein for many low income highlanders and as part of rituals and traditional medicines. It is also used as a laboratory animal since the late 18th century and still remains essential in many research areas. Thus, the conservation of this genetic diversity is a long term issue, we must take care from now on. The establishment of an embryo cryobank is an interesting way to reach this purpose. In order to produce guinea pig embryos and to apply the technique of embryo transfer, it is necessary to control the females oestrus cycle. Controlling the heat period is necessary to obtain embryos at morula/early blastocyst stage and to prepare synchronous recipients for embryo transfer. A classic treatment exists to synchronize ovulation under laboratory conditions. It consists of the implantation of a subcutaneous tube filled with crystalline progesterone over a period of 4 weeks. Synchronized ovulation is induced at any stage of the oestrous cycle within 5-6 days after removal of the implant. This treatment is demanding to set up in farming conditions and presents ethical problems regarding the use of anaesthesia and the large size of the implant (1.0 cm long and 0.4 cm i.d.). The aim of this work was to propose an alternative method to obtain synchronized ovulation in guinea pigs under farming conditions using oral progesterone in respect of animal welfare.

Nineteen multiparous guinea pigs of the Cieneguillas strain (La Molina National Agrarian University Lima-Peru) were used. Animals were housed under farming conditions (groups of 5 females together living on vegetal litter) and were fed on commercial pellets and tap water *ad libitum*. Guinea pigs were given a daily 0.1mL dose of oral progesterone (Regumate® Equine, Intervet, France) *per os* by means of a syringe for 20 days. Beginning of the heat period was determined when females presented an open vagina. Overall, 84% of the treated females (n=16) presented heat period within 7 days after the end of the treatment and the vagina was open in average 5.06+/-1.36 days after the last administration of Regumate. The distribution of females is shown in the attached graph.



These results show that treatment using oral progesterone is effective to obtain synchronized ovulation in guinea pigs. But only 63% of the treated females (12/19) presented heat period within 3-6 days after the last administration *per os* of Regumate. Such an oestrus spreading remains a problem for embryo-collection and transfer. In order to obtain a more accurate synchronization, we will adjust the dose of orally progesterone, the length of the treatment and we will use vaginal smears to have a better control of the time of ovulation.

Notes

INFLUENCE OF CLIMATIC FACTORS ON CONCEPTION RATE OF HOLSTEIN COWS REARED UNDER SEMIARID CONDITION

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This study was conducted at the Experimental Station of São Bento do Una- (ESSBU/IPA) Pernambuco, Brazil, (Latitude 08 31' 35'' and Longitude 036 27' 34.8") with the aim to study the correlation between conception rate (CR) and climatic factors such as ambient temperature, relative humidity and rainfall under semiarid condition over a period of 12 months. The herd of ESSBU/IPA is composed of 105 Holstein cows in reproductive age, with an average milk production of 7,200 kg/cow/lactation in 305 days. Females of different categories were used: Nulliparous, Primiparous and Pluriparous. Females used were in the voluntary waiting period (VWP), being kept in semi-intensive rearing, receiving a diet consisting of cactus pear (*Opuntia ficus indica* Mill), sorghum silage (*Sorghum* bicolor (L.) Moench) and protein concentrate with 18% of crude protein (CP), and mineral supplement and water ad libitum. All cows that presented estrus at the VWP were subjected to conventional AI with semen from bulls of a reputable central, and AIs were performed 12 hours after start of estrus behavior. The pregnancy diagnosis was performed by rectal palpation aided by ultrasound between the 45th and 60th days after AI. The data of monthly average temperature (°C), relative humidity (%) and rainfall (mm) were obtained from local meteorological station. The ambient temperature ranged from 19.7 to 27.3°C, the relative humidity from 52.9 to 83.3% and rainfall from 0.0 to 215.9 mm and the average CR was 44.6%. There was no correlation ($P > 0.05$) between CR and climatic parameters; temperature ($r = -0.2966$), relative humidity ($r = 0.2452$) and rainfall ($r = -0.0691$).The CR in each category was 56.00% in Nulliparous, 43.75% in Primiparous and 34.06% in Pluriparous, being recorded higher a CR ($P < 0.05$) for Nulliparous. Therefore, we conclude that in the conditions in which this study was conducted, climatic factors do not interfere with Holstein cows CR, whether reared in conditions of semiarid, and that the Nulliparous had a better result probably due to their uterus conditions.

Notes

EMBRYO DEVELOPMENT AND PREGNANCY RATE AFTER DIRECT TRANSFER OF CRYOPRESERVED IN VITRO CULTURED BOVINE EMBRYOS IN SOF MEDIUM

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The recent development of genomic selection in cattle induces dramatic changes in genetic selection strategies. In this context, breeding companies expect to increase the number of candidates per dam by means of ovum pick-up followed by in vitro embryo production (OPU-IVP) technologies. Therefore a reliable and easy applicable IVP and embryo transfer procedure is of great importance. The method should deliver good embryo development and high pregnancy rates after transfer of fresh and frozen embryos. Moreover, simplification and standardization of in vitro culture conditions limit problems due to variations of biological fluids and co-culture with epithelial cells.

The objective of this study was first to compare embryo development of bovine oocytes produced in SOF defined medium and in our reference method based on a Vero co-culture system. And secondly, to study the effect of SOF medium on in vitro survival rate after freezing and thawing and pregnancy rate after direct transfer.

Cumulus-oocyte complexes (COCs) were aspirated from slaughterhouse ovaries. Grade 1 to 3 COCs were matured in M199 with 10% FCS, FSH/LH. After a 22 h maturation period, oocytes were incubated in fert-TALP with frozen-thawed semen for 18 h. Culture was either performed on vero cell monolayers in B2-medium (Guyader-Joly et al., 2000) or in SOF_{AA} with BSA (van Wagtendonk et al., 2000). At day 7, blastocyst rate and quality were recorded (table 1). Grade 1 expanded blastocysts were selected for slow freezing (Guyader-Joly et al., 2009). In vitro survival rate was estimated for embryos cultured in SOF. Pregnancy rate after direct transfer of SOF cultured frozen embryos into synchronized heifers in UNCEIA experimental farm was diagnosed by ultrasonography on days 35 and 60.

Table 1. In vitro embryo development rates in SOF and Vero co-culture system.

IVC system	n	Fertilized (n)	Cleaved (n)	Blastocysts day7 (n)	
				Total	Grade 1
Vero co-culture	252	88.9 (224)	82.1 (207)	25.8 (65)[a]	18.7 (47)
SOF medium	655	93.7 (614)	88.4 (579)	32.5 (213)[b]	20.0 (131)

Different letters within columns differ significantly (P<0.05).

Post-thaw survival and subsequent development were assessed on a total of 98 SOF cultured embryos. Re-expansion and hatching rates of 92.8% (91/98) and 86.7% (85/98) were found respectively 48 and 72h after thawing. Direct transfer of 28 frozen embryos resulted in a pregnancy rate of 57.1% (16/28) at day 35 and this was confirmed at day 60.

These results show that the blastocyst rate after IVP in SOF medium is higher than in Vero co-culture. Good in vitro survival rates and pregnancy rates after slow freezing and direct transfer of embryos cultured in SOF medium were found. Combining IVP in SOF medium with slow freezing direct transfer technique simplifies the use of OPU-IVP in future breeding strategies, since increased numbers of pregnancies with frozen IVP embryos were observed together with easier ET procedures.

Notes

GROWTH MEDIUM EFFECTIVENESS DURING IVM OF SMALL PREPUBERTAL GOAT OOCYTES ENHANCES SURVIVAL RATES OF VITRIFIED BLASTOCYSTS

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Previous studies in bovine showed that differences in survival and developmental rates in vitrified/warmed *in vitro* produced blastocysts depend on its developmental stage (Morató et al., 2010) and the culture medium supplementation (Gomez et al., 2008). In this study we investigated the effect of growth medium (GM; Wu et al., 2006) during *in vitro* maturation of prepubertal goat oocytes on blastocysts ability to survive the vitrification and warming procedure. The Cumulus-Oocyte Complexes (COCs) were recovered from prepubertal (1-2 months old) goat ovaries by slicing. COCs with a compact cumulus and homogeneous cytoplasm were selected and classified into two categories based on oocyte diameter: <125 µm and ≥125 µm. The ≥125 µm oocytes were matured in groups of 25–30 COCs/ 100 µL drops of conventional IVM medium covered with mineral oil for 24h (**Treatment A**: TCM199 supplemented with 10% Donor Bovine Serum (DBS), 10 µg/mL FSH, 10 µg/mL LH, 1 µg/mL 17β-oestradiol and 100 µM cysteamine). The <125 µm oocytes were distributed into 2 experimental groups. **Treatment B:** COCs matured in the conventional IVM medium. **Treatment C**: COCs cultured in GM (TCM 199, 10% DBS, 0.04 µg/mL FSH, 0.04 µg/mL LH, 0.004 µg/mL 17β-oestradiol, 100 µM cysteamine, 100 µg/mL ascorbic acid and 5 µL/mL ITS: Insulin, Transferrin and Selenium) for 12h before being transferred for an additional 12h into the conventional IVM medium. After IVM, oocytes were fertilized with fresh goat sperm at a final concentration of $4x10^6$ spz/mL. Following fertilization, presumptive zygotes were cultured in synthetic oviduct fluid (SOF) for 8 days. At day 8, blastocysts were vitrified using the cryotop method described by Kuwayama et al. (2005). Treatments A, B and C were replicated four times and the results are shown in the table 1.

Table 1: *Cryotolerance assessed as blastocyst re-expansion at 3, 24 and 72h post-warming according to IVM treatment and oocyte diameter.*

Oocyte maturation treatments	*Oocyte diameter*	*Nº of vitrified blastocysts*	*% Re-expansion post-warming*		
			3h	*24h*	*72h*
Treatment A	**≥125 µm**	39	64.1 [a]	56.4	35.7 [a]
Treatment B	**<125 µm**	17	76.5 [b]	58.8	30.8 [a]
Treatment C	**<125 µm**	19	68.4 [ab]	57.9	53.3 [b]

Values in the same column with different letters (a,b) differ significantly (P<0.05).

The current study shows that the culture of small prepubertal goat oocytes in GM improves blastocyst quality assessed by the ability of blastocysts to survive vitrification and warming procedures.

Notes

INFLUENCE OF DIETARY CLA SUPPLEMENTATION ON LUTEAL MRNA EXPRESSION

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Early pregnancy loss in cattle is a major factor affecting production and economic efficiency. A functionally sufficient corpus luteum (CL) is essential for establishment of pregnancy. There is growing evidence that dietary supplementation of conjugated linoleic acids (CLA) has a positive impact on the reproductive performance as well as CL function (de Veth *et al.* 2009, Medeiros *et al.* 2010, May *et al.* 2011). This study was designed to test the hypothesis that a dietary CLA supplementation affects the mRNA expression of genes relevant for CL function.

Forty Holstein Friesian cows and heifers were randomly assigned to feeding groups that received different amounts of rumen protected CLA. Feed of the control group was supplemented with 0 g of CLA per day, group 1 and 2 with 50 g and 100 g of CLA per day, respectively. The CLA-supplement contained 10.5 % of cis9,trans11 derivate and 10.4 % of trans10,cis12 derivate of linoleic acid. Conjugated linoleic acids were added to diet from calculated day 14 ante partum. Oestrus of all animals was synchronized starting on day 59 post partum (± 3 days). Corpus luteum biopsies were taken of all animals on day 7 and day 14. The biopsies were analysed by RT-qPCR for the relative abundance of mRNA of different genes (VEGF, bCOX-2, 3ß-HSD, and STAR), which are essential for corpus luteum function.

Dietary supplementation of CLA significantly decreased relative mRNA abundance of StAR, 3ß-HSD, and bCOX-2 ($P \leq 0.05$). Additionally, day of cycle significantly increased the mRNA expression of StAR and 3ß-HSD ($P \leq 0.05$). Messenger RNA of VEGF was neither influenced by dietary treatment nor by day of cycle.

Dietary supplementation of CLA has an effect on the relative abundance of mRNA of genes with relevance for corpus luteum function.

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Notes

MATURATIONAL ENVIRONMENT AND CUMULUS INVESTMENT OF BOVINE OOCYTES CORRELATE WITH DEVELOPMENTAL COMPETENCE AND ZONA PELLUCIDA PROPERTIES AT THE SAME VALUE

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The developmental competence of bovine oocytes has been investigated due to a variety of cellular and subcellular markers. Unfortunately, these markers are often complex, time consuming and most importantly, invasive, which excludes further development of the oocyte or the embryo. The introduction of polarization light microscopy however, opens a new window for the noninvasive assessment of morphological zona pellucida properties of oocytes and embryos. The aim of this study was therefore firstly to evaluate the applicability of zona imaging to bovine oocytes and secondly to analyse different subpopulations of bovine oocytes, classified according to their cumulus cell investment as well as to their environment of maturation (in vivo vs. in vitro) with respect to their developmental competence and their zona pellucida properties, simultaneously. As an invasive technique, we analysed the zona pellucida of in vivo and in vitro derived oocytes by scanning electron microscopy to visualize alterations in morphological structure in the maturation period. Using polarisation light microscopy as a non-invasive technique, we consequently aimed to find out whether these structural changes are reflected in the birefringence. SEM analysis reveals a significantly higher proportion of in vivo matured oocytes displayed a porous zona structure with typical fine meshed reticular pores compared to in the vitro matured oocytes (31.3% vs. 100%; $p<0.05$). These differences are reflected in the zona birefringence, where we observed that the mean birefringence (CV-Mean) of in vivo matured oocytes (n= 21) was significantly lower ($p<0.05$) compared to their in vitro matured counterparts (n=247; 16.54 ± 2.41 vs. 20.76 ± 2.69). Within different COC qualities of in vitro matured oocytes a significant lower birefringence was observed for COC quality 1 (n=79) compared to quality 3 (n=77; 20.23 ± 2.69 vs. 21.75 ± 3.56). Accordingly, the thickness of the inner layer (WT-Mean) of in vivo matured oocytes was significantly thinner compared to in vitro derived MII oocytes (10.72 ± 1.48 vs. 12.39 ± 2.19). With respect to COC's quality, oocytes from quality 1 COC's displayed a significantly thinner inner zona layer compared to oocytes of quality 3 COC's (12.3 ± 1.50 vs. 12.95 ± 2.19). This is in accordance with the differences in developmental capacity. Here we observed a blastocyst rate of 39.1% for the in vivo matured oocyte, in contrast the overall in vitro matured oocytes reached a blastocyst rate of 21.6%. Within the group of in vitro matured oocytes a strong decreasing trend becomes obvious, from 27.7% for Q1 oocytes and 16.9% for Q3 oocytes. Conclusively, in vivo maturation leads to a decrease in ZPB whereas in vitro maturation overall increases birefringence. With respect to COC quality, only Q1 oocytes follow the trend of in vivo matured oocytes, whereas oocytes of quality 2 and 3 COC's increased in birefringence. In vivo maturation also leads to a decrease in the thickness of the inner zona layer in contrast to the average of in vitro matured oocytes.

To our knowledge, this is the first work, which proved successfully that COC investments as well as maturational environment are reflected in the ZPB of bovine oocytes affecting developmental capacity at the same value.

Notes

EFFECT OF SEMEN QUALITY ON FERTILITY AFTER HETERO- AND HOMOSPERMIC INSEMINATION IN CATTLE

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The objective of this study was to investigate the effects of bull semen quality on fertility after homospermic and heterospermic insemination. Ejaculates were collected during 64 weeks from 3 bulls, EDLOCK (Simmental), GLOBI (Limousin) and NEW TRANSIT (Angus) for the production of homo- and heterospermic (mixed semen, SILIAN-2) semen doses for artificial insemination. In homo- and heterospermic post-thaw semen samples sperm motility was evaluated using computer-assisted semen analysis (CASA) and DNA-, membrane- as well as acrosome integrity and the intracellular calcium concentration were analyzed by flow cytometry (FACS). For determination of homo- and heterospermic fertility in the field non-return rates (NRR) after first insemination were used. After heterospermic insemination of 237 Swiss Fleckvieh as well as 201 Brown Swiss cattle the paternity of calves originating from SILIAN-2 was verified pheno- and genotypically.

The results show that the bull had a significant influence on all parameters evaluated in the frozen-thawed semen. Most obvious were the differences between the semen of bull GLOBI and the mixed semen SILIAN-2 (all parameters) and between GLOBI and the other two bulls EDLOCK and NEW TRANSIT (8 of 11 parameters). Regarding the paternity of calves originating from heterospermic insemination with SILIAN-2, a significant effect of the dam breed was obvious. In calves born from Fleckvieh dams 58.7% were sired by EDLOCK, 26.6% by GLOBI and 14.2% by NEW TRANSIT whereas in calves from Brown Swiss dams 50.8% were originating from GLOBI, 27.2% from EDLOCK und 21.5% from NEW TRANSIT.

From our study it can be concluded that the heterospermic quality of SILIAN-2 was better than the best bull present in the mixed semen. After heterospermic insemination the breed of the dam had a clear effect on the paternity of the calves. In Swiss Fleckvieh dams most calves were sired by the Simmental bull EDLOCK and in Brown Swiss dams the Limousin bull GLOBI was most often the father.

Notes

MORPHOLOGICAL SPECIAL FEATURES OF PREIMPLANTATION BUFFALO EMBRYOS (BUBALUS BUBALIS)

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Embryo transfer in cows has a nearly 60 year´s history and today it is applied commercially in developed countries. However, in buffalo the commercial use of this technique started as recently as a couple of years ago. The pioneer studies have clearly shown that the application of the bovine embryo transfer technology directly to buffalo generally was accomplished resulting in limited success. More efforts have to be done to increase the knowledge about the basic parameters that mainly influence the success of this method in buffalos. As a result of our first experiment in the field of embryo transfer in 1987, the first live buffalo calf was born in Europe (Bulgaria) and the second in the world.

Morphological examinations have been carried out in 123 preimplantation embryos, obtained from super ovulated and inseminated buffalos (Bubalus bubalis), after non-surgical flushing and after flushing of the reproductive tract of slaughtered animals. Embryos collected between 56 to 162 hrs after the beginning of oestrus and during the utero-tubal migration were assessed by determining the morphological peculiarities as well as the developmental dynamics. Llight-microscopic observations and evaluations of native embryos showed that they have a proper spherical shape with diameter of 170 μm including a zona pellucida with a thickness of 12 μm. The vitellus in the blastomeres at the various stages of embryo development is filled with many granules and possesses high optical density, which determines the dark brown colour of embryos. A significant difference in the speed of development was also found, which exceeds that of cattle with 50-60 hours. The observation of embryos during the late morulae and hatched blastocysts stage, between the 114th and 116th hour after beginning of the oestrus, revealed that buffalo embryos develop very rapidly from the stage of compacted morula to the stage of hatched blastocyst. The process of hatching of buffalo blastocyst is completed approximately 120 hrs following oestrus detection. Hatching of blastocysts in buffalo occurs earlier - on the 6th days, unlike bovine embryos - on the 9th days after beginning of the oestrus. Other special feature are that the buffalo embryos enter the uterus at the 4th day after beginning of the oestrus and at a more advanced stage of development compared with the bovine species.

It is concluded that the measured and recorded morphological characteristics of buffalo embryos including the developmental dynamics are a helpful prerequisite for successful application of modern reproductive biotechnologies in this species.

Notes

INFLUENCE OF ESTRADIOL-17BETA AND PROGESTERONE ON CHANGES OF EXPRESSION OF *ZONA PELLUCIDA* GLYCOPROTEINS IN CANINE OOCYTES*

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The role of progesterone (P4) and estriadol-17beta (E2) in *in vitro* maturation efficiency with special relation to reach developmental or nuclear competence of oocytes is well recognized in canine species. However, still little is known about the influence of these hormones used alone or in combination on the expression of *zona* glycoproteins. Therefore, this study aimed to investigate whether genes, encoding *zona* glycoproteins, are up-or down-regulated by E2 and P4.

Oocytes were cultured in the serum-free tissue culture medium (TCM-199) for 72 h supplemented with (i) E2 (2.0 µg/ml) or (ii) E2 plus various concentrations of P4 (0.5, 1.0 or 2.0 µg/ml). Oocytes cultured *in vitro* without any hormone supplementation, were used as control. We employed the RQ-PCR and western-blot analysis to analyze the ZP2 and ZP3 mRNA as well as ZP3 and ZP4 protein expression in canine oocytes.

As a result, we found a decreased expression of ZP2 mRNA, as compared to control, after supplementation of E2 alone or in combination with each concentration of P4 ($P<0.001$, respectively). ZP3 expression was higher after supplementation of E2 alone and E2+P4 (0.5 µg/ml), ($P<0.01$, $P<0.001$, respectively). Similarly to ZP2, the dose of E2+P4 (1.0 and 2.0 µg/ml) inhibited the level of ZP3 transcript ($P<0.001$, respectively). Regarding the protein expression, we found that E2 used alone or in combination with P4 (0.5 µg/ml) has a stimulatory effect on ZP3 and ZP4 expression, ($P<0.01$, $P<0.001$, and $P<0.05$, $P<0.01$, respectively). Contrary, the dose of E2+P4 (1.0 and 2.0 µg/ml) inhibited the expression of both, ZP3 and ZP4 proteins ($P<0.001$, $P<0.05$, and $P<0.01$, respectively).

In conclusion, we demonstrated that (i) E2 used alone or in combination with P4 (0.5 µg/ml) up-regulates the expression of ZP3 mRNA as well as ZP3 and ZP4 protein in canine oocytes, (ii) ZP2 mRNA, which has been implicated as a secondary sperm receptor, is down-regulated by E2 alone or by E2+P4. Moreover, *zona* glycoproteins may be up-or down regulated by E2 alone or in combination with P4. This synergistic effect is a P4 concentration dependent manner.

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Notes

CHANGES IN THE PARAMETERS OF THE PORCINE REPRODUCTIVE SYSTEM AFTER INJECTION OF ORGANIC SELENIUM

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Organic selenium has big advantages over inorganic selenium, such as toxicity and the high bioavailability of organic selenium. Therefore the use of organic selenium supplementation in animal feed has become attractive since organic selenium is the main source of selenium for animal nutrition.

The main disadvantage of the commercially available organic selenium, selenium-enriched yeast, is to calculate and balance the dietary intake of this important trace mineral to optimize swine reproduction.

In the present work we investigated the effect of selenium organic compound selenopyran on the reproductive system of growing gilts. 18 Danube white breed gilts between 120 and 228 days of age where randomly divided into two groups. All animals received the same basal diet without selenium. The gilts of the experimental group were injected intramuscularly with an oil solution containing 0.1μg/kg live weight selenopyran (9-phenyl-symmetrical octahydroselenoxanthene) once per month. At the first and the last day of the experiment the blood samples were taken from sinus ophtalmicus for the analysis of plasma levels of selenium and oestradiol-17β. After slaughtering the morphometric analysis of reproductive organs (uterus, cervix, horns and oviducts) and the histological analysis of ovaries were performed. The concentration of the selenium in ovaries was estimated by atomic absorption spectroscopy. Data were analysed by computer software STATISTICA (Ver.6.0 of the Stat Soft Inc.).

The selenopyran treatment leads to an increase of selenium levels in blood ($P< 0.05$). No significant differences between the morphometric parameters of reproductive organs were found, but the ovaries of the gilts in the experimental group showed a higher number of preovulatory follicles. High positive correlations were found between the selenium concentration in ovaries and ovarian weights ($r=0.76$; $P<0.05$) as well as between the selenium concentration in ovaries and the serum levels of estradiol-17β ($r=0.78$; $P<0.01$).

Notes

EFFECT OF SOMATOTROPIN ON DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES SELECTED BY BRILLIANT CRESYL BLUE STAINING

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Bovine oocytes matured in the presence of somatotropin (rbST) and somatic cells show increased levels of respiratory activity which is associated with decreased levels of calcium from intracellular stores (Kuzmina et al., 2007, J Reprod Develop 53:309-316). In previous studies we demonstrated that BCB^+oocytes selected by brilliant cresyl blue (BCB) had significantly higher blastocyst development than did BCB^- oocytes (Alm et al., 2005, Theriogenology 63:2194-2205). The aim of the present study was to evaluate the influence of rbST supplemented maturation medium on the development of BCB selected bovine oocytes.

Before IVM compact cumulus oocyte complexes (COC) were incubated in BCB solution for 90 minutes. Treated oocytes were then divided into BCB^- (colorless cytoplasm, increased G6PDH) and BCB^+ (colored cytoplasm, low G6PDH) on their ability to metabolize the stain. Intensity of fluorescence of membrane-bound-calcium was fixed with a fluorescent microscope – excitation: 380-400nm, emission: 530 nm, using 40 µM chlortetracycline. The selected COC were matured in TCM 199 + 10% FCS (v/v) and 10^6/ml granulosa cells (GC) either without (control) or with the addition of 10 ng/ml rbST (experimental group). After IVM oocytes were fertilized in vitro and embryos were cultured by standard protocols. The cleavage rate including status of chromatin (cytogenetic analysis by Tarkowsky, 1966) and the blastocyst rate was evaluated. Data were analyzed by Chi^2 and Student's t-test.

Fluorescence intensity of complexes of Ca^{2+} + chlortetracycline+membrane in intracellular stores of BCB^+ oocytes was higher than in BCB^- oocytes (0.86 ± 0.063 C.U. vs. 0.67 ± 0.049 C.U). The BCB^+ oocytes yielded a significantly higher proportion of blastocysts (38.9%) by the addition of rbST in comparison to the control (19.7%), and both BCB+ oocytes had significantly higher blastocyst development than did BCB^- oocytes (19.1 and 4.2%). The number of nuclei in the blastocysts was significantly increased in BCB^+ oocytes of the rbST group (121 ± 6.56 vs. 103 ± 4.60). The addition of rbST improved the development and nuclei number in both BCB^+ and BCB^- oocytes.

Table 1. Effect of rbST on development of bovine oocytes selected by brilliant cresyl blue staining

Treatment during IVM	BCB	n oocytes	8-16 cells embryos n (%)	Blastocysts n (%)
TCM 199 + 10% FCS + 10^6 GC	+	151	94 (62.3)[a]	30 (19.7)[e]
TCM 199 + 10% FCS + 10^6 GC	-	143	70 (48.9)[b]	6 (4.2)[f]
TCM 199 + 10% FCS + 10^6 GC + 10ng/ml rbST	+	149	115 (77.2)[c]	58 (38.9)[g]
TCM 199 +1 0% FCS + 10^6 GC + 10ng/ml rbST	-	141	84 (59.6)[d]	27 (19.1)[d]

a :b;a :c ;b:c; b:d; c:d;e:f; e:g; f:g;f:d;g:d $P<0,05$ (χ^2 test)

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Notes

EFFECT OF TREATMENT WITH THE HISTONE DEACETYLASE INHIBITOR, SCRIPTAID, ON DEVELOPMENT AND QUALITY OF IN VITRO PRODUCED BOVINE EMBRYOS

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Histone acetylation and DNA methylation play a key role in the remodeling process of the genome, and are linked with gene expression and imprinting in the early embryo in mammals. Treatment of somatic cell nuclear transfer embryos with trichostatin A (TSA), a specific inhibitor of histone deacetylase, improves cloning efficiency to term. It has been showed that bovine mature oocytes express histone acetyl transferases and deacetylases and they are thought to function in remodeling of gamete genomes after fertilization to establish a totipotent state for normal development. The aim of the present study was to investigate the effect of the histone deacetylase inhibitor, scriptaid (SCR), which has lower toxicity than TSA, on embryo development, and embryo quality in bovine *in vitro* produced embryos and to determine the optimal exposure time. Presumptive zygotes (n=5184) were produced by in vitro maturation and fertilization of oocytes derived from the ovaries of slaughtered heifers. Culture of 20-25 presumptive zygotes took place in 25 µl of synthetic oviduct fluid (SOF) supplemented with 5% of FCS (C:control) as a basal medium or an additional supplementation with 100 nM SCR from day 1 to day 7 (H_{1-7}), from day 3 to day 7 (H_{3-7}) and from day 5 to day 7 (H_{5-7}) at 39ºC under an atmosphere of 5% CO_2 and 5% O_2 in air. Cleavage rate and blastocysts yield were measured at 48 h and on days 7 and 8 of culture, respectively (Day 0=day of fertilization). A representative number of blastocysts on day 7 from each experimental group were used for evaluating their quality through: (i) differential cell count and (ii) vitrification/warming. Furthermore, blastocysts from each experimental group were frozen in Liquid Nitrogen for gene expression analysis. In relation to embryo development, no differences were found between control and experimental groups (blastocyst yield on day 7 (C:31.59±2.52; H5-7:29.91±2.40; H3-7: 31.88±2.95; and H1-7:30.40±1.95). In terms of total cell number (range from 144.45±3.65 to 157.35±4.74) or trophectoderm cells (range from 104.03±3.20 to 113.88±4.01) no significant different was found between groups. However, significantly more cells in inner cell mass were observed in control group when compared with all other groups (C: 50.24±2.33; H5-7: 42.69±1.54; H3-7: 39.72±1.66 and H1-7: 43.46±2.24, $P \leq 0.05$). In terms of survival rate after vitrification, no differences were found between groups at 24 or 48 h after warming. However, more embryos (%) were re-expanded at 48 h from the H_{1-7} group (30.96±11.89) compared with the rest (C:21.39±9.31; H_{5-7}:19.58±9.25; and H_{3-7}:18.09±9.56). In conclusion, these results show that the use of SCR at 100 nM along the early stages of embryo development *in vitro* in bovine does not affect embryo production. However, the increase in embryo survival found when SCR was used for the entire culture period, can have a positive effect on embryo quality. Embryo gene expression analyses are in progress to identify potential changes at molecular level.

Notes

CONSEQUENCES OF SUBCLINICAL AND CLINICAL ENDOMETRITIS ON BOVINE EARLY EMBRYO DEVELOPMENT AND FERTILITY

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Fertility in bovine herds is decreasing especially in dairy cattle under intensive farming conditions. Several factors are involved, including high milk production, nutrition, reproductive management and uterine diseases. Recently it has been suggested that the uterine inflammation associated with subclinical endometritis may perturb early embryo development and reproductive fitness.

The aim of this study was to further investigated the influence of uterine inflammation on embryo development and fertility. For this purpose we performed gynecological examination and uterine flushings of 221 dairy cows, randomly selected in 2 large dairy herds of the Lombardy region, 40-60 days post-partum. The cows were allocated to 3 groups depending on the presence of inflammatory cells in the flushing fluid (5% threshold) and on the results of the clinical examination: CIT- (healthy cows, ≤5% neutrophils), CIT+ (healthy cows, >5% neutrophils), METR (cows with clinical endometritis). Long term effects on fertility were determined by monitoring the reproductive performances of the herd for about a year following the microflushing procedure. Moreover, in order to determine the influence of the uterine environment on early embryo development, in vitro produced bovine blastocysts (day 7) were transferred in the uterus of 7 CIT- and 4 CIT+ synchronised cows (20 embryos per cow) and collected on day 12 to determine recovery rate. Three heifers were used as control recipients and received the same number of embryos.

Results of the cytology and clinical examination indicated that 53% of the cows were CIT-, 39% were CIT+ and 8% had METR. The monitoring of the herds reproductive records in the following 1 year period indicated that reproductive efficiency did not appear to be influenced by subclinical endometritis as measured by pregnancy rate. By contrast, a significant difference in the interval from calving to conception was found in the METR group vs CIT- and CIT+.

The embryo transfer experiments resulted in a higher recovery rate from CIT+ cows (31%) vs CIT- cows (17%) ($p<0.05$). Recovery rate from control heifers was significantly higher (67%) as compared to CIT- and CIT+ ($p<0.05$).

Overall these results indicate that a) subclinical uterine disease did not significantly affect reproductive efficiency in the 2 herds involved in this study, and that b) embryo survival was lower in the reproductive tract of cows compared with heifers.

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Notes

ULTRASOUND EVALUATION OF FETAL AND PLACENTAL DEVELOPMENT IN CLONE AND CONTROL BOVINE PREGNANCIES

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Since the first success in sheep, the production of viable cloned offspring by somatic cell nuclear transfer (SCNT) in various mammals has increased significantly. The incidence of pregnancy failure and foetal death, however, is still very high, whatever the species. In cattle, most gestation losses are initially due to early foetal losses during the pre-implantation period and thereafter to abnormal placentation associated with an overgrowth of the foetus known as the Abnormal Offspring Syndrome (AOS). As a consequence, the International Embryo Transfer Society (ETS) recommends in the IETS manual that regular ultrasound (US) evaluation of the SCNT foetuses are performed throughout gestation in order to terminate pregnancy if foetal and/or maternal survival are at risk. Although US criteria such as qualitative evaluation of the placentomes, foetal movement, and foetal fluids have been proposed, there is a need to establish new quantitative criteria that could be used for the diagnosis of AOS. The objective of this study was to evaluate the use of new, quantitative parameters, for the assessment of foeto-placental development in AI and SCNT bovine pregnancies.

Twenty-two heifers of 4 different breeds were used as SCNT recipients and 11 Holstein heifers were used as AI controls (C). All SCNT foetuses were produced as previously published in the laboratory, using the same fibroblast donor. Animals were scanned every two weeks from 150 days of pregnancy, using a Voluson-i (GE Medical Systems) equipped with a transabdominal multifrequency probe (2.2-6.5 MHz). For each examination, 7 placentomes were classified from 0 to 3 according to echogenicity, general appearance and degree of oedema. Intercoastal distances were measured in a coronal view just behind the heart. Doppler velocimetry indices, pulsatility index (IP) and resistance index (IR) of the foetal umbilical artery were obtained.

So far, 26 heifers have either delivered (N= 10 SCNT and 7 C) or pregnancies have been terminated due to AOS (N=9 SCNT). Preliminary results indicate that Inter-coastal space is increasing with gestational age ($p<0.0001$) and consistently larger in SCNT vs C ($p<0.0001$) at all stages of pregnancy in the third trimester. The mean placentome classification (for 7 placentomes/examination) is significantly higher (less normal) in SCNT compared to C but does not vary according to gestational age ($p<0.0001$). In contrast, the umbilical cord pulsatility index (PI) and the resistance index (IR) tend to decrease with pregnancy ($p<0.01$) but are not different between SCNT and C.

These preliminary data indicate that there are significant differences between SCNT and C pregnancies and further analyses are on-going taking into account pregnancy outcomes and the last on-going pregnancies.

Notes

EVALUATION OF A SIMPLIFIED SUPEROVULATION PROTOCOL IN BOER GOATS

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This study was conducted to evaluate the use of a simplified superovulation protocol in mature Boer goat does. Eighteen does used were allocated into two groups during the natural breeding season (autumn). Animals of Group 1 served as controls (n=9; traditional protocol using follicle stimulating hormone alone, FSH) and the experimental animals were allocated Group 2 (n=9; simplified protocol using one shot protocol of FSH plus equine chorionic gonadotrophin, eCG). All does were synchronised for oestrous using controlled internal drug release dispensers (CIDR's) for 9 days. Does in Group 1 were superovulated with 200 µg pFSH/doe administered i.m. in 7 dosages at 12 h intervals, starting 48 h prior to CIDR removal. In Group 2 (simplified protocol) the superovulation was performed by injection of 200 µg FSH plus 350 IU eCG at once 48 h prior to CIDR removal. Does were observed for oestrous behaviour twice daily, at 12 h intervals following CIDR removal for 96 h. Cervical inseminations (with 0.1 ml fresh undiluted Boer goat semen) were performed 24 h and 48 h following CIDR removal. Embryos were surgically flushed on day 6 following the second artificial insemination. The time interval from CIDR removal to onset of oestrus was not affected by treatment. The duration of the induced oestrus period however, was significantly longer in the experimental group (Group2) compared to the traditional protocol (Group1). The simplified protocol significantly reduced the response to superovulation based on the mean number of CL's, ova and embryos. A significantly ($p<0.01$) higher number of unfertilised ova were observed when applying the simplified protocol (4.06 ± 0.9) compared to the traditional protocol (0.24 ± 0.1). However, the numbers of degenerated embryos were not affected by treatment. The total number of transferable embryos was significantly lower in the simplified protocol (0.22±0.2) compared to the traditional protocol (9.56 ± 1.9). The size of follicles were not affected by treatment however, the total number of follicles at CIDR removal was significantly lower in the simplified protocol (11.67±0.7) compared to the traditional protocol (14.83±0.7). In conclusion, the simplified protocol increased the duration of the induced oestrus period and this observation might have an implication on timing of AI, as there was higher number of unfertilised ova from the simplified protocol. The simplified protocol also reduced the response to superovulation and therefore, the traditional superovulation protocol seemed to be a more efficient superovulation protocol in Boer goats. More research with regard to the quantity of two gonadotropins when administered as a one shot and timing of AI following the simplified protocol warrant further research.

Notes

VITRIFICATION OF PARTHENOGENETICALLY ACTIVATED PORCINE EMBRYOS

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Porcine embryos are sensitive to low temperatures, so it is a challenge to cryopreserve them, especially after in-vitro production. The aim of this study was to determine the optimal developmental stage of porcine parthenogenetically activated (PA) embryos for vitrification.

Oocytes were recovered from sow ovaries and in-vitro matured for 42h. Parthenogenetic activation was made on Day 0 first by an electric pulse (1.26 kV /cm, 80µs) and then by incubation with 5µg/ml cytochalasin B and 10µg/ml cycloheximide in PZM-3 medium supplemented with 4mg/ml bovine serum albumin for 4h. PA embryos were vitrified by Cryotop on Day 4, 5 or 6, and they were time-lapse monitored for 24h after warming.

The results are summarized in Table 1. From 8h after warming, the survival rate was stable and embryos started to re-expand in all groups. Both the survival rate at 8h and the hatching rate at 24h after warming of Day 4 embryos were significantly higher than those of Day 5 and 6 ($P<0.05$), no matter whether they were morulae or blastocysts.

The results indicate that porcine PA embryos can survive well after vitrification (app. 60%), that the optimal time for vitrification is Day 4 for both morulae and blastocysts, and that 8h after warming is the optimal time-point to make an early evaluation of porcine PA embryo survival.

Table 1: Recovery of porcine PA embryos vitrified at day 4, 5 and 6 and subsequent warming.

Group	Total embryos (replicates)	Survival rate± SEM 4h after warming (no.)	Survival rate± SEM 8h after warming (no.)	Hatching rate ± SEM 24h after warming (no.)
Day 4 Morula	53 (4)	70.0±10.4 (38)[a]	66.7±12.8 (34)[a]	61.9±10.1 (32)[a]
Day 4 Blastocyst	54 (4)	68.8±13.8 (36)[a]	61.7±15.3 (32)[a]	59.6±14.9 (31)[a]
Day 5 Blastocyst	120 (8)	62.2±9.6 (73)[a]	41.7±10.6 (48)[b]	33.7±7.9 (40)[b]
Day 6 Blastocyst	53 (4)	21.0±3.9 (11)[b]	8.9±2.7 (5)[c]	2.0±1.6 (1)[c]

[a, b, c] Different letters in the same column indicate significant difference *(P<0.05)*

Notes

INCREASED CLONING EFFICIENCY USING PORCINE ADOLESCENT FIBROBLASTS WITH *XENOPUS* EGG EXTRACT TREATMENT

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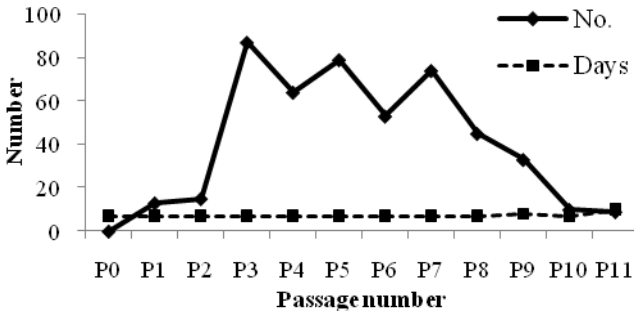
Extract from *Xenopus* eggs has a reprogramming ability on mammalian somatic cells, as also we have previously shown on porcine fetal fibroblasts (Liu et al. 2011) with induced cell colony formation and increased cloning efficiency. These effects were maintained in extract-treated cells for up to 15 passages (Liu et al. 2011). The aim of this study was to investigate porcine adolescent fibroblasts after *Xenopus* egg extract treatment with respect to their morphology and their effect after subsequent use in somatic cell nuclear transfer.

Adolescent fibroblasts were established from an ear biopsy of a 3-month Yucatan pig, and extract treatment was performed as described previously (Liu et al. 2011). Briefly, the fibroblasts grown on poly-lysine coated coverslips were permeablized by 7 µg/ml digitonin and incubated in *Xenopus* egg extract for 30 min. After resealing the cell membrane in 2 mM $CaCl_2$, the attached cells were cultured in embryonic stem cell (ES) medium for further 7 days (Vejlsted et al. 2005), defined as ExT P0. The ExT cells were subsequently passaged in ES medium. Cell clusters resulting in colonies started to form from ExT P1, and for each passage colonies were passaged when 70-80% clusters had become colonies. No colonies were observed in control cell cultures. Colony cells at ExT P6 and P8 were isolated and trypsinized into single cells, and they were used for handmade cloning (HMC). Non-treated cells grown in DMEM were used as control. Blastocyst rates were analyzed with Chi-square test (SAS version 9.2).

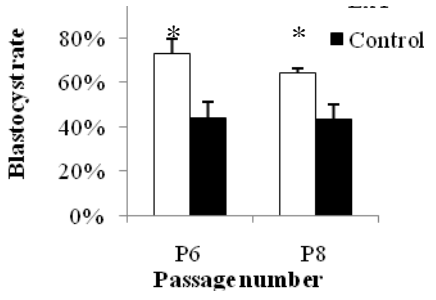
After extract treatment, no colonies formed in ExT cell before being passaged (P0). The first peak number of colonies in ExT cells was seen at P3, and then a plateau level of colony formation was maintained until P7 followed by a gradual decrease to a low level at P11 (Figure 1). Before P11, the time of colony formation for each passage was 7 days (Figure 1). In three replicates, the blastocyst rates of cloned embryos produced with colony cells used at P6 and P8 were both higher than their control (P<0.05; Figure 2). In conclusion, the *Xenopus* egg extract treatment could induce colony formation and increase *in vitro* cloning efficiency (shown as blastocyst rate) in porcine adolescent fibroblasts. However the maintenance of the effect was not as long for adolescent cell as for fetal fibroblasts, 7 vs. 15 passages (Liu et al. 2011), indicating the adolescent fibroblasts are easier to re-differentiate again after extract treatment compared with fetal fibroblasts.

Figure 1. Colony formation in extract treated porcine adolescent fibroblasts



No.: colony number in each passage;
Days: the time of colony formation in each passage

Figure 2. Blastocyst rate of cloned embryos produced with ExT colony cells or control cells



* significant difference, P<0.05

Notes

EMBRYO PRODUCTION BY *IN VITRO* FERTILIZATION OF PREPUBERTAL GOAT OOCYTES WITH CRYOPRESERVED EPIDIDYMAL SPERM OF SPANISH IBEX (*CAPRA PYRENAICA HISPANICA*)

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The Spanish Ibex (*Capra pyrenaica hispanica*) is a wild caprine phylogenetically related to the domestic goat (*Capra hircus*) and is listed as a threatened species in the International Union for Conservation of Nature (IUCN) Red list (IUCN, 2010). Epididymal spermatozoa can be retrieved from dead wild species and hunter-killed males represent an available source of sperm in species in which semen collection is difficult. Conventionally, evaluation of spermatozoa includes sperm motility, integrity of the acrosome and membrane. A heterologous *in vitro* Fertilization (IVF) assay could be a useful method to evaluate the fertility of wild species sperm because oocytes from domestic animals are easily accessible from slaughterhouses. Our aim was to evaluate the embryo development after IVF with cryopreserved Spanish ibex epididymal spermatozoa and prepubertal goat oocytes.

Prepubertal goat Cumulus Oocyte Complex (COC) were matured in *in vitro* maturation (IVM) medium (TCM199 + hormones + 10% donor bovine serum) for 24 h at 38.5 ºC in a humidified atmosphere of 5% CO_2. Thawed Ibex epididymal spermatozoa were selected by centrifugation on discontinuous gradients (OviPure®) at 1500 rpm/10min. After IVM and sperm selection, gametes were co-incubated in SOF + 2% oestrus sheep serum in a humidified atmosphere of 5% CO_2 and 38.5 ºC. At approximately 22 hours post-insemination, presumptive zygotes were denuded by gentle pipetting and transferred to 20 µL culture droplets of SOF in a humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2 at 38.5 ºC for 7 days and at day 5 the embryos were transferred to microdroplets with fresh medium. Cleavage and blastocyst rates were evaluated on days 2 and 7 post-insemination, respectively.

From a total of 147 IVM-IVF oocytes, the cleavage rate on day 2 was 26.5% (39) and the blastocysts rate on day 7 was 8.8% (13). These percentages are lower than those found by Romaguera et al. (2010) in our laboratory using ejaculated fresh semen from domestic goats (60.3 and 16.1%, respectively).

In conclusion, the low embryo development rate found could be explained because we worked with heterologous species and frozen-thawed epididymal sperm. However, this work is an example that it is possible to use in vitro fertilization of prepubertal domestic goat oocytes with epididymal sperm from wild goats and it can be used as a model for other C*aprine* wild species.

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Notes

MORPHOLOGY OF MITOCHONDRIA IN BOVINE OOCYTES WITH DIFFERENT MEIOTIC COMPETENCE DURING THEIR IN VITRO MATURATION

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Oocytes acquire cytoplasmic maturation competence after the synthesis of mRNA and proteins have finished and re-distribution of organelles, such as mitochondria, has occurred in the ooplasm. In bovine oocytes, both cytoplasmic and meiotic maturation competences closely correlate with the size of follicles from which the oocytes have been recovered. During oocyte maturation, mitochondria undergo morphological transformation from diffused through granular to clustered patterns. The present study was designed to characterize changes in mitochondrial morphology during maturation of bovine oocytes differing in their meiotic competence. Oocytes were obtained from ovaries of cyclic cows in the stagnation and regression phases, as checked by CL and follicular morphology. Only morphologically good oocytes recovered from medium (MF, 6-10 mm) or small follicles (SF, 3-5 mm) were matured, as separated subpopulations, using a standard protocol. At 0, 3, 7, 16 and 24 hours, the oocytes were denuded from cumulus cells, stained with MitoTracker Orange, fixed with paraformaldehyde and examined by confocal microscopy. Only oocytes at an adequate stage of chromatin configuration were evaluated for mitochondrial morphology. The results were analyzed by the Chi-square test. Before maturation, a significantly higher ($P<0.01$) proportion of oocytes with granulated mitochondria and a lower proportion of oocytes with diffused mitochondria were found in MF-oocytes, as compared with SF-oocytes (61.8% vs 36.7% and 38.2% vs 63.3 %). During maturation of MF-oocytes, these was a significant increase ($P<0.05$) in the proportion of oocytes with clustered mitochondria at all evaluated intervals, as shown in Figure 1.

Figure 1: Morphology of mitochondria in oocytes derived from medium follicles

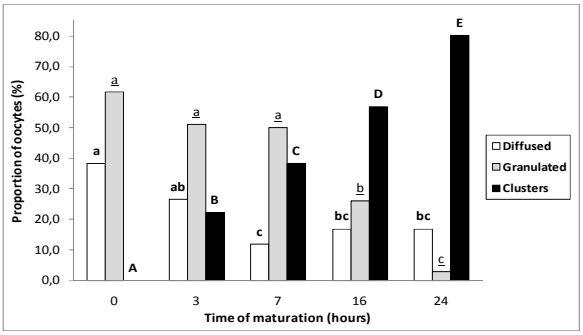
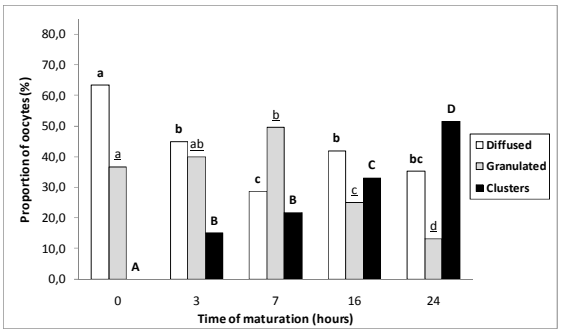


Figure 2: Morphology of mitochondria in oocytes derived from small follicles



(A-E; **a-c**; a-d) Values with different indexes above columns are significantly different ($P<0.05$)

A increasing trend in proportion of oocytes with clustered mitochondria found in MF-oocytes during maturation was also observed in SF-oocytes, but for the 3-to-7-h interval (Figure 2). After maturation, a significantly higher ($P<0.01$) proportion of oocytes with clustered mitochondria and a lower proportion of oocytes with diffused mitochondria were detected in MF-oocytes, as compared with those in SF-oocytes (80.3% vs 51.5% and 16.9% vs 35.2%).

It can be concluded that bovine oocytes differing in meiotic competence differed in the kinetics of changes in mitochondrial reorganization. The morphological development of mitochondria was accelerated between 3 h and 7 h of maturation in oocytes with greater meiotic competence, as compared with oocytes with lesser meiotic competence.

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Notes

EFFECT OF LACTATION ON CIRCULATING METABOLIC HORMONES POSTPARTUM AND EARLY EMBRYO DEVELOPMENT IN DAIRY COWS

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Infertility in dairy cattle is a multifactorial problem, which may be linked to follicle development and the quality of the ovulated oocyte, sperm transport and fertilization and/or the reproductive tract environment. The aim of this study was to examine the effect of lactation on the ability of the reproductive tract of postpartum dairy cows to support early embryo development. Twenty primiparous Holstein heifers were used. Immediately after calving, half of the cows were dried off (i.e., not-milked) while the other half entered the milking herd and were milked twice daily. At approximately 60 days postpartum, the oestrous cycles of all cows were synchronized and on Day 2, approximately sixty-five 2- to 4-cell embryos, produced by in vitro maturation and fertilization of oocytes derived from the ovaries of slaughtered heifers, were endoscopically transferred to the oviduct ipsilateral to the corpus luteum. Five days later, on Day 7, the oviduct and uterus were flushed non-surgically and the number of embryos developing to the blastocyst stage was recorded immediately at recovery and following overnight culture in vitro. In order to metabolically characterize the cows, jugular blood samples were taken twice per week from 15 days before calving to approximately Day 100 postpartum, to measure non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHBA), glucose and insulin. At the same time, body weight (BW) and body condition score (BCS) were recorded for each animal. BW and BCS were significantly different between groups for the entire postpartum period of the study. Furthermore, concentrations of NEFA and BHBA were higher ($P<0.05$) and concentrations of glucose and insulin were lower ($P<0.05$) in lactating compared to dry cows. Embryo recovery rates from lactating and dry cows were similar. Of the structures recovered, significantly more developed to the blastocyst stage in the dry than in lactating cows ($P<0.05$; Table 1) In conclusion, the reproductive tract of the lactating dairy cow is compromised in its ability to support early embryo development compared with that of matched dry cows and this may contribute to early embryo mortality observed in such animals.

Table 1. Recovery and development of bovine embryos following endoscopic transfer to the oviducts of Holstein lactating or dry dairy cows.

Cows	Embryos transferred, *n*	Recovery, *n* (%mean±SEM)	Day 7 Blastocysts, *n* (%mean±SEM)	Total Blastocysts, *n* (%mean±SEM)*
Lactating	621	393 (62.72±7.96)	101 (27.03±3.34)[a]	128 (33.05±4.29)[a]
Dry	627	403 (63.87±5.63)	165 (39.59±3.88)[b]	203 (49.26±3.72)[b]

[a, b]: values in the same column with different letters differ significantly

*Following overnight culture

Funded by Science Foundation Ireland (SFI/07/SRC/B1156 to P. Lonergan) and of the Spanish Ministry of Science and Innovation (AGL2009-11810 to D. Rizos). V. Maillo was partly supported by Short Term Scientific Mission from COST-GEMINI Action (COST-STSM-FA0702-7374).

Notes

CRYOTOP *VS* STRAW AS CARRIERS FOR THE VITRIFICATION OF RABBIT EMBRYOS: EFFECTS ON IMPLANTATION, OFFSPRING AND FETAL LOSSES

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Three key factors influence the probability of successful vitrification: cooling and warming rates, the composition of the cryoprotectant (viscosity) and sample volume. The use of the minimal volume vitrification methods enabled us to increase the cooling rate, thereby minimizing embryo exposure to the 'dangerous zone' of temperatures that cause chilling injury. Several techniques have been developed to decrease the volume of the sample, such as: OPS, electron microscope grids, cryoloops, cryotops, however, its benefits have been mainly evaluated on embryo development in vitro and not on offspring at birth.

The aim of the present study was to compare the efficacies of cryotop and straw carriers for vitrification rabbit embryos. We examined the implantation, offspring at birth and fetal losses of embryos after vitrification using both devices. Embryos of 70-72 hour after ovulation induction were vitrified in two steps. In the first step, embryos were placed for 2 min in a vitrification solution consisting of 12.5% DMSO and 12.5% EG in BM (DPBS+ 0.2% of BSA). In the second step, embryos were suspended for 1 min in a solution of 20% DMSO and 20% EG in BM. Then, embryos suspended in vitrification medium were (i) loaded into 0.25 ml plastic straws or (ii) using cryotop. Devitrification of straw was performed by immersing the central and the final section in a water bath at 20°C for 10-15 s and cryotop device was transferred stepwise into drops. The vitrification medium was removed in two steps: BM with 0.33 M sucrose for 5 min and BM for another 5 min. Thereafter, embryos were transferred into oviduct by laparoscopies. Recipient does were induced to ovulate with 1μg of buserilin acetate 60-62 hours before transfer. Later, implantation rate were studied at 12th day of gestation by laparoscopies and offspring at birth. Finally, fetal losses were calculated as differences between implanted embryos and offspring at birth.

Our results showed significant different implantation rates between both carriers: 39% *vs* 53% for cryotop and straw, respectively ($P<0.05$). No significant differences of offspring rate were observed. From the 86 transferred embryos vitrified with straw device and 96 embryos vitrified with cryotop device, 28% and 32% offspring at birth, respectively. However, fetal losses were significantly affected by the carriers employed: 16% *vs* 48% for cryotop and straw, respectively ($P<0.05$).These findings suggest that cooling velocity does not affect the efficiency for vitrification of rabbit embryos to obtained live offspring. But, our result showed as cooling velocity present a detrimental effect on embryonic development post implantation.

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Notes

FOLLICULAR POPULATION AND ESTROUS SYNCHRONIZATION IN HOLSTEIN HEIFERS

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The appropriate manifestation of estrous could be an indicator that contributes to the good reproductive performance in cattle, and could be associated with the ovarian follicular population (PF). The objective of the present study was to evaluate the effect of the population of ovarian follicles on the incidence of estrous (IE), hours to estrous (HE), size of the preovulatory follicle (SPF) and incidence of ovulation (IO). Nulliparous Holstein heifers (n=33) between 12 and 18 months of age were scanned four times with an ultrasound equipped with a 5 MHz linear transducer, to determine the population of ovarian follicles ≥3 mm of diameter, and were assigned to one of the following treatments: A) Low FP (≤3 follicles, n=17) and B) High FP (≥4 follicles, n=16). The heifers were synchronized during 8d, with 1.9 g of progesterone through a CIDR device, and 2 mg of estradiol benzoate were administered at CIDR insertion, as well as 500 µg of prostaglandins at CIDR removal. The females were scanned at the time of estrous and 6d later. The results are shown in Table 1 (mean ± ee).

Table 1. The effect of the follicular population on estrous characteristics of the estrous in Holstein heifers

Follicular population	N	Incidence of estrous (%)	Hours to estrous	Size of preovulatory follicle (mm)	Incidence of ovulation (%)
Low (≤3)	17	82.4 [a]	77.0 ± 4.11[a]	12.6 ± 0.4 [a]	88.2 [a]
High (≥4)	16	75.0 [a]	73.7 ± 6.13[a]	12.7 ± 0.4 [a]	87.5 [a]

Means with different literal are significantly different (P<0.05).

There were no differences (P>0.05) in IE, HE, SPF and IO, between the groups of heifers. The lack of differences may be due to the reduced variability of the follicular population among the groups of animals classified as having low or high number of follicles. However, there are no results published in the literature to be able to compare the present findings. In conclusion, under the conditions of this study the ovarian follicular population did not affect the estrous characteristics, the preovulatory follicle and the incidence of ovulation in Holstein heifers.

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Notes

FOLLICULAR POPULATION AND THE EFFICIENCY OF AN EMBRYO PRODUCTION PROGRAM IN HOLSTEIN HEIFERS

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One of the potential alternatives to reduce the variation in the production of embryos *in vivo* is the evaluation of the ovarian follicular population (FP). The objective of the present study was to evaluate the effect of the population of ovarian follicles ≥ 3 mm on the production of transferable embryos. Nulliparous Holstein heifers between 12 and 18 months of age were scanned in four times with an ultrasound equipped with a 5 MHz linear transducer to determine the population of ovarian follicles ≥3 mm in diameter and the heifers were assigned to one of the following treatments: A) Low FP (≤4 follicles, n=10), and B) High FP (≥5 follicles, n=9). The heifers were synchronized during 8d, with 1.9 g of progesterone through a CIDR device, 2 mg of estradiol benzoate were administered at CIDR insertion and 500 µg of prostaglandins at CIDR removal. The animals were superovulated with 300 mg of follicle stimulating hormone (Folltropin-V, Bioniche, Canada), administered every 12h during 4d in 8 decreasing doses, and inseminated with sexed semen at 12, 18 and 24h after estrous onset, they also received an injection of 100 µg of GnRH (Cystorelin, Merial, USA) at the time of the first insemination. The heifers were non-surgically flushed using standard procedures 8d after the service in an attempt to collect the embryos. The variables compared were incidence of estrous (IE), hours to estrous (HE), total structures collected (TSC) and transferable embryos (TE). The results are presented as average ± ee, in Table 1.

Table 1. Effect of the follicular population on estrous and superovulatory response

Follicular population	n	Incidence of estrous (%)	Hours to estrous	Total Structures collected	Transferable embryos (%)
Low (≤4)	10	100[a]	39.5 ± 2.9[a]	7.1 ± 2.2[a]	46.5[a]
High (≥5)	9	100[a]	42.8 ± 3.8[a]	6.7 ± 1.3[a]	36.7[a]

Means with different literal are significantly different (P<0.05).

There were no differences (P>0.05) in IE, HE, TSC and TE between groups of heifers, the results are in contrast to those obtained by Ireland *et al.* (2007). The authors found a higher (P<0.05) number of structures collected and transferable embryos in heifers with high follicular population. Probably the differences are due at least in part to the fact that the follicular class population considered in their low (≤15) and high (≥25) groups of heifers are different. In conclusion, under the conditions of this study the follicular population did not affect estrous characteristics and the production of transferable embryos in Holstein heifers.

Reference: Ireland *et al*. 2007. Human Reproduction 22:1687-1695. Corresponding author: rangelsr@correo.chapingo.mx

Notes

NUTRITION AFFECTS NATURAL OR INDUCED SEASONAL REPRODUCTIVE TRANSITIONS IN THE EWE

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In small-ruminants breeding systems, the development of "clean, green and ethical" [1] methods in reproductive management implies some manipulations of the genetic and environmental factors that control the seasonal reproductive rhythm. Amongst environmental cues, nutrition is considered as a potent factor affecting out-of-season fertility. However the development of focus-feeding practices is restricted by the lack of knowledge about how nutrition affects the different physiological states experienced by the individual.

Considering this, we've conducted three experiments on the Ile-de-France breed of sheep to determine the effects of nutrition on i) the seasonal patterns of ovarian activity (SEA), ii) the ovarian response to a treatment with melatonin implants (Melovine®; MEL) and iii) the reproductive performances in response to a ram effect (RAM). Both implants insertion and introduction of rams were realised in the late anoestrus season for this breed. For each experiment, the same model of ewes with a contrasted body condition score (BCS) between groups (restricted 'R' *vs*. well-fed 'WF') was used. In experiment iii), we also tested the effects of a nutritional supplementation (500 g of whole lupin grain) given daily before (D-6 to D-1) and after (D14 to D19) the introduction of rams (D0).

The BCS of each animal was estimated monthly. In experiments i) and ii), ovarian status was appreciated through determination of plasma progesterone in blood samples collected twice weekly via jugular venepuncture. In experiment iii), the short-term response to ram introduction (LH pulsatility and preovulatory surge) was monitored through determination of plasma LH in blood samples collected every 15 min for 6 hours and hourly for the next 72 hours. Ovulation rate was measured by endoscopy at D5 & D22 and ovarian cycles were analysed through determination of plasma progesterone in daily blood samples.

The BCS (median ± interquartile range) of the ewes in the SEA [WF (n = 10): 3.15 ± 0.36 *vs*. R (n = 5): 1.75 ± 0.42], MEL [WF (n = 9): 3.50 ± 0.63 *vs*. R (n = 8): 2.00 ± 0.58] and RAM [WF (n = 23): 3.50 ± 0.75 *vs*. R (n = 15): 1.75 ± 0.63] groups were significantly different between nutritional treatments (WF *vs*. R; $p < 0.001$) but similar within a same treatment. The ewes in the R groups i) had a shorter season of ovarian activity (-95 days; $p < 0.01$) and ii) exhibited a lower response to melatonin treatment (100 % *vs*. 50 % of induced ovarian cycles; $p < 0.05$). In experiment iii), since nutritional supplementation had no effect on the response to the ram effect, data were pooled according to BCS. The ewes in the R group had a lower mean LH pulsatility (4.48 ± 0.28 pulses *vs*. 2.67 ± 0.34 pulses; $p < 0.001$) and a similar proportion of LH preovulatory surges (WF: 91 % and R: 67 %). At D5 but not D22, ovulation rate was significantly higher for the ewes in the WF group (2.24 ± 0.31 *vs*. 1.30 ± 0.20; $p < 0.05$) and the distribution of ovarian cycles was significantly different between groups ($p < 0.05$) with more abnormal cycles for the ewes in the R group.

This study demonstrates that nutrition, through its static component, is a potent factor affecting natural or artificially-induced seasonal reproductive transitions. Thus, it has to be considered has a major factor in the development of methods to control seasonality of reproduction and improve reproductive performances (in AI or ET programs) in farm animals.

[1] Scaramuzzi, R.J. and G.B. Martin. Reprod Domest Anim, 2008. 43 Suppl 2: p. 129-36.

Notes

SURVIVAL RATE OF VITRIFIED AND WARMED *IN VITRO* PRODUCED BOVINE BLASTOCYSTS FOLLOWING CULTURE IN MEDIUM SUPPLEMENTED WITH HYALURONIC ACID

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The present study was carried out to evaluate the effect of the addition of hyaluronic acid (HA) to the embryo culture medium on bovine embryo development to the blastocyst stage as well as its viability after vitrification. Following *in vitro* fertilization, presumptive zygotes were randomly assigned to four different culture treatments: (1) SOF+10% foetal calf serum (FCS); (2) SOF+3 mg/ml Bovine Serum Albumin (BSA); (3) SOF+10% FCS+1 mg/ml HA (Block et al. Theriogenology, 71:1063-71.2009); (4) SOF+3 mg/ml BSA+1 mg/mL HA. Cleavage rate was recorded on Day 2 post insemination (pi) while blastocyst yield was evaluated on Day 7 pi. In a second part of the study, day 7 expanded and hatching/hatched blastocysts derived from the four different culture media were vitrified/warmed by the cryotop method and cultured for 24 additional hours in order to evaluate re-expansion and hatching at 3h and 24h post-warming. A sample of day 7 blastocysts, was fixed to perform the ICM/TE differential staining.

Addition of 1 mg/mL hyaluronan to embryo culture had no effect on cleavage rate at Day 2 after insemination (72.9%; 67.1% vs 71.8%; 70.5%; FCS and BSA vs HA+FCS and HA+BSA, respectively). There was no effect of the addition of hyaluronan on embryo development at Day 7 (15.0% and 15.1% for FCS and BSA, respectively) when compared to embryos cultured without hyaluronan (13.7% and 12.9% for FCS and BSA, respectively). Hyaluronan addition resulted in similar blastocyst re-expansion rates at 3h and 24h after warming (75%; 77.3%; and 62.5%; 68.2%; HA+FCS and HA+BSA, respectively) when compared to controls (74.4%; 78.0%; and 60.5%; 64%; FCS and BSA, respectively). However, there was a significant increase in the ratio ICM/TE for those blastocysts obtained from the BSA+HA (0.44±0.12) treatment compared to the BSA treatment (0.31±0.05).

In conclusion, the supplementation of culture medium with acid hyaluronic had no effect on cleavage rates, blastocyst yield or blastocyst survival rates after vitrification and warming. However, blastocyst quality, measured as the ratio of ICM / TE, increased significantly for those blastocysts obtained from the culture medium supplemented with BSA and hyaluronan.

Notes

A SYSTEM TO ESTABLISH A BIOPSY-DERIVED TROPHECTODERM CELL LINE FOR BOVINE EMBRYO GENOTYPING

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Bovine embryo typing using latest generation of high density marker chips and/or whole genome evaluation could be a solution to shorten the generation interval and to limit the cost for producing high numbers of calves used in progeny tests to achieve multi-character selection. Because there are many technical limitations of using amplified DNA with the high density markers chips, we have developed a system to produce a trophectoderm cell line from an embryo biopsy which will enable production of good quality genomic DNA without amplification. We have established a successful culture system of individual biopsy-derived trophectoderm cell lines without co-culture with murine feeder cells. Bovine blastocysts were produced by in vitro maturation, fertilization and culture in synthetic oviduct fluid (SOF) supplemented with 5% of fetal calf serum (FCS) at 39ºC under an atmosphere of 5% CO_2 and 5% O_2 in air. Hatched blastocysts on day 9-10 were used for a biopsy using a N18 scalpel. Biopsied embryos remained in culture for another 24 h to assess their survival rate (re-expansion). In the first experiment, a rapid adhesion of biopsied trophectoderm cells, which is a limiting factor for a successful establishment of a trophectoderm cell line, was obtained when 10 µl, previously gelatin-coated, were used for culture under mineral oil on 35-mm tissue culture dishes (24 10-µL droplets). In the second experiment, an optimal culture medium for rapid biopsied trophectoderm cells growth was examined using: (i) Dulbecco's modified Eagle medium (DMEM plus 4500 mg/l glucose, glutaMAX, and pyruvate) supplemented with 10% FCS, 2 mM glutamine, 1 mM MEM nonessential amino acids solution, and an antibiotic mixture containing 100 U/ml penicillin and 100 mg/ml streptomycin, as a control (C); (ii) SOF supplemented with 5% of FCS (S); and three conditioned media at a rate of 1:1 with control medium: (iii) from mouse embryonic fibroblasts (Cm); (iv) from bovine embryonic fibroblasts (Cb) and; (v) from bovine oviductal cells (Co). All media used were also supplemented with 20 ng/ml of epidermal growth factor. The efficiency of the handmade embryo biopsies (n=126) was confirmed by their high survival rate after culture for 24 h (90.5%). At day 4 of culture, biopsy adherence and cell proliferation was significantly higher in Cm group (73.8%; n=31/42) when compared with S (35.7%; n=15/42, $p<0.001$), C (51.4%; n=18/35, $p<0.05$), and Cb (39.4%; n=13/33, $p<0.01$) groups. However, Co group (60.3%; n=41/69) was different only with S group ($p<0.01$). At day 10 of cultivation, cell confluence was around 50% in all media except S group that showed cellular senescence. These results showed that the combination of a microdrop culture system on a surface treated with gelatin and the employment of conditioned media from mouse embryonic fibroblasts or from bovine oviductal cells support the growth of bovine trophectoderm cells from an embryo biopsy, which enable the production of a relatively large amount of good quality genomic DNA to perform genotyping.

Notes

EFFECT OF FAMILY ON NUMBER OF BOVINE EMBRYOS OBTAINED BY FLUSHING AFTER SUPEROVULATION

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It is already known that the number of embryos obtained by flushing cows after superovulation can show large variance between different female animals. This can partly be explained by difference in management and flush protocol, but might also be influenced by animal specific characteristics, i.e. animals that always give high a number of embryos and others that always give low a number. Merton et al. (Theriogenology 72, 885-932, 2009) showed that there is a similar difference between animals in number of embryos after in vitro production (IVP from Ovum pick-up derived oocytes). This difference is partly genetically determined, i.e. heritability of 0.21. For the number of embryos flushed, heritabilities have been estimated to be very low (0.03) to moderate (0.19).

The aim of this study was to determine if there is a family effect in the number of embryos obtained by flushing after superovulation.

Family effect was determined by evaluating the mean embryo production of daughters from different sires. A data set with information of pedigree and results of flushing of all animals that were superovulated and flushed by CRV between January 1994 and April 2011 was used. Flushes, 36,465 in total, were performed on CRV locations and in the field on both heifers and cows. The dataset contained 15,908 different donor animals of which 378 had 10 or more flushings. To investigate the family effect only sires were used (n=69) which had at least three daughters (with each 10 or more flushings).

The variation of average flush results from daughters between bulls was large (mean numbers of embryos varied from 1.9 to 14.1). An example of some sires and the mean embryo production of their daughters is given in table 1.

Table 1. Example of mean number of embryos of daughters from four different sires.

Sire	No of Daughters	Mean No embryos	SEM
A	11	1.9	0.8
B	10	4.5	1.3
C	11	10.5	1.3
D	8	14.1	2.5

The results mentioned above strongly indicate a familiar relation in embryo production through flushing.

This result can have a practical implication for logistic of a breeding program. Since there is no correlation between the number of embryos obtained by flushing and that obtained by IVP (results not shown), it can be decided to place daughters from poorly flushing families directly in the IVP program.

Notes

QUALITY OF PIGS MESENCHYMAL STEM CELLS (MSC) CULTURED WITH PLANT PROTEIN

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The aim of this study was to determine whether the plant protein (PP) can replace the FCS as a supplement in medium for MSC in vitro culture. Achieving positive results will result in replacement of an animal origin component with a plant ingredient which is safer from sanitary point of view. To assess an impact of PP on welfare of cultured cells, expression of apoptosis related genes, proapoptotic Bax, Bcl_{xs} and caspase-3, and antiapoptotic Bcl_{xl} was measured. The apoptosis level of mesenchymal cells was evaluated by western-blotting after several days of culture in two media, supplemented with 0.5% PP and 5% FCS.

The assessment of the abundance of Bax and caspase-3 were performed in four and two independent replications, respectively. The ratios of Bax and caspase-3 in relation to the loading control - actin were calculated. The assessment of the abundance of Bcl_{xl} and Bcl_{xs} were performed simultaneously in four independent replications. In this experiment the ratios of Bcl_{xl} in relation to Bcl_{xs} were calculated. The protein relative abundance (RA) of the apoptosis related genes was measured by Quantity One software (Bio-Rad) and a ratio of the optical density (OD) of the bands of estimated genes was calculated as described. Differences in proteins level were assessed using t-Student test. Differences with a probability value of 0.05 or less were considered significant.

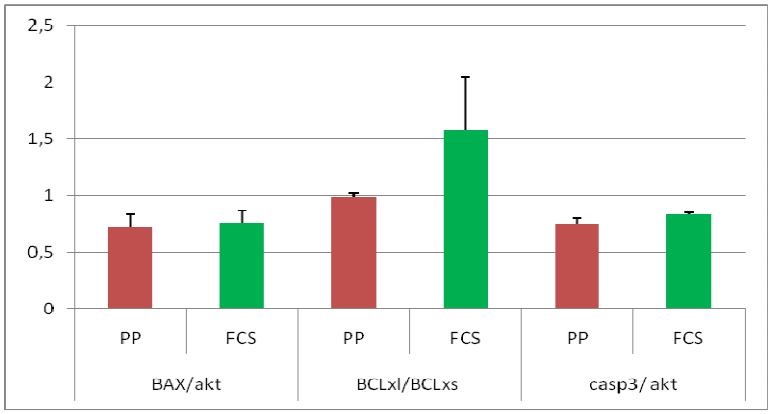

Fig.1. Evaluation of the abundance of Bax, Bcl_{xl}, Bcl_{xs}, and caspase-3 in MSC cultured with PP and FCS.

The differences between the expression of apoptosis related genes of cultured cells in PP and FCS were found not statistically significant. This means that plant protein can replace animal serum in the culture medium.

Scientific work was financed by the National Center for Research and Development; funds for science in 2009-2012 as a development project.

Notes

THE MULTIPOTENCY AND MEMBRANE INTEGRITY OF PIGS MESENCHYMAL STEM CELLS CULTURED WITH PLANT PROTEIN

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Recently, attempts were made to replace animal serum or its derivatives with safer and more standardized components for somatic cells culture media as well as other media used in animal reproduction biotechnology. The reason is an ambiguous impact of serum on a quality of cultured cells, oocytes and embryos.

The aim of the experiment was to determine the impact of plant protein (PP) used in culture medium instead of FCS on the mesenchymal stem cells (MSC) multipotency and membrane integrity measured by Sybr14 and PI staining by means of flow cytometry.

MSC were established from bone marrow (BM) collected from anesthetized gilts (n=7). MSC in vitro culture was carried out in low glucose DMEM supplemented with 0.5% PP or 5% FCS for at least 3 weeks, at humidified atmosphere of 5% CO_2 in air. To assess multipotency of BM MSCs, the expression of CD73 and CD105 antigens by flow cytometry was estimated as well as the expression of SCF and CD44 by western-blotting was evaluated.

The assessment of the abundance of SCF and CD44 was performed in three and two independent replications, respectively. The ratios of SCF and CD44 in relation to the loading control - actin were calculated. The protein relative abundance (RA) was measured by Quantity One software (Bio-Rad). Differences with a probability value of 0.05 or less were considered significant (t-Student test).

Positive fluorescence for CD73 antigen showed 28% of cells cultured in medium with 5% FCS and 34% of MSCs cultured in medium supplemented with 0.5% PP. Positive fluorescence for CD105 antigen showed 50.4% cells cultured in medium supplemented with FCS and 59.5% cells cultured in presence of PP. There were no statistical differences observed for SCF and CD44 expression between cells cultured in presence of PP and FCS.

The membrane integrity of MSCs cultured in medium supplemented with PP and FCS was estimated in four independent replicates. There were no statistical differences (t-Student test) between the number of live and dead cells cultured in medium supplemented with FCS and PP as observed by Sybr14 and PI staining (Fig. 1).

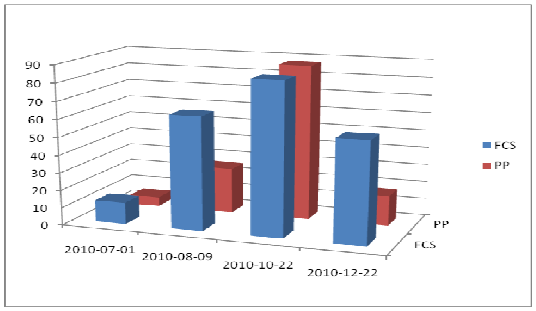

Fig1. The number of live and dead cells cultured in medium supplemented with FCS and PP as observed by Sybr14 and PI staining

In conclusion, the plant protein can be considered to replace animal serum in the culture medium however further research should be continued to determine PP affect on cell quality.

Scientific work was financed by the National Center for Research and Development; funds for science in 2009-2012 as a development project.

Notes

BIRTH OF DONKEY FOALS AFTER TRANSFER OF VITRIFIED EMBRYOS

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Most of the northern hemisphere donkey breeds are faced of the risk of extinction. Donkey embryo freezing could be an aid to preserve these genomes.

The aim of this study was to evaluate the possibility of having pregnancies and live foals after transfer of vitrified donkey embryos, and to compare day 14 and 25 pregnancy rates after intraspecies transfer of donkey and horse vitrified embryos.

Eleven day-7 donkey embryos and 10 day-6 horse embryos were recovered by uterine flushings with ringer lactate as previously described (Camillo et al., 2010). Embryos were washed 10 times in EmCareTM Holding Solution (Bio98 Srl, Milano, Italy), measured, and evaluated for morphology (McKinnon and Squires, 1988) before being vitrified using the Equine Vitrification Kit (Bioniche Animal Health, USA) and the technique described by Eldridge-Panuska et al., 2005. During the next breeding season the embryos were thawed and transferred using the non-surgical direct-transfer procedure (Eldridge-Panuska et al., 2005) into selected recipients of the same species at day 5 after ovulation. Pregnancy diagnoses were performed by ultrasound 7-8 days after ET and checked every second day until day 25, when mare pregnancies were terminated by the IM administration of 3 mg Alfaprostol (Gabbrostim®, CEVA VETEM, Milan, Italy). Pregnant jenny were not treated and checked once a week for embryo and foetus development. Pregnancy rates at 14 and 25 days in mares and jennies were compared using Fisher's Exact Test.

Mean diameter of 7-days old donkey embryos was 188±39 μm, 10/11 embryos were early blastocysts and 1/11 was a blastocyst. Mean diameter of 6-days old equine embryos was 178±38 μm, 3/10 embryos were morulas, 6/10 were early blastocysts and 1/10 was a blastocyst. All donkey and horse embryos were graded as excellent or good. Pregnancy rates were 4/11 and 3/11 in jenny and 4/10 and 3/10 in mare recipients at days 14 and 25, respectively. In the lost donkey pregnancy, an embryo proper never developed. One donkey pregnancy resulted in the delivery of a non viable foetus at day 321 of gestation while the other two resulted in the birth of healthy fillies at days 371 and 372.

This study showed that it is possible to obtain pregnancies and live foals after transfer of vitrified donkey embryos. Fourteen day pregnancy rate after transfer of vitrified donkey embryos, 36.4%, was similar to the best results after transfer of fresh donkey embryos, 50% (Panzani et al., 2008). No differences were found between pregnancy rates after transfer of donkey or horse vitrified embryos, but pregnancy rates of horse vitrified embryos were lower compared with literature (Eldridge-Panuska et al., 2005; Hudson et al., 2006). This study was based on a very small number of embryos and animals and only 2 foals were born out of 4 recipient jennies pregnant at day 14, nevertheless this is the first report of foals born after transfer of cryopreserved embryos in donkeys. More studies are needed before vitrification of embryos could be considered an opportunity for long term conservation of endangered donkey breeds.

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Notes

POTENTIAL OF EGF-LIKE PEPTIDES TO IMPROVE CULTURE OF PIG CUMULUS-OOCYTE COMPLEXES IN VITRO

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The events induced in preovulatory follicles by LH surge are mediated by production of EGF-like peptides amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC) in mural granulosa and consequently also in the cumulus cells. These peptides bind to EGF receptors of cumulus cells and in autocrine manner stimulate transcription of genes involved in regulation of meiotic resumption and cumulus expansion. The aim of this work was to assess whether FSH stimulates expression of EGF-like peptides in cultured cumulus-oocyte complexes (COCs) and to find out an effect of the peptides on activation of signaling pathways in the COCs, stimulation of cumulus expansion, oocyte maturation and acquisition of oocyte developmental competence in vitro.

FSH significantly stimulated expression of *AREG* and *EREG,* but not *BTC* in cultured COCs at 2 and 4 h after FSH addition, respectively. Both FSH and the EGF-like peptides stimulated in COCs expression of expansion related genes (*PTGS2*, *TNFAIP6* and *HAS2*), although AREG and EREG were less efficient than FSH. In contrast to FSH, AREG and EREG did not stimulate in the COCs expression of *CYP11A1* responsible for production of progesterone. AREG and EREG stimulated maturation of the oocytes and expansion of the cumulus cells, although the percentage of oocytes that had reached metaphase II was significantly lower when compared to FSH-induced maturation. Parthenogenetic development to blastocyst stage of oocytes matured with AREG and EREG or with combination of FSH and the EGF-like factors was significantly better than the development of oocytes matured with FSH or combinations of FSH + LH or PMSG + hCG. Thus, we conclude that the EGF-like factors do not reproduce all events elicited in the cultured COCs by FSH, nevertheless the oocytes stimulated with AREG and EREG possess higher developmental potential than the oocytes stimulated by gonadotropins.

To define signaling pathways that drive the FSH- and the EGF-like peptide- induced cumulus expansion and meiotic resumption, we assessed an effect of specific protein kinase inhibitors on these processes. We found that both FSH- and AREG-induced cumulus expansion was partially inhibited by PKA inhibitor (H89) and completely inhibited by PI3K/PKB, MAPK 3/1 and MAPK 14 inhibitors (LY294002, U0126 and SB203580, respectively). FSH-induced maturation of oocytes was blocked in germinal vesicle (GV) stage by H89, UO126 and SB203580 whereas LY294002 blocked maturation of the oocytes in MI. AREG-induced maturation of oocytes was efficiently blocked in GV by U0126; H89, SB203580 and high concentrations of LY294002 allowed the oocytes to undergo breakdown of GV and blocked maturation in MI. The results of this study are consistent with the idea that PKA and MAPK 14 pathways are essential for FSH-induced transactivation of the EGF receptor and synthesis of EGF-like peptides in cumulus cells whereas MAPK 3/1 is involved in downstream events regulating synthesis of expansion related genes and resumption of oocyte meiosis. PI3K/PKB signaling is important for regulation of cumulus expansion and MI/MII transition.

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Notes

CORPUS LUTEUM CHARACTERISTICS, CIRCULATING PROGESTERONE CONCENTRATIONS AND EMBRYO SURVIVAL IN BEEF HEIFERS TREATED WITH HUMAN CHORIONIC GONADOTROPHIN

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The aim of this study was to test the hypothesis that elevated progesterone concentrations resulting from induction of an accessory corpus luteum by human chorionic gonadotrophin (hCG) administration on Day 5 post estrus would lead to advanced conceptus elongation at Day 14 following embryo transfer at Day 7. The estrous cycles of crossbred beef heifers were synchronized and animals were randomly assigned to receive either of two treatments: (1) intramuscular injection of 3000 IU hCG on Day 5 after estrus (n = 14) or (2) intramuscular injection of saline on Day 5 after estrus (n = 13). Ovaries were scanned daily by transrectal ultrasonography to assess CL development. Serum progesterone concentrations were determined from daily blood samples collected from the jugular vein. In vitro produced bovine blastocysts were transferred to synchronized recipients on Day 7 post estrus (n = 15 blastocysts per recipient). Heifers were slaughtered on Day 14 after estrus and the uterus was flushed to recover the embryos. Injection of hCG on Day 5 induced the ovulation of the dominant follicle in all treated heifers, and increased the total area of luteal tissue on the ovary which was associated with an elevation ($P<0.001$) in serum progesterone concentration from Day 7 to 14. Positive associations were detected between circulating progesterone with CL area (within-day correlations ranging from, $r = 0.45$ to 0.67) and total area of luteal tissue (within-day correlations ranging from, $r = 0.65$ to 0.86) Administration of hCG did not affect the proportion of Day 14 conceptuses recovered (Table 1). However, hCG administered heifers had increased conceptus length (3.91 ± 1.23 vs 5.57 ± 1.02 mm, $P = 0.06$), width (1.00 ± 0.06 vs 1.45 ± 0.05 mm, $P = 0.002$) and area (5.71 ± 0.97 vs 8.31 ± 0.83, $P=0.02$) compared to their contempories in the control group. Although numerically greater, mean IFNT production in vitro was not different ($P=0.54$) between embryos recovered from hCG-treated and control heifers. In contrast, there was a strong positive correlation between individual embryo length ($r=0.76$, $P<0.001$) and individual embryo area ($r=0.72$, $P<0.001$) and IFNT production. In conclusion, administration of hCG on Day 5 post estrus resulted in the formation of an accessory CL and stimulated hypertrophy in the original CL the result of which was an elevation in progesterone concentrations from Day 7 onwards. These elevated P4 concentrations were associated with an increased conceptus area. Furthermore, conceptus size was highly correlated with IFNT secretion in vitro.

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Notes

SIMULTANEOUS ARTIFICIAL MODULATION OF EPIGENETIC MEMORY IN NUCLEAR RECIPIENT AND DONOR CELLS AS EFFICIENT STRATEGY USED FOR PRODUCTION OF PORCINE FIBROBLAST CELL CLONED EMBRYOS

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The present study was undertaken in order to assess the preimplantation developmental competences of nuclear-transferred (NT) pig embryos generated applying trichostatin A (TSA)-mediated epigenetic transformation of both *in vitro*-maturing oocytes and cultured adult cutaneous fibroblast cells. Cumulus-oocyte complexes (COCs) were matured *in vitro* for 20 h in TC 199 medium supplemented with 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), 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 22 to 24 h in the same medium enriched with 80 nM L^{-1} TSA. Prior to use for somatic cell cloning, the permanent fibroblast cell lines (between passages 1 and 3) that had been established from the primary cultures originating from ear skin biopsies of a postpubertal boar, were treated with 50 nM L^{-1} TSA during 24-h serum starvation. Reconstruction of enucleated oocytes was achieved by their fusion with epigenetically-modified fibroblast cells in an isotonic dielectric solution lacking the calcium cations. Electrofusion of ooplast-somatic cell couplets was triggered by two consecutive DC pulses of 1.2 kV cm^{-1} for 30 μs. Sixty to ninety minutes later, reconstituted oocytes were electrically activated using the same technical parameters as for the electrofusion. Nonetheless, the concentration of Ca^{2+} ions in the electroporation medium was increased up to 1.0 mM L^{-1}. Immediately after delayed electroactivation, clonal cybrids were exposed to 5 μg mL^{-1} cytochalasin B (CB) for 2 h, followed by their *in vitro* culture up to morula and blastocyst stages in NCSU-23/BSA/FBS medium for 144 to 168 h. Sequential *in vitro* maturation in the TSA-deprived and TSA-enriched medium contributed to the reaching the metaphase II stage by 237/249 (95.2%) oocytes as compared to 222/264 (84.1%) oocytes in a control (i.e., TSA-untreated) group. It has been also shown that the frequencies of cleaved embryos (185/216; 85.6%), morulae (129/216; 59.7%) and blastocysts (72/216; 33.3%) developing from NT embryos that were reconstructed with recipient oocytes and nuclear donor somatic cells, each of which had been exposed to TSA, were significantly higher than in the TSA-untreated group (121/193; 62.7%, 76/193; 39.4% and 38/193; 19.7%, respectively). Altogether, the improvements in not only cleavage activity of porcine cloned embryos, but also their morula/blastocyst formation rates seem to result from enhanced abilities for transcriptional reprogramming of TSA-exposed dermal fibroblast cell nuclei in an epigenomically-matured cytoplasm of recipient oocytes also undergoing treatment with TSA.

Notes

EFFECT OF FSH AND eCG ON THE GROWTH OF THE DOMINANT FOLLICLE AND OVULATION RATE OF 5/8 GIROLANDO HEIFERS TREATED WITH NORGESTOMET IMPLANTS

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The eCG and the FSH are glycoprotein hormones which can be employed in herds with low cyclicality in animals with compromised body condition score and primiparous cows to improve ovulation and pregnancy rates.

This study was conducted at the Experimental Station of Arcoverde- (ESA/IPA) Pernambuco, Brazil, (Latitude 08° 25'08 "south and Longitude 37° 03'14" west, with an altitude of 663 meters) with the aim to evaluate the effect of a single treatment with FSH or eCG on diameter of the largest follicle, on diameter of corpus luteum and on conception rate. Thirty 5/8 Girolando heifers averaging 27 month of age and with an average weight of 300 kg were used. Heifers were divided into three treatment groups. On day 0 (D0) all heifers of the control group (G1; n=10) received an ear implant containing norgestomet (Crestar®, Intervet) and 2mg of estradiol benzoate (Gonadiol®, Schering-Plough). On day 8 (D8) ear implants were removed and 500µg of cloprostenol (SINCROCIO®, Ouro Fino) was applied. Twenty-four hours after implant removal (D9) estradiol cipionate 1mg (ECP®, Pfizer) was injected and 56 hours after implant removal all heifers were artificial inseminated (fixed-timed AI). The experimental groups were basically treated as described for the control group. Additionally, heifers of group 2 (G2; n=10) received 100 IU of FSH (Pluset®, Herpate Calier) at the day of implant removal, and heifers of group 3 (G3; n=10) were treated with 500 IU of eCG (Novormon®, Schering-Plough) at the day of implant removal.

Ultrasonography was used to assess (i) the emergence of the follicular wave at D4, (ii) the diameter of the dominant follicles at D8, D10, D11, (iii) the ovulation rate one day after TAI, (iv) and the diameter of the CL 11 days after TAI. Data were analyzed by SPSS9.0 employing the analysis of variance (ANOVA).

There was no statistical difference in any of the variables analyzed. The measured ovulation rates were 80%, 90% and 90% for G1, G2 and G3, respectively (P>0.05), and the conception rates were 40%, 60% and 50%, for G1, G2 and G3, respectively (P> 0.05). After application of placebo in G1, 100 IU FSH in G2 and 500 IU eCG in G3, the increase in follicular diameter were 1.42±0.52mm, 0.86±0.33mm and 1.7±0.36mm for G1, G2 and G3, respectively (P>0.05). Although there was no statistical difference between the groups it can be seen from the data that for improvement of follicular diameter eCG was more efficient than FSH. The same tendency was seen in CL diameter 12.6±0.54mm, 12.4±0.60mm and 13.12±0.78mm for G1, G2, and G3, respectively (P>0.05). One fact that may have contributed for absence of statistical difference between the groups is the good body score condition (BSC) that the heifers presented. As it is known good BSC does not commit LH release and consequently follicular development and ovulation in cows.

Notes

PREGNANCY RATES IN CATTLE AFTER INTRAVAGINAL AND INTRACERVICAL ADMINISTRATION OF SEMINAL PLASMA IN EMBRYO TRANSFER RECIPIENTS

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Generally, seminal plasma (SP) is considered to be a transport medium for spermatozoa. But exposure to SP induces a variety of reactions in the female genital tract and seems to promote fertility, especially in species with intrauterine semen deposition. The objective of the present study was to determine if seminal plasma improves pregnancy rates when a physiological amount is placed into the caudal reproductive tract during the oestrus immediately before embryo transfer (ET). In a triple-blinded study, 4 mL SP or placebo (P: DPBS + 0.01 % gelatine) were administered into the caudal cervical canal as well as into the vaginal fundus (one-third vs. two thirds of the total amount) of dairy heifers, mimicking the presence of seminal fluid during mating (experimental groups: A, B). Group C served as control (standard ET without any further treatment; not blinded). Animals presented for ET in the area of three German artificial insemination centres were allocated randomly in equal shares into one of the three groups. They were classified according to age, BCS, lameness and further parameters, including cervix cytology, which was performed twice per animal (oestrus and ET). Seminal plasma was derived from fertile bulls, centrifuged, pooled (> 20 bulls, > 200 mL/pool) and packed. SP as well as P samples were frozen at -196°C until use. Preliminary results until May 2011 (n = 42 animals; 12-16 animals per group) show improved pregnancy rates in Group A and B (57.1 % and 56.3 %, still blinded), compared to 50.0 % in Group C. Further animals are waited out for statistical analysis in order to find out if the significantly positive influence of SP or P observed in a previous study (RDA 2011, vol. 46, suppl. 1, p 39-40) on SP or P application concurrent with artificial insemination can be confirmed for ET.

FBF Bonn is gratefully acknowledged for funding this study.

Notes

PREIMPLANTATION DEVELOPMENT OF CAPRINE NUCLEAR-TRANSFERRED EMBRYOS EPIGENETICALLY MODULATED BY TRICHOSTATIN A

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Trichostatin A (TSA) is considered to be an efficient non-specific/non-selective inhibitor of histone deacetylases (HDACs) in both cultured nuclear donor somatic cells and cloned embryos of different mammalian species. Therefore, it leads directly to enhancement of acetylation processes within the lysine residues of histones (mainly H3 and H4) that form the nucleosomal core of nuclear chromatin. As a consequence, TSA contributes indirectly to decline in the level of DNA methylation or to increase in demethylation processes within the cytosine residues of genomic DNA. The objective of our study was to examine the effect of TSA exposure on the *in vitro* developmental capability of nuclear-transferred (NT) goat embryos. *In vitro*-matured oocytes provided the source of nuclear recipient cells in the somatic cell cloning. Cumulus-oocyte complexes (COCs) were recovered by aspiration of follicular contents from postpubertal doe-derived ovaries with the aid of laparoscopic ovum pick-up (LOPU) method. COCs were cultured *in vitro* for 23 to 24 h in 500 µL of TC-199 medium supplemented with 5 mIU/mL porcine follicle-stimulating hormone (pFSH), 1 µg/mL 17-β-estradiol, 20% heat-inactivated estrus caprine serum (ECS) and 10 ng/mL recombinant human epidermal growth factor (rhEGF). Before use as a source of nuclear donor cells, the clonal cell lines of fetal fibroblasts (following 2-6 passages) were subjected to the contact inhibition of their migration and proliferative growth for 24-48 h under the conditions of the total confluency. Single fibroblast cells were inserted into a perivitelline space of previously enucleated oocytes. Somatic cell-ooplast couplets were simultaneously fused and activated with a single DC pulse of 2.4 kV/cm for 15 µs. After a 1-h delay, the reconstructed oocytes were additionally activated by exposure to 5 µM/L calcium ionomycin for 5 to 6 min, followed by treatment with 2 mM/L 6-dimethylaminopurine for 2 h. In Group I, NT embryos were exposed to 50 nM/L TSA in 400 µL of Upgraded B2 INRA medium for 24 h. Afterwards, cleaved embryos were cultured in the TSA-deprived medium, which was supplemented with 10% fetal bovine serum (FBS), for 144 to 168 h up to morula/blastocyst stages. In Group II, cloned zygotes were incubated in the upgraded B2 INRA medium, followed by culture of dividing embryos in the FBS-enriched medium. Among 102 cultured NT embryos originating from Group I, 76 (74.5%) were cleaved. The frequencies of cloned embryos that reached the morula and blastocyst stages yielded 41/102 (40.2%) and 30/102 (29.4%), respectively. In Group II, out of 70 cultured embryos, up to 39 (55.7%) underwent the cleavage divisions, but only 18 (25.7%) and 7 (10.0%) developed to morula and blastocyst stage. In conclusion, it has been found that 24-h treatment of *in vitro* cultured caprine clonal cybrids with TSA immediately after their artificial activation resulted in the relatively high morula and blastocyst yields by increasing the reprogrammability for epigenetic memory of donor cell-inherited nuclear genome in preimplanted NT embryos.

Notes

c9t11- AND t10c12-CONJUGATED LINOLIC ACIDS AFFECT BOVINE IVP EMBRYOS AT THE MOLECULAR LEVEL

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Fertility in high yield dairy cows is closely connected to the availability of energy, especially in the post partal period. It has been suggested the reproductive performance may be improved by oral supplementation with conjugated linolic acids (CLA). The two CLA that are in the main focus are c9t11- and t10c12, the latter known to inhibit lipid synthesis in different animals. Nevertheless, the direct influence on embryonic development has not been analyzed.
Therefore, the goal of the present study was to assess the effects of c9t11- and t10c12 CLA in the culture media on early embryonic development.
Embryos were cultured in vitro using a standard culture media (SOFaa). C9t11-(group 1;n=311) and t10c12-CLA (group 2; n=300) were added to a final concentration of 50 µmol l^{-1} at the beginning of culture. As the CLA were solved in DMSO a third group of embryos (n=205) was cultured in medium supplemented with DMSO only (vehicle control). A fourth group (n=155) was included in which embryos were cultured without any additives. Cleavage and development rates were assessed on day 8. Single day 8-expanded blastocysts were used for RT-qPCR. Eight gene transcripts of interest (SCD, FASN, ACAA1, ACAA2, CPT2, IGFBP2, IGFBP4, IGF1R) were analysed.
The following cleavage and development rates (mean ± s.e.m.) could be obtained. No differences could be detected among groups regarding cleavage rates (Group 1: 47.8% ± 4.0[150/311]; group 2: 51.5% ± 3.5 [151/300]; group 3: 60.2% ± 4.1[124/205]; group 4: 51.6% ± 4.6[81/155].)
The development rates were significantly different in embryos of group 3 compared to embryos of groups 1 and 2. No differences could be detected among embryos of groups 1, 2 and 4 or between groups 3 and 4, respectively (Group 1: 22.6% ± 1.8 [69/311] group 2: 20.2% ± 2.0 [58/300], group 3: 40.8% ± 3.9 [83/205], group 4: 28.5% ± 6.0 [45/155].
The relative abundance of FASN, ACAA1, CPT2 and IGFBP2 transcripts was significantly up-regulated in embryos derived from group 3 compared to those from groups 1 and 2. No significant differences could be seen among embryos of groups 1, 2 and 4, as well as between embryos of group 3 and 4.
The mRNA expression of SCD1 and IGFBP4 was significantly higher for blastocysts cultured with DMSO (group 3) compared to those supplemented with c9t11-CLA (group 1). No differences could be seen among embryos of groups 2, 3 and 4 or among blastocysts of groups 1, 2 and 4.
The relative abundance of ACAA2 and IGF1R transcripts did not differ among blastocysts of all groups.
In conclusion, that development and mRNA expression is affected by the supplementation of the culture media with conjugated linolic acids as well as DMSO. Changes in development rates and gene expression patterns that could be detected in embryos of group three (DMSO-supplemented) were partially compensated by the addition of either c9t11-CLA or t10c12-CLA.

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Notes

DYNAMICS OF NUCLEOPHOSMIN EXPRESSION DURING PREIMPLANTATION DEVELOPMENT OF CATTLE

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No reliable method for embryo quality determination has been developed up to now, the most appropriate indicator seems to be the gene expression. In this study, we concentrated on characterization of nucleophosmin, an important nucleolar phosphoprotein. Using quantitative RT-PCR, the mRNA expression of nucleophosmin from MII stage oocyte to the blastocyst stage was determined. We detected the highest level of mRNA at the oocyte stage. The mRNA amount decreased slowly from 2-cell stage to early 8-cell stage and increased at the late 8-cell stage. This increase was α-amanitin sensitive and thus represents the embryonic genome activation. The mRNA level maintained approximately the same up until the blastocyst stage. On the other hand the protein level had completely different expression pattern. We analysed the protein amount in MII oocytes, 4-cell stage embryos and morulas. In contrast to mRNA, the protein level was the lowest in MII stage oocytes. Whereas the average mRNA level decreased 4-times from MII oocytes to 4-cell embryos, the protein level slightly increased (1.2-times). Both mRNA and protein level increased between 4-cell stage embryos and morulas, but the rise was more expressive in the case of protein. Moreover we have detected a slight shift in the mobility of MII oocyte band using western blot. This likely represents the phosphorylation of the protein in MII oocytes.

Recently we have shown that the injection of nucleophosmin dsRNA causes degradation of corresponding mRNA, but the immunofluorescent analysis revealed no degradation of the protein. Hence, we performed the western blot analysis in 4-cell stage embryos and morulas. Surprisingly, we detected an expressive decrease in the protein amount in nucleophosmin dsRNA injected embryos in comparison to uninjected controls. This likely suggests that the protein is largely degraded, however, the preserved amount is sufficient for coverage of the embryo needs.

Further, we wanted to know, whether the preserved protein amount is sufficient for the proper course of preimplantation development. We analysed the developmental competence of embryos from 2-cell stage until the blastocyst stage and found only a slight difference between nucleophosmin dsRNA injected group and controls. There was a clearly noticeable decrease in number of embryos reaching the blastocyst stage in nucleophosmin dsRNA injected group, however the difference was statistically significant only at the boundary level ($p=0.05$).

In conclusion, our results show that there is a large supply of maternal nucleophosmin in bovine preimplantation embryos. Even though that under standard environmental conditions, the embryonic protein is synthesized after EGA, the stored amount of maternal protein is sufficient for preimplantation development of the embryo.

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Notes

CALVES BORN FROM BLASTOCYSTS PRODUCED IN VITRO WITH SEXED SPERM AND TRANSFERRED AS FRESH OR AFTER VITRIFICATION IN FIBERPLUGS

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The production of sex-known calves from in vitro produced (IVP) embryos is a major tool improving the efficiency of the cattle industry that can be coupled with cryopreservation. We analyzed in vivo survival rates of IVP blastocysts fertilized with sex-sorted sperm, after transfer, both as fresh or after vitrification, by using a modification of the Cryologic Vitrification Method (CVM).

In vitro matured bovine oocytes were fertilized with sex-sorted sperm (Sexing Technologies, Navasota, Texas) and cultured in SOFaaci + 6 g/L BSA. Day-7, good quality expanded blastocysts were vitrified in fiberplugs (CVM) by using vitrification solutions described in previous works (Gómez *et al*., Theriogenology 2008;69:1013-21). Briefly, embryos were washed in holding medium (HM: TCM 199-Hepes + 20% FCS). Groups of 1-8 blastocysts were exposed to HM with 10% ethylene-glycol (EG) + 10% DMSO (vitrification solution-1) for 3 min and then moved into a microdrop containing HM with 20% EG + 20% DMSO + 0.5M sucrose (vitrification solution-2; VS2). Blastocysts were then loaded into a micropipette in 0.3 to 3 µL of VS2, and put in a microdrop in the hook of the fiberplug. The sample was vitrified by touching with the hook in a super cooled block placed in liquid nitrogen. Vitrified embryos were stored in closed straws up to warming, which was performed by immersing the end of the fiberplug directly in 1.2 ml of 0.25M sucrose in HM. After 5 min, embryos were transferred into a 0.15M sucrose medium in HM for 5 min, and subsequently washed once in HM and twice in SOFaaci + 6 g/L BSA + 10% FCS. Embryos were cultured for 1 h before being transferred to synchronous mixed breed recipient heifers (1 embryo/recipient). Pregnancy was diagnosed by ultrasonography on days 40 and 62 (sex diagnosis). Data were analyzed by ANOVA. Pregnancy rates (PR) are shown in the table below. No significant differences were found in PR after transferring fresh or vitrified/blastocysts. Male and female embryos produced similar PR.

ET	N	Pregnancy rate		Sex on Day-62		Calves	Sex/
Fresh		**Day-40**	**Day-62**	**Male**	**Female**	**Born**	**Expected**
Total embryos	17	9 (61)	9 (61)	4	5	9	
Male	7	4 (57)	4 (57)	4	0	4	4/4
Female	10	5 (50)	5 (50)	0	5	5	5/5
Vitrified							
Total embryos	20	11 (55)	11 (55)	2	9		
Male	9	4 (44)	4 (44)	2	2		
Female	11	7 (64)	7 (64)	0	7		

Vitrification in fiberplugs allows obtaining pregnancy rates similar to fresh embryos, a fact that could facilitate the application of IVP technologies on farms.

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Notes

COMPARISON BETWEEN WARM AND COLD TRANSPORT CONDITIONS OF MURINE EARLY SECONDARY FOLLICLES

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Recent applications in assisted reproductive technologies have been focusing on the isolation, *in vitro* culture and preservation of ovarian tissue and isolated follicles. It has become increasingly clear that the conditions in which these vulnerable follicles are kept during various manipulations before culture have an impact on their further developmental capacity either *in vitro* or when transferred back to a recipient. In this study, we aimed to determine the best transport conditions for early pre-antral mouse follicles after isolation from the ovaries from 13-day old B6CBAF1 mice. We therefore tested both cold (13°C) and warm (37°C) transportation of murine early secondary follicles (208 and 169 follicles respectively, 4 replicates). Mean transport time was 47 min, after which the follicles were cultured individually in α-MEM based media enriched with hormones. The follicles were evaluated for follicular development on days 4, 8 and 12. Oocyte maturation was evaluated on day 13. After 4 and 8 days of culture, 40% of the medium was refreshed with standard culture medium, while on day 12 a stimulation medium (hCG, EGF) was used. An additional steroid analysis (progesteron and estradiol) was performed to study the steroidogenic capacity of day 12 antral follicles.

Binary logistic regression models revealed that there was no significant effect of transport conditions on the developmental kinetics on days 4, 8 and 12 after culture. There was neither an effect on the nuclear oocyte maturation (extrusion of the first polar body). No differentiation could be made between the two follicle groups based on steroid analyses. Mean percentages of a good follicular development for respectively cold and warm transport were 72% vs 75% on day 4 (follicular-diffuse or diffuse stage), 74% vs 72% on day 8 (diffuse, diffuse-antral or antral stage), 74% vs 71% on day 12 (diffuse-antral or antral stage) and 69% vs 67% on day 13 for oocyte maturation.

These results suggest that follicles, before *in vitro* culture or transfer to a recipient, may be kept in either warm or cold conditions, in an adjusted incubator. Since the preservation of ovarian tissue, follicles and oocytes is very topical and research in this area is evolving very quickly, this information is of potential use for both fertility research groups and clinical settings for assisted reproduction.

Notes

BOVINE OOCYTE DEVELOPMENTAL FAILURE IN RESPONSE TO ELEVATED NEFA CONCENTRATIONS: CONTRIBUTION OF THE MITOCHONDRIAL B–OXIDATION

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Elevated concentrations of non-esterified fatty acids (NEFA) in the blood are known to alter the follicular micro-environment. While it is clear that such alterations impair the development of the oocyte and alter the quality, metabolism and viability of the resultant embryo, there is no etiological explanation for these observations. In this study, we explored the potentially unifying hypothesis that reduced oocyte developmental competence is attributable to increased mitochondrial function. We therefore evaluated the effect of elevated NEFA concentrations during oocyte maturation in the presence of an inhibitor of the mitochondrial fatty acid oxidation. The inhibitor used was β-mercapto-acetate (β-MA), a competitive inhibitor of 3-hydroxyl CoA dehydrogenase, a component of the β-oxidation pathway.

During a serum-free maturation period of 24h, bovine COCs were exposed to maturation medium (0.75% BSA) supplemented with 1) physiological NEFA concentrations = CONTROL (150 µM of total NEFA, i.e. oleic, stearic and palmitic acid), 2) elevated stearic acid concentrations = HIGH SA (75 µM SA), 3) elevated NEFA concentrations = HIGH COMBI (425 µM of total NEFA), 4) CONTROL + 0,1mM β-MA, 5) HIGH SA + 0,1mM β-MA, 6) HIGH COMBI 0,1mM β-MA. Following IVF using semen from a bull of proven fertility, zygotes were cultured in SOF (+ 5% of FCS) medium. Cleavage (48h pi) and blastocysts rates (8 days pi) were evaluated. In total, 649 oocytes were fertilized (3 replicates).

The CONTROL and HIGH SA exposed oocytes showed a reduced post-fertilization development when β–oxidation during oocyte maturation was inhibited. This was in line with our expectations, since β-oxidation is the primary energy generating pathway during oocyte maturation. In contrast, the developmental potential of the HIGH COMBI exposed oocytes significantly improved following inhibition of the β-oxidation during oocyte maturation suggesting that the developmental failure of oocytes exposed to elevated NEFA during maturation, is the consequence, at least partly, of an excess in mitochondrial oxidative activity.

n (%)	CONTROL	CONTROL + β-MA	HIGH SA	HIGH SA + β-MA	HIGH COMBI	HIGH COMBI + β-MA
Oocytes	120	116	92	111	109	101
Cleaved	96 (80.0)	89 (76.7)	72 (78.3)	85 (76.6)	93 (85.3)	82 (81.2)
Blastocysts from oocytes matured	27 (22.5)	20 (17.2)	18 (19.6)	16 (14.4)	14 (12.8)*	21 (20.8)$^{\$}$

Superscripts *,$: *P*=0.01

In conclusion, these data reinforce our hypothesis, showing that β-oxidation plays a crucial role in the induction of developmental failure in NEFA exposed oocytes.

Notes

ANTI-MUELLERIAN HORMONE (AMH) CAN HELP TO PREDICT FOLLICULAR GROWTH IN MARES

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Anti-Muellerian hormone (AMH) is a glycoprotein that is expressed only in the gonads. While secretion of AMH from the testicular Sertoli cells is essential for male fetal sex differentiation during embryonic development, in adult females it is produced only from granulosa cells of preantral and small antral follicles. Therefore it is used as an endocrine marker for this pool of small gonadotropin responsive follicles, which are also termed the ovarian follicular reserve in humans. Woman with high AMH plasma levels indicating a great ovarian follicular reserve can get lower FSH doses and have a better ovarian response after a stimulatory treatment. Agreeing findings are recently published also for cows, but only little is known for equine.

The aim of our study was to determine AMH levels in equine plasma and to describe their relationship to follicular growth in mares.

After weekly repeated ultrasound guided follicles aspiration sessions, we chose 4 mares, out of 12 oocyte donor mares, which had constantly a high number of follicles between the aspiration sessions (11.1 ± 1.1 follicles) and 4 mares, which showed constantly low numbers of new grown follicles (6.2 ± 0.5 follicles). Plasma AMH levels were measured with the Active MIS/AMH ELISA kit (DSL-10-144400; Beckman Coulter, USA) in blood samples, which were taken from each mare at five consecutive follicle aspiration sessions.

Mares with many new grown follicles between consecutive follicle aspirations had significant higher plasma AMH levels (1.2 ± 0.05 ng/ml) compared to mares with a lower number of new grown follicles (0.6 ± 0.04 ng/ml; $p < 0.001$). Individual AMH levels remained at a constant level during the observed period in each mare. While we found a significant positive correlation between AMH levels and the number of weekly new grown follicles ($r = 0.67$), the number of recovered oocytes per aspiration session showed no relationship to plasma AMH levels.

We present for the first time quantitative AMH analysis in equine plasma and show that AMH concentrations have a significant relationship to follicular growth in mares. High AMH concentrations in the plasma of mares indicate that a greater number of follicles will be available from that animals in a follicle aspiration program. Because induction of superovulation is still a problem in mares, AMH concentrations possibly could be a useful diagnostic marker especially in equine to compile individual superovulatory treatment protocols, like it is done in human medicine.

In conclusion, plasma AMH levels can be used to predict the number of available follicles for a follicle aspiration program in mares. Therefore it could be also an important marker to calculate the ovarian follicular reserve in mares with regard to assisted reproductive technologies.

Notes

IN VIVO SURVIVAL OF RABBIT MORULAE AND EARLY BLASTOCYST VITRIFIED IN DIMETHYLSULFOXIDE-ETHYLENGLYCOL VITRIFICATION MEDIA WITH DEXTRAN AND POLYNYLALCOHOL

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Vitrification of embryos is being increasingly important for cryopreservation in mammals. Substantial advances were made to decrease the toxicity of cryoprotectants by applying less toxic and more permeable chemicals, increasing cooling and thawing rates using both new carrier tools and devices producing vacuum in the liquid nitrogen. In order to reduce the amount of permeable cryoprotectant used, large molecular weight polymers can be added to vitrification media. Several of these molecules have been studied, such as polyvinylalcohol, polyvinylpyrrolidone, ficoll or dextran. Polymers can enhance the properties of vitrification medium preventing osmotic injury, coating the cells, protecting the cell membrane, serving as bulking agents or increasing the tendency of the solution to supercool.

The aim of this work was to assess the effect as vitrification medium of the 1% (w/v) polyvinylalcohol, 10% (w/v) dextran, 15% (v/v) dimethylsulfoside and 20%(v/v) ethyleneglycol on the *in vivo* survival of rabbit embryos.

Nulliparous New Zealand White females belonged to a selected line were used as donor and recipient does. Donors were inseminated with a pool of semen from males of same line. Two-hundred eighty eight morphologically normal embryos of 70-72 hour after ovulation induction were vitrified or were used as fresh control to transfer into oviducts of 24 recipient does (12 embryos by female). Recipient does were induced to ovulate with 1μg of buserilin acetate 60-62 hours before transfer.

After warming, 100% of vitrified embryos were transferable, and 22 recipients became pregnant and give at birth (11 from each group, 91.7%). *In vivo* survival rate at birth in pregnant does was 34.8% vs 49.2% from vitrified and fresh embryos, respectively ($P<0.05$).

In rabbits is well known the essential role of mucin coat and *zona pellucidae* in the embryo development and implantation, therefore, the cryopreservation media and procedures must avoid, in addition of injuries in embryo cell, damages on rabbit embryo coats. In accordance with our *in vitro* previous study, addition of dextran and polyvinylalcohol seem to protect embryos against cryoinjuries by prevent crystallization in the transition phase between solid and liquid during warming process, and 100% of vitrified embryos were undamaged after warming. In spite, vitrification media based in 35% of permeable cryoprotectants (dimethylsulfoxide and ethylenglycol) with dextran and polyvinylalcohol polymer can be used with *in vivo* successful results, it did not improve results from other vitrification media or procedures used by our laboratory. Further assays have to make any necessary adjustments to achieve *in vivo* survival rate of fresh embryos.

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