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European Embryo Transfer Association

29ème COLLOQUE SCIENTIFIQUE

29th SCIENTIFIC MEETING

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Dr. Tom McEvoy

Special Celebration

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Istanbul, Turkey, 6th and 7th September 2013

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Tom McEvoy is a native of County Kildare in Ireland and grew up there on a small farm in Kilberry, a few miles north of Athy where he attended the Christian Brothers School (CBS Athy) and encountered Brother SC O'Toibin whose enthusiasm for science helped sow seeds of interest. Two other influences back then were Pat Sheridan, the local Agricultural Instructor and family friend, and Tom's younger brother Pat who suggested sensibly that the farm at home wasn't big enough for both of them: Pat still farms there to this day!

In 1983, Tom graduated from University College Dublin (UCD) with an Honours BAgrSc Degree, his years there having been opportunities to learn from Professor Ian Gordon and Dr (later professor) Maurice Boland, both of whom would be AETE medallists themselves, in 1995 and 2011, respectively. Professor Gordon's enthusiastic and animated teaching style were particular influences that attracted Tom to both in vivo and in vitro aspects of reproductive physiology, a 'pairing' from which he has not yet detached himself.

Also in 1983, Tom was fortunate to be chosen for a post-graduate studentship at The Agricultural Institute's (An Foras Taluntais) Belclare Research Station in County Galway, under the direct supervision of Dr Joe Sreenan, a pro-active scientist whose contributions to embryo transfer (ET) and related research were already appreciated internationally. Others there who'd be influential included Dr Seamus (JP) Hanrahan, Dr Michael Diskin, Dr Dermot Morris and Tony McDonagh. Professor Ian Gordon, a Scot from the eminent Sir John Hammond's 'Cambridge School', was Tom's university supervisor. Because of a commitment to participation in the National Colleges Ploughing Championships later that year, Tom spent the first few weeks of his postgraduate studentship at UCD's Lyons Estate, combining his preparation for that 'macro-manipulation' contest with laboratory-based learning of procedures for micro-tool forging (akin to a blacksmith moulding miniature glass instruments) and micro-manipulation, skills that would be employed and improved at Belclare in the years that followed. Tom spent a total of seven years at Belclare (not always doing PhD studies!) and, under the expert guidance of Joe Sreenan, enjoyed a range of opportunities to flourish as an embryologist in the laboratory as well as being involved in animal-based research related to superovulation, egg collection, embryo transfer, twinning and, occasionally, immunisation. The opportunities included various overseas visits, notably to INRA at Jouy-en-Josas in France for training related to gene transfer in fish, to Sudan as part of a team involved in embryo transfer to Kenana cattle, and (beyond the Iron Curtain) to Sofia in Bulgaria as a participant in a three-week molecular biology training course organised by the United Nations' International Cell Research Organization (ICRO). On the home front, key links would include University College Galway (Dr Frank Gannon's and Dr Mike Kane's laboratories, in particular), the Ovamass cattle IVF laboratory in Fethard, County Tipperary (Dr KH Lu), and Salmon Research Trust of Ireland (SRTI; Dr David Piggins) research stations at Cong and Newport, County Mayo.

Occasional farm-visit activities included participation in embryo collection from mares at Kildalton, County Waterford (with Michael Diskin) and from then-exotic cattle in Tandragee, County Armagh (with Tony McDonagh).

Embryo bisection was Tom's first focus and, such was the 'buzz' in seeing identical twin calves emerge nine months later from eggs encountered as morulae or blastocysts, rumour has it that if Joe had not insisted research involved moving on to new challenges, Tom might still be happily bisecting embryos to this day! Instead, taking Joe at this word it seems, Tom's career has since been one of varied activities, typical of a pioneering inclination awakened under Joe's influential and foundational supervision. Ultimately, most of Tom's post-graduate research and training while at Belclare involved studies related to gene-injection, both in mammals and fish. The latter, involving Dr Frank Gannon and colleagues at UCG, culminated in the publication of a paper in Aquaculture (1988) reporting the first-ever case of Atlantic salmon (Salmo salar) expressing a foreign gene transferred via microinjection. Other original research findings from those Belclare days were published on topics such as embryo bisection and, with great friend Caroline McCaffrey, embryo co-culture. In 1990, Tom submitted his PhD dissertation to the National University of Ireland on the same September day as he left his native land to take up a post-doctoral appointment at the Rowett Research Institute in Aberdeen, Scotland. Consequently, Tom's PhD viva was held in Edinburgh, the external examiner being Dr Ian Wilmut (AETE medallist in 2003).

The postdoctoral appointment in Aberdeen introduced Tom to South American camelids and to Dr Clare Adam and Deirdre Bourke, two scientists who already were pioneering ET-related research among these species. A steep learning curve ensued in coming to terms with alpaca, llama and guanaco reproductive anatomy and physiology. A seemingly necessary part of the initiation – i.e. being the recipient of gut contents spat out by an unimpressed llama – left Tom with an aroma that, although distinctly different, was as memorable as that picked up a quarter century earlier when wee Tom, in a mis-timed experiment, had managed to jump into (rather than all the way across) a dip-tub full of sheep on his grandfather Patrick Holden's farm in Ouragh, County Carlow. It was said subsequently that, since then, there were no flies on Tom. Maybe so, but an Aberdeen llama expressed its opinion in a not-to-be-forgotten manner that, for a time at least, attracted flies and laughter in equal measure! As well as such mishaps, several years of research among alpacas, guanacos and llamas contributed to developments in camelid semen processing, AI and embryo culture. Among the team's successes was the establishment of pregnancies following transfer of llama and guanaco embryos to llama recipients. Original reports of camelid research at RRI were complemented by review papers that aimed to widen awareness of the reproductive physiology and embryology of these fascinating creatures.

During just over four years spent at RRI, Tom also worked closely with Dr John Robinson (later Professor and FRSE), then Head of the Animal Reproduction Department. John and his wife Margaret, both natives of County Down in Ireland, provided unequalled hospitality that echoed an ancient Irish tradition of fostering where, historically, a son was 'farmed out' to another family that could help broaden his horizons and experience. They both deserve tremendous credit and thanks for all that followed. Meantime, science-wise, as well as his ongoing camelid research Tom became involved in applying an in vitro culture dimension to projects within John's department that were investigating factors influencing ovine reproductive competence. That work, which continued after John and Tom had moved across the road to work at SAC (Scottish Agricultural College), initially explored effects of nutrition and exogenous hormones, notably progesterone, on developmental competence of oocytes and embryos from superovulated donor ewes. Subsequently, effects of dietary nitrogenous compounds on embryo development would be manifest as upregulated metabolism in embryos and ultimately, in a minority of cases, increased lamb birthweights.

From 1995 onwards, Tom became involved in almost a decade of in-depth in vivo and in vitro studies of the large offspring syndrome (LOS) in sheep, as well as in beef cattle research relating to superovulation and ovum pick-up (OPU). The LOS research was a collaborative effort involving personnel at Roslin Institute, RRI and SAC, among them Ian Wilmut, Lorraine Young, Charlotte Maltin, Cheryl Ashworth, Peter Broadbent and Kevin Sinclair. While asynchronous ET

had been implicated, Tom's particular interests were in effects of donor diet and in vitro culture, the latter especially in terms of impacts of reliance on serum. As well as original research findings being published in a range of journals (most notably Young et al. 2001; Nature Genetics 27: 153-154, cited more than 450 times to date), various review papers from the team have alerted a wider audience not only to the unfolding and fascinating developmental realities of ruminant livestock embryology but also to the need for great care and vigilance because mammalian oocyte and embryo exposure to in vitro culture and/or manipulation can incur long-term adverse repercussions. The marvel never to lose sight of, according to Tom, is that development occurs at all! Inherent resilience, he insists, underpins this reality on which both the ET industry and in vitro research studies depend. Ironically, of course, it is because of this same resilience that aberrant development rather than (in some respects more straight-forward) outright embryo loss can be a consequence of sub-optimal culture. One statistic suggesting that perhaps too few of us give due credit or adequate attention to embryo resilience is that, in June 2013, a search of the US NIH's PubMed website listed 11,097 results for 'embryo sensitivity' but only 35 for 'embryo resilience'. Incidentally, among the latter was a paper (McEvoy TG, 2003; Reprod Dom Anim 38: 268-275) presented by Tom at UCD ten years ago.

At SAC, the cattle research in which Tom was involved was in conjunction with Dr Peter Broadbent and his team, notably David Dolman, Robert Watt and Callum Angus-Meldrum. In due course, through collaboration with Dr Hal Thompson of the University of Glasgow, the research provided important data, in terms of animal welfare, on effects of repeated OPU on ovarian tissues and epidural injection sites. Other cattle research, this time with emphasis on dairy herd infertility and with input from Dr John Rooke, included SAC collaborations with Professor Bob Webb and his teams at Roslin Institute and, subsequently, University of Nottingham. On the home front, laboratory-based research (thanks to expertise of Mary Staines, Alison Ainslie, Margaret King, Karen Mackie and Gilbert McCallum) facilitated various stepwise advances in bovine / ovine embryo culture and cryopreservation, both in conventional and defined media, often as part of PhD projects for post-graduates from across Europe (Frances Alink, Netherlands; Vanessa Moreira, Portugal; Alex Reis, Portugal; Graham Coull, Scotland; Ali Galip Onal, Turkey). Laboratory-based investigations also contributed to studies as varied as gender preselection, seasonality and in-depth analysis of the lipid and fatty acid content of cattle, pig and sheep oocytes. Drs David Cran, Bill Dingwall, Bill McKelvey, Linda Mitchell and Brian Speake were key to those advances.

Among other beneficial collaborations, which Tom sees as crucial to various efforts and achievements throughout his career to date, were those involving enthusiastic post-doctoral visiting scientists including Ylva Brandt (Sweden), Catherine Carolan (Ireland) and Mehmet Kuran (Turkey). Likewise, links with Professor Henry Leese and Dr Roger Sturmey have been rewarding not only in terms of elucidating a revised understanding of embryo metabolism norms, consistent with Professor Leese's 'Quiet Embryo' hypothesis, but also in helping to increase awareness of fatty acid roles and importance in embryo development and energy provision. Ties to industry also have been significant, formally with feeding and/or breeding companies (such as Cogent, Genus, Harbro Ltd, Innovis, North Eastern Farmers, Ovamass Ltd) and informally by various routes, such as SAC's "Success through Knowledge", linking science to practice at International workshops and via advisory booklets. Drs Colin Morgan and John Vipond expertly facilitate this knowledge exchange/transfer (KE/KT) with whom and to where it most matters. Research-wise, recent project themes with emphasis on farm-level relevance included ewe prolificacy (e.g. Inverdale studies) and dietary-mediated (e.g. fish oil / feed intake) effects on reproductive function. John Hunton, Peter Kenyon, James Mylne, Dr Julie Finch and Dr Maureen Wood were key participants.

Unfortunately, it is not possible here to name everyone who helped Tom do the work that has, to his surprise, led to this Pioneer Award. All his mentors, co-authors (on more than 150 publications), colleagues and line-managers, in Belclare, RRI and SAC (now SRUC, Scotland's Rural College) are duly acknowledged. Governments, Funding Agencies and various Trusts, nationally and Europe-wide, also have provided indispensable support. In his own words (but even here including input from Bernard de Chartres!), Tom has written:

"*It is all too seldom said that we see a little further because we stand 'on the shoulders of giants' and I've been extremely fortunate throughout my career, in both formative and subsequent years. Names of key mentors have changed over the years but, always and advantageously, 'there be giants' among the guides, supervisors and collaborators I've known, foremost among them being farming-wise my name-sake father and late uncles Mick Holden and Denis Hickey, and career-wise Ian Gordon, Mike Kane, Henry Leese, John Robinson, Joe Sreenan and Ian Wilmut.*"

Last but by no means least, Tom is totally indebted to his darling wife Anne whom he met in *Macra na Feirme* (the 'Young Farmers' organization which dramatically enhanced his PhD years in Galway!); to his late parents Tom and Ellen McEvoy, God rest them; sisters Mary, Margaret, Cecile and Helen; and brother Pat who has not only kept the home fires burning but also always provides a great welcome whenever Tom finds time to return to his roots in the short-grass county where, at clan gatherings, precious catch-up encounters with 18 nieces and nephews help keep his feet firmly on the ground.

Ian Kippax BVSc. MRCVS
AETE Board member, UK

LIVESTOCK EMBRYOS: *IN VIVO* AND *IN VITRO* SENSITIVITIES AND RESILIENCE

MCEVOY TG

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Introduction

Seventy-five years ago, Walton and Hammond's classical Shetland x Shire and reciprocal cross-breeding study demonstrated the extent to which the maternal uterine environment – the cradle of embryonic development – can facilitate or hinder development (Walton & Hammond, 1938). Just over a decade ago, and availing of the intervening progress in embryo transfer technology for application to livestock, Allen and colleagues' studies of pony and thoroughbred embryos confirmed that genotypic blueprints of in vivo-derived embryos rely greatly on environmental influences to achieve their potential in terms of phenotype. Where constrained, offspring birthweight reflects the limitations of surrogate dams, with foals delivered being of reduced birthweight and exhibiting features typical of having been physically as well as physiologically restricted in utero (Allen et al, 2002). Embryo transfer technology has also been used to investigate the legacies of less natural origins, notably of in vitro production which, in contrast to aforementioned in vivo equine studies, sometimes but not always saw significant numbers of ruminant (cattle, sheep) embryos either succumb inexplicably or survive and seemingly refuse to be constrained by the uterine capacity of their surrogate dams (Behboodi et al, 1995; Mayne & J McEvoy, 1993; JD McEvoy et al, 1995a; McEvoy et al, 1998a; Reichenbach et al 1992; Walker et al, 1992). In what eventually was referred to as the Foetal Oversize phenomenon, or Large Offspring Syndrome (LOS), their developmental trajectory meant that phenotypic outcomes often far exceeded breed-specific birthweight norms. Here was both sensitivity and resilience in action but, in the early days, we knew neither why nor how.

Developmental intrigues

The author's first introduction to LOS was a caption and front-page photograph, in the Irish Farmers Journal, of calves born to dairy cows at Hillsborough Research Centre, Co. Down, following transfer of embryos that had been produced in vitro in a laboratory at Fethard, Co. Tipperary in the late 1980's. That story, while initially seeming to be a great success despite the unusually high birth weights that were being reported (and some readers initially presumed those data were typographical errors!), actually but inadvertently indicated that those transferred embryos had developed in an utterly unprecedented manner that would interest and intrigue many scientists in due course. For the time being, because the news was of calves born alive and well and because seemingly no one yet even guessed what damaging developmental 'iceberg' lurked out of sight, the intriguing questions did not surface. Certainly, more harmful and lethal manifestations of the syndrome, reflecting various insults to ruminant embryos' developmental sensitivities, were not on the wider scientific community's radar until a paper by Walker et al (1992) sounded an alert at that year's Annual Conference of the International Embryo Transfer Society.

Eventually, although not until some years after moving to Scotland in 1990, but then during almost a decade of collaborations involving various research groups in the UK, the author was privileged to be involved in investigating the large offspring syndrome (LOS) phenomenon. The syndrome encompasses many other aberrant developmental outcomes too, although in some laboratories it was not deemed to be an issue unless somatic cell nuclear transfer was practised (Gordon, 2003b). In the author's experience, however, the 'large offspring' phenomenon was very

real, the title very apt and, frankly, no exaggeration. In LOS sheep studies conducted at SAC and The Roslin Institute, for example, the familiar requirement for designation of LOS was that birthweight (or Day 61 or Day 125 ovine foetal weight, when welfare implications precluded development to term) be more than three standard deviations above corresponding mean birth or foetal weights of control counterparts (Sinclair et al, 1999). Nevertheless, in some of our studies, where in vitro conditions were engineered to induce/generate LOS (with a view to better understand and thereby eventually avoid or correct the phenomenon and its causes), as many as two-thirds of the foetuses or lambs derived from particular in vitro culture 'treatments' did match that criterion (Sinclair et al, 1999). These extremes are not typical and, following culture and transfer (whether of cloned or non-manipulated eggs), various other findings have been reported. For a cross-section, see: Lane and Gardner, 1994; Keskintepe et al, 1995; Brown and Radziewic, 1996; Hill et al, 2000; Kuran et al, 2000; Bauersachs et al., 2009; Mansouri-Attia et al, 2009).

When eventually a range of intriguing LOS-related questions were tackled, the term 'epigenetics' (Bird, 2002; Young and Fairburn, 2000; Young et al, 2001) would feature prominently and, of course, it is known now that epigenetic alterations or aberrations can play a part in other phenomena associated with embryo resilience and sensitivities, both in vivo and in vitro. Critical sites and stages, where relevant processes such as DNA demethylation and histone tail modifications elicit their effects in reprogramming cell fates, are in the egg immediately after fertilization and, subsequently, in the embryo/foetus during establishment of primordial germs cells, the progenitors of mammalian gametes. (Seisenberger et al, 2012). These undoubtedly are phases of acute developmental sensitivity and so great care is needed when considering possible effects of procedures such as, for example, intracytoplasmic sperm injection (ICSI) that "may alter epigenetic regulation in such a manner that these effects persist in the course of development" (Kohda and Ishino, 2012).

Before moving on to more particular issues in terms of livestock embryo sensitivities and resilience, it seems fitting to note that one advantage of involvement in research across a range of species is that, again and again, nature springs a surprise or, perhaps more accurately (at least in this author's case), alerts the observer, at least partially, to what has been going on for millennia (maybe embryochronological aeons?) in particular species. Three South American camelid species (or sub-species) – alpaca, guanaco and llama – confounded a then younger scientist's expectations by displaying a range of features not found in the ruminant rule-book. Although gestation lasts nearly a year, their concepti are long-since hatched as early as 8 days post-ovulation, and the 'window' for maternal recognition of pregnancy has already arrived. Both ovaries are equally active but only the left uterine horn carries the cria to term. Some of what was learned in the course of a few years in their midst has been recorded (Bourke et al, 1995; McEvoy et al, 1994) but, as applies also to topics that follow in this paper, many intriguing questions remain and that is the inherent beauty of this discipline!

Manipulations, monozygotics and transgenes

While livestock oocyte/embryo sensitivities and vulnerability have been focal in much of the author's research post-PhD, in contrast and with mixed results, the work of earlier years relied greatly on embryo resilience as Day 2 and Day 7 in vivo-derived bovine ova were subjected to microinjection and bisection, respectively. During those days too, as well as the work among cattle and with their embryos (McCaffrey et al, 1991), a lot was learned about laboratory mouse, rat and rabbit reproduction, semen collection, cryopreservation and (most crucially) in vitro culture and media formulation through time spent at University College Galway in the expert and enthusiastic company of Michael T Kane, an innovative embryologist with close ties to Bob Foote and Barry Bavister (see, for example, Kane & Foote 1971; Kane and Bavister 1988), and all of this thanks to Joe Sreenan, who not only was a pioneer of cattle ET studies (when Ray Newcomb and others were

similarly occupied) but also, as a front-runner in terms of in vitro advances (Sreenan et al, 1968; Gordon and Lu, 1990; Gordon, 2003a), was someone whose PhD thesis laid crucial groundwork for the eventual bovine IVM- and IVF-derived twin-pregnancy success of Lu et al. (1988) in University College Dublin.

At Belclare, the resilience of recently fertilized bovine ova subjected to centrifugation (13,000 x *g* for 5 min) to facilitate pronuclear visualisation and subsequent gene-injection was confirmed to the extent that, following oviductal transfer to surrogate recipients via mid-ventral laparotomy, 21 of 66 subsequently developed to term and although none was transgenic, the pertinent finding in the context of this review was that all were phenotypically normal (McEvoy and Sreenan, 1990a). Incidentally, absence of a transgenic animal among the offspring was consistent with the then-expected "0 to 2%" incidence of transgenic calf production following gene transfer via microinjection (Gannon et al, 1990). Joe Sreenan's laboratory, in collaboration with the laboratory of Frank Gannon at UCG and colleagues at the Salmon Research Trust of Ireland in County Mayo, did however have notable success in being first to transfer genes to ova of Atlantic salmon (*Salmo salar*; McEvoy et al, 1988), albeit using a distinctly different two-stage process for microinjection (Chourrout et al, 1986), as illustrated and discussed in a subsequent review that considered whether transgenic animals might have a role in food production (McEvoy et al, 1992a). In terms of resilience of fish ova subjected to the physical process of microinjection, the stage at which injection occurs can, as for mammals, be a determining factor influencing not only survival but also the extent to which, and how the transferred DNA subsequently persists (extra-chromosomally being possible) in a range of fish species (Chourrout et al, 1990; McEvoy et al, 1992b; Walton et al, 1987).

The resilience of Day 7 bovine embryos is now well-appreciated and, given that we now know enucleated oocytes can withstand reconstitution via electrofusion and undergo reprogramming, it is not easy to appreciate that some decades ago researchers were just discovering that morulae and blastocysts could be bisected successfully and subsequently transferred (without either zona pellucida or agar-chip protection) with a view to producing monozygotic twin calves and lambs. A prelude to blastocyst-stage bisection was the more intricate work of scientists such as Steen Willadsen, who devised protocols for micromanipulation of earlier stage embryos (2-, 4- and 8-cell) and demonstrated the inherent resilience exhibited by 4- to 8-cell embryos when subjected to blastomere separation and subsequent chimaeric or other recombination. At Belclare and elsewhere, various procedures for zona-free demi-embryo generation from bovine morulae and blastocysts were investigated and revealed the readiness and alacrity with which these recovered from the trauma of bisection to continue development in the short-term and, in the case of most of those subsequently transferred to recipient heifers, to neonatal and later life stages (Kippax et al, 1991; McEvoy and Sreenan, 1990b; Ozil et al, 1982; Williams et al, 1984).

In days before laser surgery, ophthalmic microsurgery blades allowed bisection of embryos so that, for example, the subsequent availability-on-demand of identical offspring could be anticipated to enable researchers to conduct experiments with far fewer livestock than otherwise required. A case in point arose some time after the author had moved to Scotland and, in a mini-project, bisected embryos that resulted in production of three sets of identical twin calves destined for use in research at the University of Edinburgh's Centre for Tropical Veterinary Medicine. Allen (2009) also noted that identical twin equine embryos are valuable in terms of research, with the best prospect of their production being through transfer of monozygotic demi-embryos to separate recipients. Resilience of embryos, in recovering from trauma of incision and premature zona pellucida removal, underpins such practical options and, in its time, such resilience was considered notable. Later, of course, the far more invasive protocols required for oocyte enucleation and subsequent somatic cell nuclear transfer eventually made such incisive interventions seem near-benign but successes in both cases rely on and reflect inherent oocyte / cytoplast / embryo resilience

(McEvoy et al, 2003, 2006). Interestingly, in recent times the value of monozygotic twins for research has been attributed not to their similarities but to their differences. Whereas previously it was presumed that the similarity of genotypic identicals could help researchers effectively avoid much of the incidental variation normally encountered in research studies (and thereby reduce the requisite 'n' per treatment), it is now perceived that their differences can offer crucial insights into epigenetic factors and influences that underlie diseases or other limitations differentially evident in adult-stage monozygotic twins (Coolen et al, 2011; Czyz et al, 2012; Talens et al, 2012). Farm managers in the 1980's queried the author's claims that, despite overt coat colour differences (e.g. eye patches present or absent), twins were truly monozygotic; appreciating that 'seeing is believing', the question is answered most helpfully by visual evidence apparent in conjoined twin specimens from museums (see, for example the 1951 paper of Johansson and Venge). Nowadays, insights into diseases of humans and other mammals are accruing from the fact that far greater and more profoundly influential (fateful, even) differences than those affecting coat colour are manifest in some cases (Coolen et al, 2011; Czyz et al, 2012; Talens et al, 2012). Referring to this, Zwijnenburg et al (2010) noted that discordant monozygotic twin pairs are informative in respect to, for example, post-zygotic mutagenesis and that analysis of such twin pairs may represent an elegant approach to identify genes of relevance to inherited disorders.

Biopsy procedures have recently come back into the spotlight on account of emerging evidence that there can be repercussions in terms of subsequent development of mammals from embryos subjected to the process at an early stage of development. Rather than being a practice that can be carried out with impunity, there can be problems in terms of subsequent hatching and/or exposure of genotypes sensitive to in vitro culture conditions (Sugawara and Ward, 2013). Of particular note is the report by Yu et al (2009) who compared the development of mice from either control (intact) or biopsied embryos and found that there was what they termed 'a potential high risk' of neurodegenerative disorders among mice from biopsied embryos As a result, recent years have seen a call for closer scrutiny and vigilance following interventions such as PGD (pre-implantation genetic diagnosis), that compromise the zona pellucida. Incidentally, in the context of natural fertilization procedures, it is noteworthy that Bedford's overview of mammalian fertilization has repeatedly referred to the inherently 'resilient' zona pellucida of eutherian mammals, especially in terms of both its protease-insensitivity and elasticity, highlighting the prominence and importance of those properties (Bedford, 2008). Earlier reviews and studies (Holm and Callesen, 1998; McEvoy et al, 2000b) noted that in vitro-derived embryos of livestock species have not always retained the zona pellucida's 'natural' properties. For example, Holm and Callesen (1998) noted that while the zona pellucida tends to be a virtually impregnable barrier to pathogens in vivo, it became 'sticky' and less resistant to proteases in vitro. A practical consequence of zona 'stickiness' which prompted concerns regarding stubborn viral/pathogen adherence to the zona pellucida among the livestock ET and import/export community about that time, was that the then-standard trypsin washing procedures came into question and researchers including Dinkins et al (1999) undertook to investigate more aggressive enzymatic processes than those that could be relied on to sanitise in vivo embryos. It has been noted, of course, that prevention of contamination is better than cure (McEvoy et al, 2000b), especially in circumstances where micromanipulation (and its associated violation of the zona pellucida) is practised as a clinical tool (Cohen et al, 2000). In terms of options when applying biopsy procedures to livestock species, it is notable in the context of this review that, in a study of 300 embryos (grade 1 morulae or blastocysts) biopsied and subsequently subjected to cryopreservation before ET, Cenariu et al (2012) found significant effects of biopsy method on embryo resilience as manifest by subsequent 30-day pregnancy incidences, these being 57%, 43% and 31% for biopsy by needle, biopsy by aspiration and biopsy by microblade, respectively. The authors concluded that, because biopsied embryos had had to undergo cryopreservation prior to ET, eventual success was associated with the extent to which zona pellucida structural integrity was preserved during biopsy, the needle method being least damaging in that respect.

Nutrition and nutrients / toxins and trace-elements

During the past few decades, awareness of discrete nutrient-level as well as gross feeding-level effects on developmental competence has grown steadily both in terms of in vivo and in vitro resilience and sensitivities of livestock oocytes and embryos. That same awareness has not always brought enlightenment except perhaps insofar as revealing the complexities and intricacies of nutrient, and indeed metabolite, effects – whether by virtue of absence, adequacy or excess provision – on developmental outcomes, limitations and legacies. Much has already been written by, among others, Robinson and colleagues about ruminant nutrition and its influence on reproduction among both spontaneously ovulating and superovulated livestock. Selected overviews include those by Ashworth et al (2005, 2009), McEvoy and Robinson (2002; 2003), McEvoy et al. (2001a), Robinson (1996), Robinson and Symonds (1995), Robinson et al. (2002a; 2002b; 2006), Sinclair and Agabriel (1998) and Sinclair et al (2003).

Ten years ago, a list of 'priorities for the future' (McEvoy and Robinson, 2003) drafted in the context of conservation biology and applicable to more than mammals (notably egg-laying birds and reptiles) included:

- Feed for fitness and recognise role of body reserves
- Beware of overfeeding – consider growth targets
- Evaluate dietary adequacy in egg-laying species
- Keep diets safe and use suitable feeding regimens

All of these apply equally emphatically today and one in particular, not solely because of its role in stimulating the author's interests in lipid and fatty acid and antioxidant roles, warrants additional mention. Egg-laying species are required to provide a pre-packed feed reserve of sufficient adequacy to sustain their offspring for weeks rather than days. While mammalian embryos have a miniature 'starter-pack' of nutrients within the oocyte cytoplasm (i.e. nanogrammes of lipid, for example; McEvoy et al 2000a) and very soon graduate to absorption / incorporation of nutrients from the maternal (or surrogate dam's) oviducto-uterine milieu, their avian and reptilian counterparts rely for weeks on whatever accompanies them within the notably larger (60 g for poultry, of which lipid is 10% by weight; Etches, 1996) shell-protected egg. A key concern, therefore, has been that captive birds, for example, can be compromised through dietary limitations that could reduce the resilience of developing chicks that, in turn, are captives of their own protective cleidoic shell up to time of hatching (Speake et al, 1999; McEvoy and Speake, 2001). Of paramount importance in ensuring adequate and suitably fortified dietary provision to such creatures is the antioxidant vitamin E, the many roles of which were reviewed in an avian context about that time by Surai (1999). The expertise of Brian Speake, in particular, offered an opportunity about that time to turn attention to microscopic mammalian ova in order to characterise, more completely than before, the lipid and fatty acid composition of oocytes from cattle, pigs and sheep. Following initial reports in 1997 and 1998 (see, for example, Coull et al, 1998) the findings (McEvoy et al, 2000a) were published just one year after the landmark report of Ferguson and Leese (1999) who showed that in vitro culture conditions could lead to near-doubling of the triglyceride content of bovine blastocysts. At the time, given the minute amounts of lipids available for analysis, it was reassuring that the triglyceride content recorded via very different methods in each study were comparable. That reassurance led to a long overdue surge of interest in the lipid and fatty acid composition of livestock oocytes and embryos, not least because of growing awareness that the lipid content of mammalian eggs, whether in vivo- or in vitro-produced, apparently had a major influence on their sensitivity or resilience during and after conventional cryopreservation. Insights into fatty acid roles are also emerging (Sturmey et al, 2009).

Elegant work by Nagashima et al (1995) had demonstrated that physical removal of lipid from porcine 2-4-cell stage and 4-8-cell stage embryos (following centrifugation) enhanced their subsequent cryo-survival and facilitated derivation of live piglets following post-thaw transfer to surrogate sows. As noted by Nagashima et al (1995), such enhanced resilience augured well for cryopreservation of embryos from those mammals, including several endangered species that naturally contain large amounts of lipid. It also was viewed by some as an option to counter the detrimental effects of lipid accumulation in vitro which was found to aggravate the sensitivity of, among others, bovine and ovine embryos, to conventional cryopreservation protocols. While that same sensitivity usefully prompted various researchers to explore vitrification as an alternative to conventional cryopreservation (Moreira et al, 2005), it would not have been good practice merely to accept lipid accumulation as a would-be norm for in vitro embryo production in the knowledge that delipidation technology could somehow bail us out. Among others, Abe et al (1999, 2002a, 2002b), Crosier et al (2000, 2001) and Reis et al (2003, 2005) have investigated in vitro lipid and fatty acid accumulation by ruminant embryos and its effects on structural characteristics as well as viability. A notable finding from some of this work was the value of vitamin E, or a water-soluble analogue of this antioxidant, in helping embryos to be more resilient when challenged in this way (Olson and Seidel, 2000; Reis et al, 2003; Moreira et al, 2005). Earlier, and again more recently, in a context where contrasting ewe diets have been used as a means to alter fatty acid and lipid profiles in vivo, or to generate contrasting sera for parallel investigations in vitro, distinctly different outcomes were recorded for mostly- saturated (Kuran et al, 1999) and polyunsaturated fatty acid-rich (PUFA-rich; McEvoy et al, 2012) supplement scenarios. In the latter study, it was concluded that fortification with antioxidant and moderate rather than excessive fish oil inclusion (i.e. not more than 3% w/w) was a prerequisite to allow oocytes and embryos with only limited inherent resilience to survive in milieux (in vivo and in culture) where risk of damage by reactive oxygen species (ROS) was accentuated by dietary provision of PUFA-rich fish oil or in vitro presence of its derivative sera, respectively (McEvoy et al, 2012). ROS per se will not be considered further in this review but, in anticipation of later remarks about heat stress, it is interesting to remember the message of Hansen (2009) that "many effects of elevated temperature on gametes and the early embryo involve increased production of reactive oxygen species". In contrast to the fish oil-based study, calcium soaps of (mostly) saturated fatty acids were found to be not only benign vis-à-vis embryos but beneficial in terms of enhancement of luteal function and progesterone provision (Kuran et al, 1999).

Benign environments and absence of stressors or damaging agents are major goals when considering embryo development and seeking longer-term optimal outcomes. These 'goals' may not seem too much to ask but attaining them can be a major challenge, whether in vivo or in vitro. In each circumstance, awareness of embryo 'needs' can be helpful, of course, but correctly inferring the implications of particular parameters (e.g. metabolism or amino acid flux in vitro) can be difficult (Barnett and Bavister, 1996; Bavister, 1995). To some extent, appreciation of the concept of quietness (i.e. all being well) in terms of, for example, embryo metabolism has taken some time to evolve but, thanks in particular to the efforts and insights of Henry Leese, there is growing awareness that metabolic busy-ness, or indeed upregulation of particular genes (e.g. gadd153 or CHOP-10; Fontanier-Razzaq et al, 2001) on the part of embryos can often be indicative of distress or damage and of associated efforts on the embryo's part to counter the stress or repair the harm being done (Leese, 2002; Leese et al, 2008). This is not solely an in vitro phenomenon. For example, in a study where dietary urea was used to elevate (cytotoxic) ammonia concentrations in sheep used as embryo donors, indices of metabolism among retrieved embryos were almost 3 times those recorded among control counterparts (McEvoy et al, 1997) and, as in other in vivo 'stress' scenarios (including heat stress), reflected a detrimental loss of 'quietness' (Leese et al, 2007). Along similar lines, Powell et al (2006) noted that, among ovine zygote donors, diets associated with elevated plasma urea concentrations had a part to play in inducing long-term aberrant consequences, notably altered expression of IGF2R, a developmentally important imprinted gene.

Studies in cattle, featuring in vivo and in vitro dimensions, have further explored dietary-modulated nitrogen metabolism and its implications for fertility (Armstrong et al, 2001; Elrod, 1992; Elrod and Butler, 1993; Sinclair et al, 2000a, 2000b)

Quite apart from causes of and cures for adverse effects exerted on oocytes and embryos in vitro, many of which have featured previously in specific reviews or original reports (Gordon, 2003b; Maxfield et al, 1998; McEvoy et al, 1998a, 2001b; Peterson and McMillan, 1998a, 1998b; Peterson and Lee, 2003; Sinclair et al, 1999, 2000c; Young et al, 1998, 2001) consideration also has to be given to both donor and recipient in terms of their management, status and well-being, regardless of the procedures for which they are required. While it is promptly recognized that great expertise and care is needed if oocytes are being harvested via transvaginal ovum pick-up (McEvoy et al, 2002; Reis et al, 2002), equally, something as seemingly mundane as timing of artificial insemination needs to be optimised for best results, because otherwise oocyte sensitivity to aging could undermine results (McEvoy et al, 1996). Finally, of course, diet needs to be safe and so great care is required to avoid exposure to toxins or deficiencies of discrete micro-nutrients such as cobalt or methionine (McEvoy et al, 2001a; Mitchell et al, 2007; Sinclair et al, 2007; Rooke et al, 2009). An in vitro extension of this requirement is that culture media need to be safe, something that has driven research into contrasting systems, not all reliant on serum (Kuran et al, 1998, 2001, 2002; Reis et al, 2002; Rooke et al, 2012).

Latitudes, Seasons and Temperatures

Among factors which normally influence livestock embryo production are season-of-year and diet, with associated elements including day-length, temperature and body condition impinging on, if not actually determining, reproductive competence of different species. In vitro studies underline the fact that mammalian embryos are sensitive to increased temperatures (for example, murine: Gwazdauskas et al, 1992; equine: Mortensen et al, 2010; bovine: Hickman et al, 2013). Much work has been done with cattle and Hansen (2004, 2009) has investigated and reviewed effects of temperature and, in particular, heat stress on livestock, including Zebu cattle. Sensitivity and resilience are considered to be stage-dependent with, for example, expression of heat-shock protein (hsp) genes being a requirement for best survival. Experience of heat-stress is more acute in non-Temperate latitudes and the author's appreciation of the issue was accentuated during participation in a cattle breed improvement project in Sudan during the 1980's (Sreenan et al, 1986). This was at a time when Universities and Governments in many countries worldwide were keen to avail of the new livestock technologies with a view to upgrading breeding stock, either by importation and ET of 'foreign' breed embryos to native cattle or by cross-breeding with 'foreign' livestock imported on the hoof. The thinking (and some evidence) that came to the fore was that the former option was preferable, not only because imported adult stock were vulnerable in more extreme environments but also because ET afforded the option of generating more robust newborn calves that had acquired immunity from their surrogate dams. Often, however, although such breeding programmes in various countries tended to provide headlines and kudos to partners, the feeling among Belclare's team at the time was that, as for counterparts nearer home, Sudan's livestock owners and managers might have gained as much or more from mundane but pragmatic improvements in livestock nutrition, herd management and within-breed genetic selection. As a consequence, a feature of that team's involvement in Sudan (at Khartoum and Wad Medani, the latter just 14 degrees north of the equator), wisely insisted on by Joe Sreenan, was that for every seminar or TV interview with technology & overseas genetics as a focus, another would focus on native resources (nutritive and genetic) and how these were being or could be exploited in the best possible sense of that word. After all, there was little prospect of widespread adoption of AI and ET if liquid nitrogen and electricity were not consistently available in rural areas and, moreover, where nomadic pastoral farming dominated. Better too, to upgrade the native Kenana and Butana breeds

(already the best Zebu dairy cattle in Africa) with their inherent resilience than to divert scarce funds to pamper heat-stressed *Bos taurus* breeds (Rocha et al, 1998) from northern climes.

Returning to more familiar latitudes, environmentally-driven sensitivities in maritime regions of North-western Europe are rarely related to heat stress among livestock embryos. Instead, the major determinants are daylength-related factors that influence the innate circannual variations typical of long- and short-day breeders. In general, although seasonal limitations can and do influence inherent reproductive capability, such issues are not significant for commercial farmers in tune with the seasons. Even so, research interests and alternative farming possibilities have prompted studies to investigate which components of the reproductive process are down-regulated and which retain their inhererent functionality or competence typical of the breeding season. The key question that has been asked, in the context of this review, is whether livestock embryos per se (or their unfertilized progenitors) retain resilience that could facilitate, for example, out-of-season breeding and oocyte/embryo collection. Such studies require careful decision-making in terms of how they are conducted because investigations in 'real time' (e.g. peak season data collected in October; other data later) can be difficult to manage and interpret, even if using within-animal comparisons, on account of the fact that some factors, notably age (and perhaps body condition score and diet quality), inevitably change during the study and they, as much as 'month', may be influential. The alternative approach, which compares out-of-season sheep with others that are 'melatonin-treated' to remain cyclically active, overcomes many difficulties by ensuring 'control' and 'treated' ewes are investigated concurrently (McEvoy et al, 1998b) but then the question that arises is how natural the 'melatonin-treated' cohort is in terms of reproductive physiology.

Among studies that have looked in 'real time' at peak- versus late-season influences on developmental competence of oocytes/embryos was one involving mature Bluefaced Leicester x Scottish Blackface (Mule) ewes to compare embryo development following superovulation at peak-season (October) and again during transition to anoestrus (April), using the same donor animals each time (Mitchell et al, 2002). All semen (pooled from Suffolk rams) had been collected before the study began in October, was stored frozen and subsequently thawed prior to artificial insemination in each month of the study. A standard superovulation protocol (9 mg oFSH administered in 8 equal doses at 12 h intervals) also was used. Results of the study indicated that there was a greater incidence of fertilization failure in April compared to October, perhaps as a consequence of the fact that the LH surge elicited in April did not emulate that recorded in October or because of associated effects on ovarian follicular function. Embryos retrieved in October and in April were evaluated in terms of their morphology and developmental status at collection. Then, following cryopreservation, morulae and blastocysts were subsequently thawed in order to facilitate in vitro laboratory-based developmental studies of collected embryos at the same time. These demonstrated that, although parameters at collection did not differ, post-thaw development and metabolic indices of glucose uptake and de novo protein synthesis were inferior among April embryos, indicating functional differences consistent with a sub-optimal endocrine milieu perhaps as much as several weeks to months before ovulation had occurred. This time-scale is plausible because it is now appreciated that developmental competence of bovine and ovine oocytes is not solely a reflection of contemporary ovarian status at ovulation but also a consequence of influential factors such as diet from the time of follicular recruitment from the primordial pool. Sensitivity, therefore, pre-dates ovulation and fertilization.

Progesterone, prolificacy and counter-intuition

Picking up the theme that 'sensitivity' pre-dates ovulation and fertilization, it is notable that, among superovulated sheep, improved progesterone or progestagen analogue provision during pre-ovulatory priming with intravaginal CIDR and/or sponge devices can facilitate optimal blastocyst production, especially if feed intake level, and perhaps also shearing at housing for example, steps

up hepatic clearance of the hormone from circulation (McKelvey and Robinson, 1986; Thompson et al, 1990; Parr et al, 1993a & 1993b; Scudamore et al, 1992; McEvoy et al 1995b) but that no advantage should be expected to accrue if there is already adequate provision (Scudamore et al, 1993). Sensitivities are not confined to sheep and cattle nor indeed to pre-ovulatory 'windows' but tend to be peri-ovulatory (Ashworth et al, 1989; Forde and Lonergan, 2012; Forde et al, 2013) and also have been noted in pigs, as indicated, for example, by the report of Ashworth et al (1999). An important consequence of this work with ruminant species was that it became apparent that management procedures appropriate to non-superovulated, spontaneously ovulating livestock did not necessarily apply, without adaptation, to those undergoing superovulatory regimens prior to embryo collection. Thus, whereas nutritional 'flushing' might be relied on to enhance ovulation incidence and litter-size among non-supervoulated ewes in moderate body condition by virtue of stimulating ovarian activity (Rhind et al, 1989), it became apparent that provision of additional feed could be both counter-productive and pointless in other circumstances, by virtue of compromising progesterone availability (hence counter-productive) without any ovarian stimulus as pay-off since exogenous gonadotrophin provision would preclude its too subtle ovarian stimulus (hence pointless).

Years later, in a different context but on the other side of same coin, so to speak, it would again become apparent that the aforementioned "management procedures appropriate to non-superovulated, spontaneously ovulating livestock" would not be applicable in another ovine scenario, that relating to an 'Inverdale' fecundity mutation found on the X chromosome and conferring a prolific phenotype to heterozygous carrier ewes. Appropriately enough, on this occasion the research, with UK Home Office approval, was partly conducted at commercial farms in the north of Scotland (Aberdeenshire and Ross-shire) and the outcome would endorse management practices already being relied on as far away as New Zealand. Those practices would, for quite some time yet, be considered counter-intuitive here, not least because of effectively contradicting the familiar 'flushing' recommendation for spontaneously ovulating ewes. Perhaps "Less is More" could be the mantra to explain the so-called 'negative flushing' practice that is relied on by New Zealand flockmasters with prolific Inverdale ewes. The work conducted in flocks of Scottish Blackface and North Country Cheviot ewes bred by AI to Texel rams carrying the Inverdale *FecX*^{I} mutation demonstrated that, unless carrier ewes were restricted in terms of their feed intake about the time of conception, eventual lamb birthweights would be severely compromised. Briefly, on the basis of findings from that and similar studies elsewhere, the scenario is that embryos and/or uterine environments of such ewes are extremely sensitive to circulating progesterone concentrations (or factors reliant on progesterone) despite the fact that such ewes, because the mutation affects ovarian follicular architecture and functionality, tend to be less capable producers of the steroid hormone. Non-carrier 'control' ewes with one or two corpora lutea in the Scottish study, for example, registered higher mean progesterone concentrations (6.6 ng/ml, n = 19) in peripheral circulation than Inverdale-carrier flockmates with 1 or 2 corpora lutea (3.3 ng/ml, n = 38; Alink et al, 2006). Thus, to retain and optimise progesterone concentrations in ewes that are inherently poor at producing the steroid hormone, feed restriction is relied on to enhance likelihood of each developing embryo/entity/conceptus achieving sufficient placental support to ensure delivery at a 'fighting-chance' birthweight. The resilience of those not having adequate placental support in utero can sometimes encourage survival despite low birthweights but many succumb and survivors almost invariably contend with long-term legacies of a sub-optimal or severely compromised start in life, analogous to problems associated with discrete nutrient deficiencies (see, for example, Sinclair et al, 2007).

Closing remarks

Although the main focus of this review has been on resilience and sensitivities of oocytes and embryos in various situations, both natural and contrived, it would be remiss not to also acknowledge the inherent resilience of spermatozoa and the fact that such a crucial quality has facilitated advances in gender preselection via so-called semen sexing. Having shared a laboratory with David Cran almost two decades ago, the author was fortunate twice-over in being privileged also to share in an early milestone event which, following an earlier report from a bovine sex preselection field-trial (Cran et al, 1995) saw Scottish Blackface-cross lambs of pre-determined gender (all female) born after low-dose insemination of ewes with Suffolk ram spermatozoa that had been flow-cytometrically sorted to yield X chromosome-bearing paternal gametes (Cran et al, 1997). Nowadays, in the context of cattle production, it is widely acknowledged that, where feasible, 'sexed semen' offers notable welfare benefits as well as far better than 50:50 odds in terms of predicting offspring gender, thanks largely to the inherent resilience of livestock spermatozoa (Roberts et al, 2008). Meanwhile, 'what if' scenarios in terms of analogous sexed semen' applications to the sheep industry (McEvoy, 2000) still await associated advances that could facilitate non-surgical approaches to low-dose insemination of ewes. In this regard, it is encouraging that some progress has been made towards the sought-after goal of doing so transcervically via means that neither jeopardise animal welfare nor, in view of the pharmacological and/or physical 'interventions' relied on during the process, compromise post-insemination prospects for embryonic development (Robinson et al, 2011).

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Commercial Bovine Embryo Transfer Activity in Europe 2012

by Hiemke Knijn, The Netherlands

Commercial Bovine Embryo Transfer Activity in Europe 2012

General

Country	Name Collector	N° of approved ET teams	N° of teams provide ET data
Austria	G Wetchy	-	-
Belgium	P Vercauteren	6	4
Bosnia & Herzegovina	T Markovic	1	1
Croatia	M Matkovic	1	1
Czech Republic	P Bucek	3	-
Denmark	H Callesen	12	9
Estonia	J Kurykin	1	1
Finland	M Mikkola	4	3
France	C Ponsart	21	13
Germany	H Cramer	39	18
Greece	S Foteini	2	2
Hungary	F Flink	4	3
Ireland	P Lonergan	5	2
Italy	G Lazzari	-	-
Luxembourg	J Westphal	2	2
The Netherlands	JG Derksen	-	-
Norway	E Kummen	2	2
Poland	J Jaskowski	-	-
Portugal	J Chagas e Silva	8	5
Spain	J de la Fuente	11	9
Sweden	A Tidström	3	3
Switzerland	R Saner	6	4
Turkey	E Emsen	**-**	**-**

Bovine In vivo embryo production

Country	N° of flushed donors	N° of embryos collected	N° of transferable embryos	N° of transferable embryos/flush
Austria	45	334	207	4.6
Belgium	1025	6179	4985	4.9
Croatia	5	91	50	10.0
Czech Republic	34	345	206	6.1
Denmark	508	4737	3565	7.0
Estonia	3	18	5	1.7
Finland	390	4482	2626	6.7
France	6414	56044	32985	5.1
Germany	2436	25982	16498	6.8
Greece	3	-	19	6.3
Hungary	99	1300	875	8.8
Ireland	495	4901	2682	5.4
Italy	2240	20429	13893	6.2
Luxembourg	143	1954	1120	7.8
The Netherlands	5688	33640	31874	5.6
Poland	168	1360	914	5.4
Portugal	155	1685	965	6.2
Spain	584	5478	3147	6.9
Sweden	36	241	114	3.2
Switzerland	459	5107	3536	7.7
Total	18086	157487	105212	5.8

Bovine In vitro production

Country	N° of OPU sessions	N° of oocytes collected	N° of transferable embryos
Czech Republic	1	12	2
Estonia	37	627	51
France	338	2414	639
Germany	1268	5562	3900
Italy	61	1340	234
Italy (Buffalo)	113	1195	177
The Netherlands	3543	28587	3239
Total	5361	39737	8242

Bovine In vitro production "Slaughtered donor"

Country	N° of oocyte donors	N° of oocytes collected	N° of transferable embryos
Estonia	37	627	51
France	1	20	-
Italy	134	1354	271
Italy (Buffalo)	212	1586	75
Portugal	1125	10841	741
Total	1509	14428	1138

Embryo Technologies in Bovine

Country	N° of biopsied embryos		N° of sexed embryos		N° of genotyped embryos	
	In vivo	**In vitro**	**In vivo**	**In vitro**	*In vivo*	*In vitro*
The Netherlands	992	--	-	-	992	-
Total	992	-	-	-	992	-

Bovine Embryo Transfer

Country	N° of transferred embryos				
	In vivo fresh	In vivo frozen	In vitro fresh	In vitro frozen	*Total*
Austria	46	97	-	-	143
Belgium	1122	3576	-		4698
Croatia	7	22	-	-	29
Czech Republic	129	32	-	2	163
Denmark	2011	928	-	-	2939
Estonia	5	5	2	-	12
Finland	844	2810	-	-	3654
France	14778	15416	337	299	30830
Germany	5834	11169	2021	891	19915
Greece	6	6	-	-	12
Hungary	167	417	-	-	584
Ireland	973	1058	550	-	2581
Italy	-	-	-	479	479
The Netherlands	4847	17043	3011	652	26453
Norway	-	45	-	-	45
Poland	437	346	-	-	783
Portugal	227	639	28	10	904
Spain	1047	875	-	-	1949
Sweden	15	95	-	-	110
Switzerland	727	2146	-	24	2897
Total	32993	56576	7052	2357	93291

Embryo Activities in other Species (I)

Country	Sheep		Horses	
	N° embryo produced	**N° embryo transfers**	**N° embryo produced**	**N° embryo transfers**
Bosnia & Herzegovina	2	2	-	-
Czech Republic	-	13	-	-
Finland	-	-	5	1
Greece	116	-	-	-
Italy	-	-	91	74
Poland	-	-	1	1
Portugal	-	-	0	0
Switzerland	-	-	7	7
Turkey	-	250	-	-
Total	118	265	104	83

Embryo Activities in other Species (II)

Country	Goat		Swine	
	N° embryo produced	**N° embryo transfers**	**N° embryo produced**	**N° embryo transfers**
France	406	406	**-**	**-**
Total	406	406	-	-

INVITED LECTURES

STRATEGIES FOR SUPEROVULATION, EMBRYO PRODUCTION AND TRANSFER IN SHEEP AND ALPACAS

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Introduction:

Several reviews on embryo production and transfer have been done for sheep (see Cognie et al., 2003; Gonzales-Bulnes et al., 2004; Gonzales-Bulnes, 2007; Vivanco-Mackie, 2001) and alpacas (see Vivanco, 1999; Vaughan, 2012). The present paper will try to complement the reviews done already and to show how the embryo transfer technologies in sheep and alpacas have been developed and applied at commercial level with the aim to produce consistent and cost effective results in the field; it also discusses the present limitations and suggests some necessary areas of improvement.

The main objective of embryo production and transfer is to achieve a substantial increment in the reproductive rate of the females but also of the males since several offspring can be obtained from a single artificial insemination dose through embryo production and transfer. Current applications of embryo production and transfer are mainly for:

- Generation of sires for genetic improvement schemes (dam to sire pathway)
- International movement of genetic material
- Multiplication of exotic breeds introduced to a specific region/country
- In genetic rescue and/ or conservation efforts
- As an instrument for sanitary control
- To correct fertility problems

Other less frequent but very important use of embryo production and transfer is to produce the breeding females (dam to dam pathway) of the next generation (flock/herd replacements). This use is less frequent due to the cost of embryo production making this application less cost effective.

The embryo transfer industry has seen substantial increments in the number of cattle and horse embryos transferred in all continents in the past 5 years whereas in small ruminants the progress of its application has been more modest, however in 2011 there has been a substantial increment (66%) in the reported number of embryos transferred in sheep in comparison with the embryo transfers reported for 2010. The number of reported embryo transfers in sheep for 2010 was 29,078 transfers being for 2011 a total of 48,319. The country with more reported sheep embryo transfers in 2011 is Australia with 45,500 sheep embryo transfers (94% of the total global embryo transfers in sheep for that year) followed by New Zealand (2,458 sheep embryo transfers), Mexico (1,272) and Argentina (1,139); seven other countries have reported sheep embryo transfers in 2011 but the quantities are very small (see IETS Statistics and Data Retrieval Committee Report for 2012). The number of embryos transferred in alpacas in 2011 has not been reported, an estimation will be in the range of 200 to 400 embryos and likely to have been done mostly in Australia, New Zealand and Peru.

Small ruminants share a number of common features and consequently are considered as a distinctive group, however, alpacas are quite different in their reproductive physiology and

consequently very few strategies can be extrapolated from sheep or goats to alpacas being necessary the development of specific strategies for them.

To be able to increase the use of the embryo transfer technology in sheep and alpacas this has to be cost effective, that means the benefits from applying the technology have to be larger than the costs; this implies a good level of efficiency in the embryo production and in the embryo survival to term.

Commercial sheep embryo transfer has been around for more than 4 decades and still is considered a costly exercise in relation to the individual value of the animal; the high cost is mainly due to the relatively low and variable transferable embryo yields which have been improved lately but still need to be optimized to achieve economic justification for routine use in genetic improvement programs. In alpacas, the technology is relatively new and there are still several aspects that need to be developed to higher levels of efficiency.

1. Sheep embryo production and transfer

1.1. IN VIVO embryo production in sheep:

The most critical factor affecting the whole efficiency and cost of the IN VIVO embryo production technology is the strategy for superovulation. After more than four decades of commercial application of IN VIVO embryo production the yield of embryos has improved but not at levels that are necessary for more cost effective use of the technology and still there is a large variation in responses. Our failure in achieving a precise control of the superovulatory process suggest that there is still too much we do not know about follicular development and ovulation.

What is involved in superovulation?

Follicular development up to mature stage and subsequent ovulation can only occur if a sequence of physiological events is happening at the right time and pace. The estrus cycle and the ovarian cycle have to be synchronised as occurs in nature (see Chemineau et al., 1986), follicles develop up to pre ovulatory stage only during pro-estrus and estrus and ovulate at the end of the receptive estrus or early metestrus (Bearden and Fuquay, 1980).

a) Estrus synchronisation of donors:

The estrus cycle in the ewe is in average 16 to 17 days, the length of the receptive estrus varies from 24 to 36 hrs., the pre-ovulatory LH surge is initiated about 4 to 6 hours from onset of estrus and it lasts about 6 hrs., ovulation occurs between 20 to 24 hours after onset of the pre-ovulatory LH surge (see Bearden and Fuquay, 1980).

Estrus synchronisation in sheep is achieved by affecting and controlling the functional life of the corpora lutea of the ovary either by induction of luteolysis (only during the breeding season) with Prostaglandin F2 alpha (Willadsen, 1979) or any analogue injected during the diestrus (one injection on day 9 post estrus if previous estrus date is known or two injections at an interval of 7 days if previous estrus date is not known) or by the induction of an artificial luteal phase either by administering progesterone (i.e. CIDR-G) or progestagens (i.e. F.G.A, M.A.P., Norgestomet) as intravaginal devices mimicking the luteal phase of the estrus cycle. Progesterone and progestagens can also be applied as daily injections or as subcutaneous implants but this is less practical. Ten to 12 days of treatment with progestin is sufficient in the ewe (Crosby et al., 1991; Ainsworth and Wolynetz, 1982). Synthetic progestagens are more potent and effective than progesterone. At LambXL New Zealand, our initial progestational treatments for donors used one CIRD for 11 days, later we had to use two CIDR per donor replacing the first CIDR for a new one

on day 8 (day zero day of CIDR insertion) before starting the gonadotropin injections for superovulation otherwise some ewes will come on heat while still with the CIDR on. Table 1 shows the estrus distribution of a typical group of ewes at the Australian Texel Corporation, (ATC) Australia, subjected to superovulation and being synchronised with 2 CIDR. It can be seen that the highest proportion of ewes come on heat at 28 hr. post CIDR removal (60%) and that by 36 hrs. 95% of the ewes are already on heat. With our regime at ATC we had 95% of the treated groups showing 100% ewes on heat as per the typical distribution of table 1.

It is important to note that in sheep the interval between pre-ovulatory LH surge and ovulation time is practically a physiological constant with duration of about 20 hours (Jabbour et al., 1991). The interval between onset of estrus and onset of the preovulatory LH surge that is normally constant and of 4 to 6 hours duration in non-hormonally treated ewes becomes highly variable in superovulated animals depending on the level of oestrogen generated by the many follicles and affected negatively by the stress imposed on the animals. Since the interval from CIDR removal to onset of estrus is variable and since the interval from onset of estrus to LH surge could be variable we considered of high risk to do "fix time" artificial inseminations (AI) in superovulated donors, we always used inseminations over detected estrus, we think that fix time insemination in superovulated ewes should be used only when you know very well how the estrus distribution is for a particular breed in a particular season with a particular regime. We used in all cases intrauterine laparoscopic AI with minimum 50 million motile sperm per ewe distributed in each of the two uterine horns.

Both at LambXL, New Zealand and at ATC, Australia we used vasectomized harnessed teaser rams (10% over the number of ewes in the group) for estrus detection. The use of teasers has the additional advantage of inducing LH secretion in the ewes as part of the "male effect", this improves follicular maturation, oestrogenesis and ovulation (see Cognie, 1990).

Table 1. Typical distribution of interval from CIDR removal (8 AM day zero) to onset of estrus in superovulated Texel ewes synchronized with two CIRD (Australian Texel Corporation, ATC, Autumn 1994).

Time of the day when the ewes were detected on estrus	Interval from CIDR removal to onset of estrus	% of the population of ewes showing heat at the particular interval	Cumulative % of ewes on heat	Actual insemination time based on detected estrus	Expected ovulation time
8 PM, day 0	12 hrs.	2.5 %	2.5%	8 AM, day 1	8 PM, day 1
8 AM, day1	24 hrs.	30.0 %	32.5%	5 PM, day 1	8 AM, day 2
Noon, day 1	28 hrs.	60.0 %	92.5%	8 PM, day 1	Noon day 2
8 PM, day 1	36 hrs.	5.0%	95.0%	8 AM, day 2	8 PM day 2
8 AM, day 2	48 hrs.	2.5%	100.0%	5 PM, day 2	8 AM day 3

b) Which are the right hormones at the right timing and at the right levels?

There is plenty of evidence that shows that FSH and LH (gonadotropins) are the hormones regulating antral follicles growth and maturation. Pre antral follicles are not responsive to gonadotropin administration and still reminds as a complete enigma the mechanisms that make the primordial (pre antral) follicles initiate growth and commit themselves to development.

Understanding and taking control of such mechanisms should increase substantially the yields not only in superovulation but also in oocyte recovery.

The population of follicles we are dealing with in superovulation is the population of follicles already committed for growth and that are gonadotropin dependant, we want "all of them", instead of "only one or two dominant follicles" (as is the case in nature for most breeds of sheep), to reach pre-ovulatory stage and be ovulated. In other words, we want to enhance recruitment, development, maturation and ovulation of follicles and counteract any negative effects on this process from the existing mechanisms established by nature "precisely to avoid superovulation".

Follicles grow in waves through the estrus cycle but only during the pro-estrus and estrus is that the follicle(s) destined to ovulate will escape atresia and continue its growth and maturation reaching pre-ovulatory stage (Guilbault, 1998). We then have to focus on understanding what is going on during proestrus and estrus and how we can manipulate the physiological events in such a way that we overcome the negative regulators and increase the response to gonadotropins.

Work from Campbell et al. (1995) suggest that there are three fundamental levels of control of development and selection (selection to go up to ovulation) of the follicles during the normal pro-estrus and estrus:

- Gonadotropins for follicular development and maturation
- Factors produced by the ovulatory follicle which suppress development of other follicles through gonadotropin-dependent mechanisms
- Intrafollicular factors within the ovulatory follicle, which enhance or attenuate the actions of gonadotropins.

Findlay et al. 1996, indicate that even when there is a role for autocrine and paracrine factors in folliculogenesis, only FSH and LH (gonadotropins) are essential for follicular growth and development.

The role of FSH:

The role of FSH is to stimulate follicular growth through promoting granulose cell division and the formation of antral fluid and in cooperation with LH regulate oestrogenesis in follicular cells. FSH once bound to its receptor in the surface of the granulose cells of the ovary activates a series of intra-cellular events that effect the expression of genes crucial for cell proliferation and cell differentiation (see Hillier et. al., 1996). Everybody seems to agree and recognise the role of FSH and on the necessary use of FSH in superovulation. All protocols for superovulation use a source of FSH or products with FSH-like activity.

Findlay et al. (1996) describe two mechanisms by which superovulation can result:

- Mechanism A: is based on "prolongation of the viability" and "decreasing atresia" of gonadotropin-dependent follicles (2-5 mm diameter in sheep) by exposing them to FSH for a prolonged period of time (through the late luteal phase and in the following follicular phase). This means there is no increase in the pool of existing gonadotropin-dependant follicles but only an action of "rescue" of these follicles allowing them to go ahead instead of become atretic. This type of mechanism is the one operating when we administer exogenous gonadotropins or when immunization against inhibin is used (see Monniaux, 2012). It should be noticed that FSH support is needed not only through the late luteal phase but also through the follicular phase (pro-estrus and early estrus) and it is here where most of the superovulatory regimes fail since in most of the protocols there is a fixed number of FSH injections stoping the applications well

before onset of estrus leaving the many selected follicles without FSH during the most critical stage of development and differentiation.

- Mechanism B: Is based in the "possibility" of increasing the size of the pool of gonadotropin-dependent follicles for further development. This mechanism, may or may not involve increased levels of FSH. Trials in heifers (Gong and Webb, 1993) have demonstrated that this mechanism is observed when bovine somatotropin (bST) is used in the superovulatory regime which results in the increase of total number of follicles of 2-4 mm whereas applications of FSH alone does not influence the entry of follicles into that group. Increasing sharply the energy intake for a short period before the superovulatory treatment has also resulted in the increment of small follicles (lower than 4 mm) in cattle. Monniaux (2012) suggest that the increased number of small follicles responsive to gonadotropin obtained from application of bST and/or from the increased nutritional level are effects mediated by enhanced plasma concentrations of IGF-I and insulin.

  The utilization of mechanism B in commercial superovulatory strategies is not so common yet but I think offers a great potential.

Since In sheep it has been demonstrated that the superovulatory response to exogenous FSH treatment is higher when the number of small follicles (2 to 3 mm diameter) present in the ovary at the initiation of the FSH treatment is high (Brevion et al., 1992), some strategies have been developed to increase the number of small follicles and decrease the number of larger ones at the start of the FSH treatment, one strategy include the administration of GnRH agonist or antagonist to supress gonadotropin secretion before the start of the superovulatory treatment (Cognie et al., 2003), this increases the pool of follicles of 2-3 mm and decreases the number of larger follicles, increases ovulation rate but also the percentage of unfertilized ova and degenerated embryos so it needs still to be optimised; other strategy is based in the control of the initiation of the follicular wave by inducing ovulation and subsequently accompanying the follicular development of the new follicular wave with exogenous FSH from the start of the wave at the early luteal phase (Gonzales-Bulness et al., 2004) or right after ovulation (Rubianes and Menchaca, 2003) in this way all the follicles are small at the start of the gonadotropin treatment; these are strategies that should have great impact once optimised. The increment of the pool of small follicles (2-3 mm) and the absence of large dominant follicles at the start of the FSH treatments is recommended in the new superovulatory regimes, however in sheep there is no clear beneficial effect on ovulation rate in treatments where the dominant follicle has been eliminated or there is absence of dominant follicles, this could be due to the suppressive effects of progesterone and progestagens on the expression of dominance effects of the dominant follicle (Gonzales-Bulness et al., 2004) since most of the synchronization regimes in sheep have high levels of progesterone or progestagens during the time of induced follicular development with FSH applications.

The role of LH:

The granulosa and theca cells of the follicle co-operate in a functional interplay with the purpose of producing oestradiol. Both FSH and LH regulate this interplay. As the follicle develops it acquires the capability of oestrogen production. In the natural ovarian cycle during the mid-follicular phase, the dominant follicle becomes increasingly dependent on LH. The receptors of LH are always present in the theca cells but in the granulose cells they only develop after FSH stimulation. LH stimulates the synthesis of androgen in the theca cells; this androgen passes from the theca to the granulose cell where it is transformed by aromatisation into oestrogen. Both FSH and LH regulate aromatisation of androgen in the granulose cells (see Fortune and Quirk, 1988; Hillier et al., 1996).

The ability of the dominant follicle to very rapidly become the first one synthetising oestrogen is the key for its survival as dominant follicle. Oestradiol is the primary regulator of FSH secretion by the pituitary consequently as the oestradiol level increases the FSH secretion falls to levels too low for

other follicles to continue development and so they undergo atresia. The ovulatory follicle continues development even with lower levels of FSH because at that stage has experienced maturational changes that increase its sensitivity to gonadotropins and is increasingly dependent on LH (Hillier et al. 1996).

The key role of LH is to transform the developing follicle into an oestrogenic follicle; only oestrogenic follicles that are mature enough to be highly sensitive to gonadotropins can undergo final maturation and ovulation. This crucial role of LH has been very rarely taken into consideration when deciding strategies of superovulation. One elemental aspect to consider is that LH action comes after FSH priming; the follicle has to reach certain level of development before it can become oestrogenic. Wrong timing of administration (before FSH priming) and/or excessive dosage level of LH have resulted in atresia of follicles or early luteinization. This does not mean that LH is not necessary for superovulation, at the contrary it cannot be normal pre-ovulatory follicular development and normal oocyte maturation without LH oestrogenic activity but LH administered at the wrong time and in excessive levels will disrupt the normal process of follicular development, maturation and ovulation.

Another important aspect to consider is that the role of LH cooperating with FSH for achieving follicular oestrogenesis is a different role of that of LH for ovulating the pre ovulatory follicle. For the oestrogenesis the levels of LH are basal and gradually increase in pulsatile mode, whereas that for inducing ovulation LH is secreted massively constituting what is called the LH pre-ovulatory surge. Ovulation is initiated by the positive feedback effect of oestrogen on the brain and the pituitary gland. The brain releases GnRH that stimulates the pituitary for the release of the LH peak. The LH peak or surge starts about 14 to 24 hrs. after the serum oestrogen reaches peak concentration (see Healy et al. 1996). The LH surge initiates luteinisation and the beginning of progesterone production by the granulose cells of the dominant follicle. LH is also responsible for the resumption of meiosis in the oocyte and the synthesis of prostaglandins that are essential for follicular rupture. Ovulation occurs 24 to 30 hours after onset of LH surge when the follicle ruptures and the oocyte is released from the ovary (Healy et al.1996). Improper timing and dosage of LH in superovulatory regimes causes these changes to occur prematurely and is the cause of those many controversies that lasted for decades between scientists, practitioners and drug companies regarding the role of LH in superovulation. The truth of the matter is that LH is as important as FSH for ensuring normal follicular growth, follicular and oocyte maturation and ovulation. LH at the proper level should be used after FSH priming and when the follicle has reached certain level of development. The level of LH for ensuring proper oestrogenesis and follicular maturation should be mild and should be present through the late follicular phase. Only after the animal has reached onset of estrus is that a preovulatory surge of LH could be induced, this is totally different than the mild level used during the late proestrus.

Much of the controversy on the role of LH came because the products used initially to induce superovulation were either pituitary extracts with high content of LH or eCG also with relatively high content of LH like activity. These products were used from the start, that is, from the first injection for recruitment onwards without ensuring previous follicular development, consequently a very random response was achieved which depended on the stage at which the follicles were at the time when the treatment started and in many cases early luteinisation and atresia was evident, there were many trials recommending different ratios LH:FSH but at the end it was evident that with low levels of LH or no LH at all the ovaries “looked better or nicer”, there was no evidence of large non ovulated or atretic follicles or luteinized follicles, this induced to believe that “LH was not needed for superovulation”, a product for superovulation had to be absolutely free of LH. Obviously this did not result in great improvement since the oestrogenic activity of the follicles was not optimum consequently affecting the positive feedback of oestrogen on the brain and reducing the level of endogenous LH needed for follicular maturation and ovulation (Price et al., 1999). This situation

was more dramatic when any level of stress was involved due to the inhibitory effects of ACTH and glucorticosteroids in LH secretion (see Moberg, 1984). Due to the erratic responses with "pure FSH" many superovulatory strategies were then recommending to give a shot of eCG in conjunction with the pure FSH, they almost got it right, but they were recommending to do this at the beginning of the FSH treatments!, so why use a pure source of FSH and then "contaminate" the pure source of FSH with a source of LH given at the beginning of the treatments? The source of LH and at a mild dose level should be given at the end of the luteal phase (see Cognie, 1999) and after the follicle has developed up to a certain stage due to the injections of pure FSH or FSH with very low (basal) level of LH that were injected before the LH source.

The role of autocrine, paracrine and other mechanisms

There has been great progress in the last years in identifying several factors and their functions on follicular regulation. The eventual use of this knowledge in defining strategies for superovulation should improve our chances of increasing responses and diminishing variation. Several ovarian steroidal and non-steroidal factors facilitate the mitogenic action of FSH during recruitment and increase the LH-induced androgen and oestrogen synthesis in the dominant follicle (Hillier et al., 1996). Most of these factors are involved in ensuring the dominant follicle reaches very quickly oestrogenic activity and continues development even at lower levels of FSH (once oestrogenic activity in the follicle is established).
Moniaux (2012), describes the intra- follicular regulations for the selection of ovulatory follicles indicating that changes in the IGF-I system are thought to be essential for follicular selection and development until the preovulatory stage; it is also shown the participation of the Bone Morphogenetic Protein (BMP) system in the regulation of the ovulation rate and it is mentioned that in this system the oocyte itself could play a role in regulating the ovulation rate as proposed by McNatty et al. (2005). The Anti-Mullerian Hormone (AMH) whose expression is restricted to the granulosa cells of growing follicles is proposed by Moniaux (2012) as an ideal endocrine marker of the size of the ovarian pool of growing follicles (gonadotropin-responsive follicles) in such a way that the poor responders could be detected and discarded from a superovulation program based on AMH levels in blood, this should decrease between animal variability in ovulation rate and increase the average number of embryos produced; this however could have consequences in reproductive traits such as reproductive life span of the females in a herd subjected to selection of donors based on AMH levels since we will be selecting the females that "burn" their reserve of follicles very quickly.

c) How we developed our regimes for commercial superovulation:

Very much work has been reported in relation to superovulatory regimes and/or hormonal strategies for sheep. The simplest treatments have been the ones based on a single injection of eCG (due to its long life in circulation post injection), dosages varied between 1500 to 2000 IU depending on breeds and age of the donors. The eCG injection was given at about 48 hrs. before the suspension of the progestational phase (Armstrong and Evans, 1983). Results with eCG were variable, observing at time of embryo flushing many non-ovulated follicles. Other regimes included Horse Anterior Pituitary Extract (HAP) or HMG (human menopausal gonadotropin) with results also variable and with similar non-ovulatory or atretic follicles present at time of flushing (Shiewe et al., 1985). All these products were mainly crude extracts high in levels of active LH or LH like activity.

The purification and commercial availability of FSH from porcine or ovine origin marked the initiation of new superovulatory strategies. The FSH is given mainly as repeated injections every 12 hours (due to the short life of FSH in circulation) either as constant or decreasing dosage (Torres et al., 1987), constant dosages are simply a waste of drugs since the follicles are more sensitive to FSH at the end of the developmental stage and are shifting sensitivity towards LH . The treatments are

initiated at about 3 days before the end of the progestational phase, the last injection of FSH given either at the end of the progestational phase (i.e. CIDR or sponge out) or 12 hours later (Maxwell et al., 1990). Remy et al. (1991) have found a loss of sensitivity (or development of refractoriness) with reduction in the ovulatory response in sheep and goats subjected to repeated superovulatory treatments with porcine origin FSH (pFSH) due to production of antibodies against pFSH , this was not observed when ewes and does were treated with ovine origin FSH (oFSH) (Baril et al., 1992). We have subjected at LambXL New Zealand and at the ATC Australia to each donor to 7 cycles of superovulation and embryo flushing per year (4 in the Autumn and 3 in the Spring), most of the donors going for at least 3 to 4 years, some completing 6 years on this system and we used mainly pFSH, on those conditions we did not find any decline of the ovulation rate, as can be seen in table 8 the number of previous treatments/flushing had no significant effect on the ovulation rate.

The LH/FSH ratio was a matter of several discussions. We have discussed earlier in this paper the relative roles of both hormones. In general commercially available drugs with high FSH and low LH content gave better responses (see review by Maxwell et al., 1990; Chupin et al., 1987) because they avoided early oestrogenisation and early luteinisation of the follicles but if those drugs, and especially the ones totally free of LH, were not used in conjunction with other drugs supplying LH at the right time, the responses were poor (McMillan and Hall., 1990). The use of eCG injected at the time of the first injection of purified FSH did not improve results significantly (McMillan and Hall, 1990; Greaney et al., 1991; Rangel-Santos, 1991). Table 2 shows the results we obtained at LambXL with 3 commercial drugs with different LH/FSH ratios, supplementing two of them (the ones with the lower LH content) with eCG at the first FSH injection. Regimes combining FSH with LH at the first injection of the gonadotropin treatment were practically no different from using just FSH-P (with high ratio LH/FSH).

Table 2. Mean transferable embryo production per donor ewe obtained with 3 different regimes using drugs with high and low LH content. (Donor ewes: Danish Texel 1.5 years of age). Data LambXL 1990

Variable	FSH-P: 34 mg Armour, 7 decreasing injections	OVAGEN: 15.2 ml 7 decreasing injections 300 iu PMSG given at 1st FSH injection	FOLLTROPIN V: 12.8 ml 7 decreasing injections, 100 i.u PMSG given at the first FSH injection
N° of treated ewes	14	54	100
N° of flushed ewes	13 (92%)	47 (87 %)	85 (85%)
Mean transferable embryos per ewe flushed ± SD	4.0 ± 3.08	2.6 ± 5.2	4.45 ± 3.1
Mean transferable embryo per treated ewe ± SD	3.71 ± 2.8	2.3 ± 5.0	3.78± 2.65

* CIDR removed at the 6th injection.

Based on these results and after reviewing physiological studies of that time (Driancourt and Fry, 1988; Jabbour et al., 1986; Maxwell and Wilson, 1989) and confirmed later as seen earlier in this article, we realised that LH or drugs with LH like activity should not be administered at the beginning of a superovulatory treatment since the follicles are not LH dependant but up to the end of the follicular phase, besides an early administration would result in induction of early luteinisation and atresia, so we decided that it was necessary to use a source of FSH with low content of LH for the initial FSH injections during the late luteal phase, but then at time of CIDR out when the intense follicular phase starts we needed a source of LH to mimic the gradual increase in LH pulses of the proestrus, eCG was the obvious choice since it has LH like activity but also

FSH like activity and a relatively long life in circulation so will provide a sustained LH support from CIDR out to onset of estrus. Other authors had used hCG ,LH or GnRH at the end of the luteal phase, in all those cases they had as a consequence an early (before onset of estrus) surge of LH instead of a mild increase in LH level, that did not result in better performance (Wright, et al., 1981; Chupin et al., 1987; Walker et al., 1989).

Another hypothesis that we developed and was confirmed later by the different findings referred in the first part of this paper, was that we needed to continue FSH administration until onset of estrus if we wanted to support the many dominant follicles that we had created with the superovulatory regime because those multiple dominant follicles were producing oestrogen and reducing the secretion of endogenous FSH. Most of the regimes used at that time will recommend a fixed number of FSH injections either 6 with the last one at time of CIDR or sponge out or 7 with the last one 12 hours post CIDR or sponge out.

In a simple experiment (table 3) we continued injecting a pure source of FSH (Ovagen) post CIDR out until the ewes were on heat, some ewes received 8 or 9 injections some just 7 depending on when they came on heat. The ewes that received FSH up until onset of estrus produced higher number of transferable embryos.

Table 3. Effect of continuation of FSH injections post CIDR out until onset of estrus for superovulation of Texel ewes

	Injected with FSH up to onset of estrus Inclusive	Suspension of FSH injections when observed in estrus	Received fixed number of injections, the last one 12 hrs after CIDR out
Interval between last Ovagen injection and onset of estrus	0 hrs	12 hrs	24 hrs
	N mean ±SD	N mean ±SD	N mean.± SD
Total number of transferable embryos per treated ewe	19 3.4±0.6 (a)	43 1.3±0.3 (b)	6 0.3±0.3 (b)

Means with different letters in parenthesis are different (P≤ 0.05)
Data: LambXL 1991.

To test our hypothesis regarding the need of injecting eCG as source of LH at the end of the FSH treatment, we did a very small trial with Rommey ewes, just to see the tendencies before we used the regime in a larger scale in our Texels. We injected one group of ewes with 200 i.u of eCG at the beginning of the FSH treatment (Folltropin V: 2.8, 2.8, 1.4, 1.4, 1.0, 1.0, ml, each injection 12 hrs apart, CIDR out at 6th injection, continue injecting after CIDR out 0.8 ml of Folltropin V every 12 hrs. until onset of estrus) and another group with same regime but the 200 i.u of eCG injected at the time of CIDR out instead of at the beginning of the FSH treatment. Table 4 shows the results that confirmed our hypothesis; the group with eCG at CIDR out doubled the yield of the ones with eCG at the beginning of FSH treatments. We then applied this strategy in all our donors and we doubled the yields of transferable embryos per donor per session from 3.0 to 7.0 embryos per treated or programmed ewe. Table 5 shows a typical result for our embryo transfer operations for the spring and autumn seasons at ATC, Australia. It can be seen that using this strategy our average with more than 1500 ewes programmed in each season was more than 6 embryos per donor programmed. These kind of averages were obtained from 1991 onwards in our commercial operations.

The ewes were programmed 4 times in the autumn and 3 times in the spring using the following calendar:

Day	Time	Activity
Zero	AM	Put CIDR IN
7	AM	Replace CIDR
	PM	1st Folltropin V injection
8	AM	2nd Folltropin V injection
	PM	3rd Folltropin V injection
9	AM	4th Folltropin V Injection
	PM	5th Folltropin V Injection
10	AM	6th Folltropin injection, CIRD OUT, Teasers in, eCG
	PM	check heats, inject Folltropin V to ewes not in heat
11	AM	Inseminate, check heats, inject Folltropin V to ewes not in heat
	PM	Inseminate, check heats, inject Folltropin to ewes not in heat
12	AM	Inseminate all remaining ewes
18	AM	Starve ewes
	PM	
19	AM	Embryo Flushing, Prostaglandin
26	AM	START A NEW ROUND ,CIDR IN, etc.

The dosage of Folltropin V varied with breed and age and with body weight of the ewes. In all cases we used decreasing doses. The results per breed using this strategy are shown in table 6 for ewes of 1.5 years of age or more. The results with 7 months old ewe lambs are offered in table 7.

Table 4. Effect of the application of eCG at time of CIDR out in Romney ewes superovulated with Folltropin V in comparison to application of eCG at time of first injection of FSH (Folltropin V).

	200 u.i PMSG at time of first Folltropin V injection		200 u.i PMSG at time of CIDR out at 6th injection of Folltropin V	
	N	mean ± SD	N	mean ± SD
Interval from CIDR out to onset of oestrus hrs.	5	21.6 ± 9.90	5	25.6 ± 9.90
Number of ovulations per ewe	5	3.6 ± 1.88	5	9.6 ± 1.88
Recovery rate	4	76.3 ± 30.80	5	54.8 ± 52.67
Fertilization rate %	4	93.8 ± 72.16	4	100.0 ± 72.16
Mean number of transferable embryos per ewe flushed	4	3.0 ± 0.72	4	7.0 ± 1.63
Mean number of embryos per ewe programmed	5	2.4 ± 0.64	5	5.6 ± 1.47

*Regimen: Folltropin-V: 2.8, 2.8, 1.4, 1.4, 1.0, 1.0 ml, CIDR-out, continuation of 0.8 ml Folltropin V every 12 hrs until onset of estrus
Data: LambXL 1991.

Table 5. Results obtained during spring and autumn seasons at ATC using the superovulatory strategy as described in table 4 (Folltropin V decreasing doses plus eCG at CIDR out, injections of FSH continue until onset of estrus), all breeds pooled together (Danish Texel, Finnish Texel, Finnish Landrace)

	Spring 1993		Autumn 1994	
	N	mean ± SD	N	mean ± SD
N° of ovulations/ewe	1544	10.16 ± 5.63	1190	9.45 ± 5.71
Recovery rate %	1385	77.02 ± 26.70	1139	76.10 ± 30.11
Fertilization rate %	1363	94.27 ± 16.89	1089	90.87 ± 23.81
Transferable embryos per ewe treated	1596	6.30 ± 5.23	1213	6.13 ± 5.39
Percentage (%) transferable embryos over total embryos	1339	85.55 ± 25.12	1039	88.27 ± 22.79

Data. Australian Texel Corporation (ATC)

Table 6. Performance of donor ewes of different breeds superovulated with Folltropin V decreasing dosages, eCG at time of CIDR out and Folltropin V up to onset of estrus.

Breeds	Danish Texel		Finish Texel		Finnish Landrace	
Variables	N	Mean± SD	N	Mean ± SD	N	Mean± SD
No of ovulations/donor	523	7.1± 4.1	433	11.0 ± 5.5	234	11.9 ± 7.0
Recovery rate (%)	508	78.0± 30.8	415	72.9 ± 29.1	216	77.7 ± 30.0
Fertility rate (%)	480	90.7± 24.0	407	93.2 ± 20.4	202	86.5 ± 28.7
Transferable embryos per donor treated	536	4.7± 3.97	438	7.0 ± 5.4	239	7.8 ± 7.0
Percentage (%) transferable embryos over total embryos	458	88.0± 23.18	393	90.0 ± 20.4	188	85.1 ± 26.0

Data: Australian Texel Corporation (ATC), autumn 1994.

Table 7. Performance of ewe lambs born in New Zealand in Spring 1992 and used as superovulated donors in the Autumn of 1993 in Kirra Australia at 7 months of age using the strategy of Folltropin V decreasing doses, eCG at CIDR out and Foolltropin V up to onset of estrus.

	Danish Texel		Finnish Texel		Finnish Landrace	
	N	Mean± SD	N	Mean± SD	N	Mean± SD
No of ovulations per ewe lamb	42	6.71 ± 3.82	27	9.3 ± 4.65	12	6.16 ± 3.69
Recovery rate %	37	74.08 ± 28.63	25	64.38 ± 32.76	9	75.11 ± 34.25
Fertilization rate %	37	95.27 ± 11.70	24	96.70 ± 10.78	8	100.00 ± 0.00
Transferable embryos per ewe lamb programmed	45	4.02 ± 3.71	27	5.55 ± 4.01	12	4.00 ± 3.74
Percentage (%) of good quality embryos over total embryos	37	85.93 ± 22.49	24	94.00 ± 20.46	8	86.45 ± 18.00

Data: ATC. 1993.

d) **Factors that affected the production of transferable embryos:**

Table 8 shows the results of analyses of more than 4 thousand embryo-flushing operations at LambXL, New Zealand. It should be noticed that this study was made before we introduced our new superovulatory strategy, that means all the regimes used were either with FSH products used alone (either FSH-P, Ovagen or Folltropin V) or in combination with eCG "at the wrong time" (at the first injection of FSH) or including GnRH, Neutra-PMSG, HCG, etc., most of the regimes included one more FSH injection after CIDR out.
Results in table 8 are expressed as the percentage of the total variance observed in each parameter that is due to each specific factor affecting the parameter or variable (i.e. from table 8, the percentage of the total variability observed in ovulation rate within a year due to breed effect is 8.9%. Only the percentages for the significant ($p \leq 0.05$ or $p \leq 0.01$) effects are given. Non-significant effects are coded as NS. Interpretation of the results should take into account that these effects are for this specific population and for these specific conditions of management. Other populations in other conditions might show totally different responses to the effects analysed. Also significance of an effect means simply that the percentage of total variability that is given in the table is due to that effect; it does not mean that is important enough. For example, from table 8, the effect of the inseminator on the ovulation rate is significant but the percentage of total variability in ovulation rate that is explained by differences between inseminators is 0.5%, this means that we are very certain that the inseminator affects in 0.5% the ovulation rate, but we might consider that 0.5% is too low to be worried about that effect.

The most important factor affecting significantly the percentage of transferable embryos (which is the most important result of a superovulatory exercise since number of "good embryos in the dish" is the bottom line) was the donor breed (prolific breeds like the Finnish Landrace produced significantly higher number of embryos). The results show that besides breeds, the factors that are related with strategy of superovulation are the ones affecting the yield of transferable embryos. The interrelationship between factors and of the factors with the strategy of superovulation are not shown in the analyses since it is an analyses only of single effects, this is reflected in the low coefficient of determination (R^2) of the model.

In addition to the factors analysed in table 8, other factors of critical importance on determining responses to superovulation are the nutritional balance and general condition of the donors (Smith and Steward., 1990). As management practice both at LambXL and ATC we subjected the donors to delousing, drenching, vaccination, etc. at least 8 weeks before the superovulation program started. The nutritional level was increased from maintenance (100%) to 130% gradually in a period of 6 weeks and keeping it at that level through the program.

Early regression of the corpora lutea is seen at times of energy deficiency, this is corrected by supplements such as lupines (Jabbour et al., 1991). Our observations in sheep (empiric since we have not done scientific trials on this), show that any kind of stress (i.e. sudden drop of glucose in the blood for prolonged starvation before surgery, use of dogs in the yards, etc.) has a severe influence in the life span of the corpora lutea. Whatever the reason, luteal regression is caused by release of prostaglandin F2 alpha. Some authors have used prostaglandin blockers (i.e. Flunixin 2.2 mg/Kg; Battye et al., 1988). In Australia at ATC we observed certain level of early luteal regression; this was associated with the prolonged time the animals were under dry forages and was corrected with a supplementation of 3000 IU of vitamin A.

Stress not only is related with early luteal regression but with failure to express receptive estrus and failure to ovulate. Glucocorticoids and ACTH are the most powerful blockers of LH affecting follicular maturation, onset of estrus, LH surge, ovulation and corpora lutea formation (see Moberg, 1984). A stress free management system will ensure at least 50% of the success of the program.

Table 8. Factors affecting the different variables recorded during LambXL embryo flushing operations in the period 1987-1990. Values are expressed as percentage of the total adjusted variance observed for each variable that is explained by each factor analysed.

Variables / Factors	Ovulation rate	Recovery rate	Fertilization rate	Percentage of transferable embryos
Breed of donors within years	8.90	26.41	NS	29.90
Interval CIDR out to onset of estrus	11.37	6.60	34.94	15.96
Hormonal regime	1.35	1.86	11.93	4.04
Farm	2.36	7.64	NS	NS
Season	NS	1.21	NS	NS
Number of previous flushing	NS	4.19	43.03	NS
"Quality of CL"	10.02	33.57	NS	17.90
Presence of follicles >5mm	65.26	12.57	NS	12.36
Interval onset of estrus to insemination	NS	NS	7.88	7.37
Inseminator	0.51	NS	NS	-
Embryo flushing method	-	NS	-	-
Rams	-	-	NS	NS
Age of embryo	-	NS	-	7.37
Embryologist	-	NS	-	10.16
Surgeon	-	4.69	-	-
Number of observations	4789	4041	4577	3768
R^2	24.7	11.6	20.50	14.10

From: H W Vivanco, et al. 1994

e) Later modifications in our superovulatory regime:

In the period leading up to 2004, embryo transfer programs by Macquaire Artificial Breeders, Australia, had mainly been conducted on the introduced breeds which included Dohne, South African Mutton Merino, Dorper and Damara. The Australian Merino was the only home bred breed involved in embryo transfer programs. Regression of the Corpora Lutea (CL) had been noted to occur infrequently in these breeds during this period. Continuing the flushing procedure on ewes that were noted to have regressing CL was discontinued very early in the period due to extremely poor recovery rates on ewes that had been flushed. The occurrence of regressing CL was estimated to be less than 1%, and was not considered to be a major problem. In 2004, mainly as a result of international inquiries, other breeds of Australian sheep were being programmed for export orders. The breeds involved here were Poll Dorsets, White Suffolks, Texels and East Friesians. Particularly

in the Poll Dorset breed, regressing CL was a major problem. Of 301 Poll Dorset ewes programmed, an average of 15% of ewes experienced regressing CL, with some programs experiencing the condition in up to 75% of ewes programmed. The other breeds also experienced regressing CL, but did not suffer the catastrophic rates noted in the Poll Dorsets. This comment is tempered by the relatively small numbers of donor ewes programmed from the other breeds.

The occurrence of Regressing CL was thought to be as a result of the stress of undergoing a laparoscopic procedure at insemination, but this was an unconvincing explanation as the regression was noted to occur after day 4 post AI. All perceived stresses were theoretically addressed and eliminated. Adjustments included heavier sedation at the time of AI, housing the ewes outdoors in larger numbers in close proximity with shelter and increasing feeding events to twice daily, with an increase in total amounts fed. Subsequent programs revealed that these procedures did not reduce the incidence of regressing CL, so we introduced some new procedures which included:

1. The CIDR change on Day 7 was altered to a CIDR addition on Day 7, resulting in the donor ewes having 2 devices in place after Day 7.
2. Extend the Progesterone Phase by 1 day i.e. start the hormone therapy a day later.
3. Inject Luteinising Hormone 12 hours prior to AI (5ml Lutropin)
4. Insert a CIDR 3 days post AI
5. Change PG administration from the Day 7 to Day 1.
6. Administer NSAIDs 3 & 5 days post AI (3ml Flunixin)

Inclusion of these changes resulted in an immediate response. The number of donor ewes undergoing CL regression had not changed, but embryo recovery rates in these donors were similar to ewes not experiencing CL regression. The changes introduced into the program seemed to result in the maintenance of a favourable uterine environment for embryo retention and development. Embryo development and quality was not noted to be any different from donors not affected by regressing CL, and the pregnancy rates also were not detectably different. The main benefit was considered to be the maintenance of circulating progesterone levels as a result of application of a CIDR 3 days post AI. With this in mind, further refinements to the program removed the use of Lutropin and NSAIDs, as it was considered that their inclusion was not necessary. Subsequent programs that did not incorporate their use supported this view. As a result of the development of this treatment regime, the Australian meat breeds such as the Poll Dorset and White Suffolk are achieving similar results to the balance of the sheep breeds Macquaire Artificial Breeders deals with. Table 9 shows the results with the new superovulatory regime.

Table 9. Performance of donor ewes of different breeds (Dohne, Poll Dorset, Dorper, East Friesian, Texel, White Suffolk) superovulated with the new superovulatory regime from 2005 onwards.

Period	N° of Donors programmed	Total embryos collected	Mean total embryos per donor	Total transferable embryos	Mean transferable embryos per donor	Percentage of transferable embryos (%)
2005-06	729	5,867	8.05	4,637	6.36	78.97
2006-07	396	3,566	9.01	2,649	6.69	74.00
2007-08	186	1,608	8.65	1,165	6.26	72.45
Total	1,311	11,041	8.42	8,451	6.45	76.60

Data: Macquaire Artificial Breeders, Dubbo, NSW, Australia.

The new regime allowed us to confront the regressing CL incidence and maintain similar performance in all breeds, however the mean number of transferable embryos was not improved substantially (compare table 6 with table 9), which is a challenge for us, being necessary to test new strategies like the GnRH antagonist treatment before superovulatory treatments and/or the synchronization of the follicular wave to start FSH treatments at the early luteal phase.

1.2. Embryo survival post transfer:

A series of factors affect the proportion of embryos that survive up to lambing. The sources of variation more frequently identified as the more important ones affecting embryo survival are breed and age of donors, embryo quality as reflection of regimes, embryo handling from flushing to transfer, the embryo age and developmental stage at time of transfer, embryo transfer techniques, the nutrition and general condition of the recipients, the breed and management of recipients to term (see review by Rangel-Santos, 1991; Baril et al., 1993).

LambXL analysed early in 1987 (Aspinal, 1987) the effects of breed of embryo and number of embryos transferred per recipient, age of donor, age of embryo and stage of development according to embryo age, the condition of the recipient, the method of estrus synchronisation of the recipient, the side of transfer, the interval from CIDR out to estrus of the recipient and the degree of synchronisation between the donor and recipient estrus.

There was no difference between single and twin transfers within breeds but there was difference between breeds, embryos from one year old donors survived significantly less than embryos from adult donors (50% versus 60%), expanded blastocysts and hatched blastocysts at day 7 survived better than younger embryos, recipients (Romney Marsh) lighter than 71 Kg at time of transfer had significantly lower pregnancy and survival rate of the embryos, this was also true for recipients heavier than 80 Kg, there was no influence of synchronisation method of the recipients on embryo survival, there was no difference between single or twin ipsilateral or contralateral transfers, there was no effect of the time interval between CIDR out and onset of estrus of the recipient on the embryo survival. Recipients on heat within plus or minus 24 hours from the embryo donor heat had high embryo survival than the ones on heat plus minus 36 to 48 hours from donor's estrus.

In later evaluations (table 10) we found an influence of number of embryos transferred per recipient and of embryo quality in embryo survival to term. It can be noticed that within each embryo quality, twin transfers increased pregnancy rates but reduced embryo survival rates.

Table 10. Influence of the embryo quality in the pregnancy rate and the embryo survival to term of Texel embryos according to the number of embryos transferred per recipient.

	Pregnancy rate %		Embryo survival %	
No and quality of embryos transferred/recipient	N	Mean	N	Mean
1 Grade A embryo	181	78.00 (b)	142	78.00 (a)
2 Grade A embryos	239	92.00 (a)	478	70.00 (b)
1 Grade B embryo	73	68.00 (b)	73	68.00 (b)
2 Grade B embryos	90	80.00 (b)	180	51.00 (c)

Means with different letters are different (P≤0.05)

data: LambXL. Kiwitea. NZ, 1990

As mentioned for the donor's case, reduction of stress is crucial in recipient management as well as proper nutrition through the program up to lambing.

Efforts at LambXL and ATC to increase embryo survival included also hormonal treatments to recipients. Table 11 shows different alternatives tested. The use of CIRD (inserted to the recipient at time of embryo transfer, replaced on day 13 and removed on day 56 of pregnancy) had no effect in increasing embryo survival. The other treatments show a good potential but need to be tested more extensively. The application of eCG or hCG at time of embryo transfer is based on evidence of their luteotropic effects (see Vivanco, 1996).

At the very end the parameter of most importance in an embryo transfer program is the number of offspring produced per donor programmed and the total number of offspring generated per donor ewe per year. We used embryo bisection very successfully and our efficiency of lamb production increased in 30% (table 12, see also Vivanco et al., 1991; Vivanco and Graeney, 1992).

The efficiency of lamb production increases with experience. Without including embryo bisection, the total number of lambs produced per donor programmed per round of ET increased from 1.26 lambs in autumn 1987 at LambXL to 3.98 lambs in autumn 1994 in ATC. The total number of lambs produced (with 7 flushes per year) per donor per year in 1994 for the ATC was 27.86.

Table 11. Different hormonal treatments applied to recipient ewes for improvement of embryo survival to term

Trial N°	Treatment within trial	No of recipients	No of embryos transferred	No of foetuses scanned	Embryo survival %
I	300 u.i. hCG at transfer	91	181	65	35.91
	Control	89	177	51	28.80
II	CIDR*	645	1180	747	63.33
	Control	727	1279	801	62.65
III	CIDR*	640	1024	736	71.87
	Control	643	1157	804	69.05
IV	CIDR*	993	1787	1004	56.16
	Control	49	80	40	50.00
	200 u.i PMSG at transfer	22	44	29	66.00

Data: LambXL, ATC. See also Vivanco H W. 1996
*: CIDR at transfer, replaced day 13, removed day 56 post transfer

Table 12. Survival to term of bisected (demi) and whole Texel embryos

Type of embryo	Number of embryos transferred	Number of foetuses	Survival of the demi embryos %	Survival of the whole embryos %	Total survival, of the original embryos %
Demi embryos	1410	710	50.3	-	100.6
Whole embryos	1252	771	-	61.6	61.6

Data: LambXL.1991. Vivanco H.W. et al.1991

Our work in LambXL New Zealand and the ATC Australia was based on the transfer of fresh embryos; work at Macquaire Artificial Breeders, Australia, was done with fresh, chilled and frozen embryos, the last ones mainly for the export market. Initially the embryos were frozen using glycerol but from 2005 the method of freezing was using Ethylene Glycol for direct transfers. The results for the local market in Australia are shown in table 13; results of embryo survival of embryos produced in Australia and transferred in South America are shown in table 14.

Table 13. Survival to term of fresh, chilled and frozen sheep embryos (mixed breeds) in Australia

Period	2005-06	2006-07	2007-08
Embryos transferred fresh	898	1,130	122
Pregnancies for fresh embryos	591	743	83
% pregnant for fresh embryos	65.81	65.70	68.03
Embryos transferred Chilled-warmed	901	607	36
Pregnancies for chilled embryos	650	397	25
% pregnant for chilled embryos	72.14	65.30	75.00
Frozen/thawed embryos transferred	361	148	158
Pregnancies for frozen/thawed embryos	208	106	110
% pregnant for frozen/thawed embryos	57.62	71.62	69.62

Data: Macquaire Artificial Breeders, Dubbo, NSW, Australia

Table 14. Survival to term of frozen/thawed East Friesian and Dohne embryos frozen in Australia and transferred in South America.

Country, geography and year transferred	N° of embryos transferred	N° of recipients pregnant	% pregnant	N° of lambs born	% born over transferred
Peru, 4,200 meters elevation; 2010	197	97	49.30	91	46.20
Uruguay, sea level, 2005	720			490	68.00

Data Vivanco International SAC.

Embryo transfer in sheep is an effective method for increasing the reproductive rate of the females, its use has been cost efficient in many applications, and further improvements in the art of superovulation should increase its cost effectivity.

2. Alpacas embryo production and transfer

2.1 Some physiological aspects of Alpacas reproduction:

Alpacas are animals with induced ovulation, the females will ovulate only in response to a stimulus and only if at the time the stimulus is applied the ovarian follicle is at the adequate stage of development (Bravo et al., 1991). The stimulus can be the simple presence of the male (Vivanco et al., 1985), the actual mating and its duration (Vivanco et al., 1985; Sumar, 1985), the ovulation induction with the application of exogenous hormones (Rodríguez, 1959; England et al., 1969; Leyva et al., 1977; Bravo et al., 1997; 2000) or induction by "ovulatory factors" present in the seminal plasma (Adams et al., 2005; Ratto et al., 2006). Alpacas ovulate 30 hours after induction of ovulation if they have a follicle of at least 6 mm of diameter (Adams and Ratto, 2001; Bravo et al., 1991), the CL is formed 2-3 days after mating, regressing 11-12 after mating if conception has not occurred (Adams et al. 1989). Alpacas do not express what is known as "estrus cycle" (interval between two spontaneous estrus), but they present what are known as "follicular waves" of development and regression of ovarian follicles (Adams et al. 1990; Bravo et al. 1990; Vaughan et al. 2004). The ovarian follicles in alpacas (in females that have not been exposed to males) starting at puberty show continuous waves of development and regression that overlap in certain portions of the wave, that means there is a segment of time in which some new follicles are developing and some old follicles are regressing. The follicular wave can be divided into three phases: one of initial development, another one of frank development and maturation and the third one of regression. The initial development of the new wave overlaps with the regression phase of the previous wave (Bravo et al., 1990).

In alpacas, the follicular development from initial stage (2 to 3 mm of diameter) to a developed stage of 8mm of diameter takes 4.8 days then it takes 5 more days for the mature follicle (8 to 12 mm) to start regressing, the regression lasts 4 days; the development of a subsequent follicle wave starts 2 to 3 days after the initiation of regression of the dominant follicle of the previous cycle; consequently the interval between follicular waves in alpacas is of 11 to 12 days. The development of follicular waves is alternated between the ovaries in 81% of the cases (Bravo et al., 1990); this is in average, existing obviously individual variations, for instance Vaughan et al. (2004) find that 39% of the alpacas show 12 day intervals between follicular waves but 32% show waves up to 16 days of interval existing a direct correlation between the length of the wave interval and the

maximum size of the mature follicle. At the initiation of the follicular wave there are several follicles developing but only one of then develops into a dominant follicle and an inverse relationship is established between the diameter of the dominant follicle and the number of follicles developing. Observations by Vaughan et al. (2004) show that the rate of development of the dominant follicle is consistent and that it reaches the ovulatory size at 10 days from emergency independently of the subsequent interval between waves, consequently we can predict the optimum time for mating if we have control over the emergency of the follicular wave.

2.2 Control of the ovarian cycle in Alpacas:

It is important to know the stage of the follicular wave in which a female is in order to control the wave; one way is to monitor by ultrasound examination and follow the wave development for at least 14 days so we know when the follicular emergency starts. Another way to control the initiation of the follicular wave is by inducing ovulation and subsequently once the CL has functionality 3 to 5 days after mating or 2 to 3 days after ovulation regression of the CL is induced injecting PgF2α (Fernandez-Baca et al., 1979; Gamarra et al., 2007). Others have tried to control de ovarian cycle inducing an artificial luteal phase with progestagens initiating a new follicular wave about 5 to 7 days after the finalization of the induced luteal phase (Alberio and Aller, 1966; Chaves et al., 2002), or by ablation of the dominant follicle either by hormonal or mechanical means (Ratto et al.,2003).

From the observations of Vaughan et al. (2004) we know that we can expect a preovulatory mature follicle at maximum follicular development 10 days alter the initiation of the wave, then we hypothesized that if we control the initiation of the wave and stimulate the ovary with gonadotropins to achieve the development of multiple follicles and accompany them from the initiation of the wave up to preovulatory stage 10 days after the initiation of the wave we should have superovulation.

When doing ovarian stimulation for multiple ovulation in alpacas we need to take into account that the follicles produce sufficient estrogen to stimulate secretion of ovulatory levels of LH post copulation or ovulation stimulus, only when the follicles reach between 6 to 12 mm of diameter, then ovulation occurs with the subsequent formation of a functional CL; but when the follicles are small (4-5 mm) there is not enough endogenous production of LH as response to an ovulatory stimulation and so the females do not ovulate; in the other extreme if the follicles are too old and they have already initiated the regression process after reaching maximum development, even when in these cases there is sufficient production of endogenous LH as response to the ovulatory stimulus, the females do not ovulate and the follicles are luteinized and the CL that are formed are not normal and have a short life span of only about 5 days (Bravo et al., 1991). It is then extremely important to supervise follicular development during superovulatory stimulation in such a way that the mating or ovulation induction is done at the right moment, not before, not after. Bravo et al. (1991) also demonstrated that llamas produce higher levels of LH than alpacas in the different developmental stages of the follicular development; this could explain the better results obtained in llamas in their response to superovulatory regimes in comparison with alpacas.

It seems that the clue for an adequate response to hormonal ovarian stimulation for multiple ovulation in alpacas is in the ability to control the initiation of the follicular wave and to manage the time of induction of ovulation in such a way that this is done when the follicles are at optimal pre-ovulatory stage and so the adequate response to the levels of endogenous or exogenous LH is achieved. The levels of FSH used are less critical and can be optimized by titriation according to response, however it should be taken into account that there exists large individual variation in response to a determined level of FSH (Bravo et al. 1990).

2.3 The development of superovulatory strategies for alpacas:

Most of the work on superovulation in South American Camelids (SAC) have been done in Llamas, very few trials have been done in alpacas, however, most of the commercial application of MOET for SAC is done in alpacas due to its importance in fiber production in their home region in the Andes and the high value of each individual in countries outside the Andes where they have been introduced in the last 2 decades.

The very few reports in Alpacas superovulation (Novoa and Sumar, 1968; Correa et al., 1992, 1994; Novoa et al., 1968, 1999) used different superovulatory strategies and the results have been very variable in terms of number of transferable embryos per donor subjected to the superovulatory treatment. The initial strategies were to mimic sheep superovulation protocols injecting FSH within an artificial luteal phase, Correa et al. (1992) used a progestational phase of 12 days and treated 4 alpacas with FSH injections 3 days before the end of the progestational phase and 4 alpacas with eCG applied at the end of the progestagen treatment, they had an average of 2.5 CL and 1.1 transferable embryos per donor treated; later the approach was not to use a progestational phase, Novoa et al. (1999) selected by ecosonography alpacas with follicles of less than 7 mm diameter, injected 500 to 750 i.u. eCG on day zero and hCG (700 to 1000 i.u.) on day 5 at time of mating, collected embryos by laparotomy, their average was 1.7 embryos per alpaca treated.

Our own first trials (Gamarra et al., 2007) to develop superovulatory regimes tested different approaches, we trialed FSH treatments 3 days before ending a progestational phase of 9 days and treatments without progestational phase but without knowing the stage of the follicle at the start of the of FSH injections given 5 to 7 days after prostaglandin and continuing for 3 days until mating; results showed that FSH treatments at the end of progestational phase do not differ from FSH treatments without progestational phase; the ovulation rate we obtained with both strategies was low probably due to the presence of dominant follicle at the start of the FSH injections since we did not control the wave emergency neither the presence of dominant follicle. Miragaya et al. (2006), report that the presence of a dominant follicle of 5 mm of more at time of gonadotropins injections, induces the development only of the dominant follicle and not of the cohorts.

The injection of gonadotropins within a natural or artificial luteal phase, that means when progesterone is acting, was informed as causing poor superovulatory responses (Bourke et al., 1992, 1995; Aller et al., 2002). Adams et al. (1990) demonstrated that when the CL is present there are fewer follicles and the size of them is smaller, so there is a clear limitation to the follicular development in SAC in the presence of progesterone. This was corroborated by Chaves et al. (2002) who showed that the injection of exogenous progesterone at any stage of the ovarian cycle in SAC inhibits the follicular development. This effect is independent of the presence or functionality of the dominant follicle since gonadotropic treatments when is acting the exogenous progesterone accompanied by injection of Estradiol Benzoate to limit the development of the dominant follicle, resulted also in poor superovulatory response in llamas (Aller et al., 2002). Our observations (Vivanco et al., 2010) partially agree with this, we found that if the gonadotropin is injected within a progestational phase but at the final part of the phase (last 3 days) the production of transferable embryos is low, but if the gonadotropin is injected at the beginning of the luteal phase when a new follicular wave is emerging after ovulation, then the results are very satisfactory. In our trial comparing different strategies to take control of the initiation of the follicular wave (Vivanco et al., 2010), the group where we selected by ecosonography the donor alpacas having follicles of 8 mm or more and induced ovulation in them by mating with vasectomized males plus an injection of GnRH right after mating, ovulated and formed a functional CL; then we treated them with 1,000 i.u. of eCG on day 2 after mating; on day 7 they were injected with prostaglandin, on day 8 were mated twice with fertile males and injected again with GnRH and the embryos were collected on

day 15 (see table 15). This group had a new wave of follicles developing after ovulation and were stimulated with eCG when they were forming a functional CL, the follicles developed during the time the CL was active; in this group the average number of CL per donor was 6.0 and the average number of embryos per donor was 2.77 and was the highest in comparison with the groups where the gonadotropin treatment was applied in the later part of the progestagen phase and in comparison with the groups that were injected after the progestational phase was ended. Later repetitions of this protocol have consistently given us relatively good results (table 16).

Table 15. Superovulatory strategy for alpacas (Vivanco et al. not published)

Day	DONORS	RECIPIENTS
Day 0	Selection of alpacas with follicles of 8 mm diameter or more. Application of GnRH (0.0084 mg. of Buserelin acetate) to selected females and natural mate them with vasectomized male (PM)	
Day 2	Application of 1000 i.u. of eCG (AM)	
Day 7	Application of PGF2α (0.25 mg.of Cloprostenol) (AM)	
Day 8	Verify if follicles are at 8mm or more, perform First mating with fertile male and application of 750 UI hCG (AM)	Application of GnRH (0.0042 mg. of Buserelin acetate) + natural mating with vasectomized male (PM)
	Second mating (PM)	
Day 14	Embryo collection (mid day)	Embryo transfers (mid day)

Table 16. Superovulatory responses of alpacas treated with eCG at the initiation of the follicular wave and in presence of a functional CL.

TRIAL/YEAR	N° of donors	Total CL/ donor Mean ± SD	Total embryos collected/donor Mean ± SD	Transferable Blastocyst collected/ donor Mean ± SD
I, 2010	10	6.70 ± 2.20	3.52 ± 3.01	3.00 ± 2.87
II, 2012	26	6.00 ± 2.01	2.50 ± 2.28	1.50 ± 2.00
III, 2013	17	6.88 ± 2.15	4.18 ± 5.11	3.80 ± 4.87
IV, 2013	20	6.81 ± 3.22	3.69 ± 3.42	3.13 ± 3.24

Data: Vivanco et al. not published. Trial IV was performed this year one month after trial III.

Vaughan has done very large numbers of embryo collections in alpacas, both with and without superovulation, she mentions (Vaughan, 2012) that she has treated 1,600 donors for superovulation, the donors had been induced to ovulate and to form a CL with an injection of Buserelin and had received FSH treatments (she does not give any indication of the actual regime used), she obtained an average of 6.6 ovulations and 2.6 embryos collected per donor.

For superovulation is not enough to have several follicles developing, it is of same importance to achieve that all developing follicles mature and ovulate and that we obtain high fertilization rate. We know from the work by Bravo et al. (1991) in llamas and alpacas that if we induce ovulation when the follicles are of less than 4 mm there is no secretion of LH and consequently there is no ovulation, and also in the other extreme, if the matured follicles start regressing, even when there is enough LH, there is no ovulation but luteinization of follicles. It can be seen that it is critical to stimulate the ovulation at the right timing when the follicle is between 8 to 12 mm in diameter (Aller et al., 2003, recommend to do the mating when the follicle has more than 7mm), for achieving that, it is necessary to monitor the follicular growth during the period of gonadotropin induced development and induce the ovulation by mating, or by injection of hCH or GnRH or mating plus hormonal treatment when the follicle has more than 7 mm diameter (Vivanco et al., 2010; Vivanco et al., 2011).

Ratto et al. (1997) informs that mating after 36 hours from the last injection of FSH in llamas that received gonadotropin treatment for 5 days, increased the ovulatory response in comparison with llamas that were mated immediately at the end of the 5 day gonadotropin treatment; the fact that the llamas that did not receive gonadotropin treatment in the last 36 hours before mating had a more prolonged period from initiation of treatment to mating, makes the data difficult to interpret since the effect is confounded , the question still remains about what would have happened if instead of stopping the treatment during those 36 hours the treatment would have continued.

Many authors report the use of two matings or AI services in alpacas treated for super ovulation, inseminating or mating the second time at 12 to 24 hours from the first insemination or mating (Aller et al., 2002; Ratto et al., 1997; Gamarra et al., 2007; Vivanco et al.,2010; Vivanco et al., 2011), this is done with the aim of increasing the total availability of sperm for fertilization of the many oocytes produced and has no influence in ovulation rate since Bravo et al. (1992) demonstrated that both in llamas and alpacas, the pituitary does not respond secreting LH to the second copulatory stimulation or to a second injection of GnRH if the second mating or injection is done within 24 hours from the first stimulation.

There are conflicting reports about the preference on the use of FSH or eCG. Ratto et al., (1997, 2006) find that the use of eCG seems to be as effective as FSH or the combination of FSH and eCG for the stimulation of multiple follicular development but not on the number of ovulations; on this parameter the treatments with FSH result in higher ovulatory rate (Correa et al. 1997); also Agüero et al. (2001) observed less sexual receptivity in alpacas when eCG is used and suspects that eCG induces early luteinisation. Miragaya et al. (2006) indicates that there is a high incidence of cystic follicles in females treated with eCG if no previous treatment with progesterone was applied. However, our own results (Vivanco et al., 2010; Vivanco et al., 2011) show better results for the superovulatory protocol using eCG than FSH in alpacas.

Regarding the ovulatory stimulus in alpacas or llamas under superovulatory treatment, due to the fact that the majority of studies have used natural mating to fertilize the treated females, the mating with normal entire males has been the main stimulation used, sometimes in combination with an injection of hCG or GnRH (Bravo et al. 1991, 1992; Aller et al. 2002; Ratto et al.1997; Correa et al. 1997; Gamarra et al., 2007; Vivanco et al., 2010; Vivanco et al., 2011).

The ovulatory stimulation used for recipients is similar to the one described for females induced to ovulate for artificial insemination, (mating with a vasectomized male or GnRH injection or hCG injection or combination of mating with vasectomized male plus GnRH or hCG). The recipients are induced to ovulate same day as the donors. Ratto et al. (2006) have not found difference between mating only, injection of 5 mg of LH only (without mating) or injection of 50 ug of GnRH without

mating, in the proportion of llamas that ovulated (80 to 90%) and in the interval between induction of ovulation and ovulation time (29 to 30 hours). We (Gamarra et al., 2007) have induced ovulation in 112 recipient alpacas using mating with vasectomised male in combination with 10 ug of GnRH injected intramuscularly immediately after copulation and found at inspection 7 days after that 82.14% of the alpacas had ovulated, 3.57% with twin ovulations, 50% of the ovulations in the left ovary and 50% ovulations in the right ovary.

Results from all studies reviewed show that we have achieved adequate techniques for control of ovulations in recipients, with consistent repeatable results. In donors in the other hand the results are still too variable and some more research is needed in order to achieve an adequate strategy for super ovulation (induction of multiple follicular development with a high ovulatory rate of fertile oocytes translated into normal transferable embryos). Our results to date (Vivanco et al., 2010; Vivanco et al., 2011 and not published data of table 16) show that we are already obtaining near 4 transferable embryos per donor alpaca, this is a very significant improvement from the initial rates obtained only 5 years ago, so with more support for research and technological development we should be able to reach higher rates of embryo production comparable to other domestic species.

2.4 The IN VIVO embryo production in alpacas without superovulatory stimulation:

Some authors have chosen to collect the embryo formed after a normal ovulation and mating within a normal ovarian cycle without gonadotropin stimulation. Taylor et al. (2000) collected successively 22 llamas in a period of 2 consecutive years, monitoring the ovaries by ultrasonography, mating the llamas by natural mating and injecting 2 mg of LH at mating time that was done when the follicle had 10 mm diameter or more; the recipients received only the LH dose. In total 99 collections were made obtaining 67 embryos (0.68 embryos per collection) and transferred 49 embryos that resulted in 18 pregnancies (36.7%). They collected on day 7, 8 and 9 post mating and found that the recovery rate and the pregnancy rate increased in direct correlation with the interval between mating and embryo collection. All the embryos collected were hatched blastocysts which confirm the observation by Del Campo et al. (1995) that indicates that the embryos in SAC arrive to the uterine horns at very advanced stage in comparison to other species. Taylor et al. (2001) used the same strategy (collection without stimulation for super ovulation) in alpacas collecting an alpaca for two consecutive times in a period of 37 days achieving 100% recovery rate and 100% pregnancy rate when transferring the alpaca embryos to llama recipients.

The demonstration by Taylor of the feasibility of this system and the very important observation of Vaughan et al. (2004) in the sense that the follicular development in the first 10 days from emergence of the follicular wave is consistent and continuous regardless the subsequent interval between follicular waves, has permitted to develop the "Single Embryo Flushing System" and consists in collecting embryos from the alpaca female every 10 to 12 days without hormonal stimulation of the donors. Vaughan (2012) informs that she has collected 822 alpaca donors that had in average 1.2 ± 0.3 CL, obtaining 667 (67% recovery rate) embryos at an average of 0.81 embryos per donor. Vaughan (2012) indicates that the donors for single ovulation flushes may be synchronized to allow several animals to be flushed in the same day, by inducing ovulation in all females 10-12 days before mating day and inducing luteolisys of the CL 24 hours before mating; donor females are mated once and flushed 7 or 8 days later. With the current improvements in the yield of transferable embryos per superovulated donor (table 15), it seems that the single-ovulation flushes need to be reevaluated for the convenience of their application in genetic improvement programs where the maximization of the reproductive rate of elite females needs to be achieved.

2.5 The techniques of embryo flushing and embryo transfer:

The technique for embryo flushing that is most used in domestic SAC (alpacas and llamas) is the catheterization of the uterus by transcervical route ; the technique is described in detail by Taylor et al (2000); the size of the Foley catheter used in llamas by Taylor et al. (2000) was of 20 Fr.g , same as in alpacas (Taylor et al. 2001); we (Gamarra et al. 2007) use in alpacas a Foley catheter of 14 Fr. g with a 5cc inflatable cuff but we reduce to 12 Fr. g for some alpacas with small cervical lumen. Other technique for embryo flushing in SAC is the surgical embryo recovery by laparotomy, used since the very early attempts for embryo collection (Novoa and Sumar, 1968). We have used surgical embryo collection in alpacas but have discontinued its use because we did not find a significant improvement in embryo collection rate.

For transferring the embryos, the most used technique is also the non-surgical technique via transcervical catheterization of the uterus, is very similar to the technique used in cattle, packing previously the embryos in 0.25 cc straws and using catheters and pistolettes commercially available for 0.25 cc (Taylor et al. 2001). We have used for embryo transfer the non-surgical transcevical method and the surgical laparoscopy aided technique exteriorizing the tip of the left uterine horn; we only transfer the embryos in the left uterine horn of alpacas independently of which ovary has ovulated, since most of the pregnancies are carried in the left uterine horn even when the alpaca ovulated in the right ovary (Fernández-Baca et al., 1979). The pregnancy rates we achieve are higher for the surgical laparoscopy aided technique.

2.6 Pregnancy results:

From the observations of Bravo et al. (1990) we can see that in alpacas the CL starts regressing very early (11-12 days after mating, 9 days after ovulation), so if an embryo is not sending signals to the uterus of the recipients by day 8 or 9, it is very likely that regression will occur and the pregnancy will not be established (Aba et a., 1997; Del Campo et al., 1995). We have compared transferring embryos to recipients that were induced to ovulate the same day than the donors, one day before than the donors and one day after the donors; this last group gave us the highest pregnancy rates (44.5%) when the embryos were transferred by surgical laparoscopy aided method. We collect the embryos on day 6.5 after mating, 91% of the structures recovered are transferable embryos at the stage of hatched, expanded blastocyst; this fast development of the embryos is probably related with the need of sending as soon as possible messages to the uterus for the maternal recognition of pregnancy. Vaughan (2012) reports 41.8% pregnancy rate for 3,963 recipients receiving embryos produced by superovulation and 42.4% pregnancy rate (553 recipients transferred) for embryos produced for by single-ovulation flushing.

Cryopreservation of in vivo produced alpaca embryos:

There are very few reports of cryopreservation of SAC embryos, most of them for llama embryos. There is no report of successful cryopreservation of alpaca embryos (successful in terms of pregnancy and live births generated with frozen/thawed embryos). We have performed recently a trial for freezing and vitrification of alpaca embryos; the embryos were at the hatched expanded blastocyst stage, were frozen using an Ethylen Glycol plus Sucrose protocol developed by us and the vitrification method was as described by Skidmore et al. (2005) for camels. Two out of 22 frozen/thawed embryos transferred are surviving to date (70 days post transfer) but none of the 13 vitrified embryos transferred.

3. Final Comments:

It would seem unnecessary to continue the development of in vivo embryo production strategies in sheep and alpacas when there is now the in vitro alternative, however we need to take the view that both systems should complement each other and that there is room for both in the industry.

In Vivo embryo production has some advantages over in vitro embryo production because of the very low capital investment necessary to implement the in vivo embryo production system and because of the "robustness" of the embryo produced that results in higher pregnancy rates (being the recipients cost the highest cost of any embryo transfer operation, better pregnancy results in lower costs) and lower incidence of abnormal gestations and/or births in comparison with the in vitro system.

The in vitro system has also some advantages, the most important ones being the higher number of embryos that can be produced per donor per year and the possibility to generate offspring from pre pubertal animals reducing dramatically the generation interval.

The efficiency of both systems depends fundamentally on the number of antral follicles that we can have available in the ovaries either for superovulation or for oocyte recovery, this points as our first priority to do more research in ovarian physiology in order to increase this available number of antral follicles.

In sheep, the in vitro system requires invasive methods for harvesting oocytes from live donors , the frequency of the ovum pick up is limited due to this fact and because of the seasonality of ovarian activity in some latitudes, so the use of an invasive method to produce less embryos per surgery than with in vivo system seems not justified unless is for juvenile oocyte aspiration (with gonadotropin stimulation) and embryo production that is performed once in the life time of a ewe lamb and harvests large amounts of oocytes for embryo production (Earl et al. 1995). Juvenile in vitro embryo production and transfer has a significant impact on reduction of generation interval and has been used intensively in Australia to achieve fast genetic changes in wool diameter improvement in Merino Sheep and also in the dissemination of the Inverdale Gene.

In alpacas, significant progress was made in the in vivo embryo production in the last 10 years, currently we can expect 4 embryos per donor programmed and 45% pregnancy rates this would permit the cost effective use of female alpacas as genetic resource; the main limitation at the moment is in the efficiency of utilization of selected males. Male alpacas have very low sperm concentration per ejaculate and very low ejaculate volume compounded by the high viscosity of the ejaculate that limits the manipulation and dilution of the ejaculate; as a consequence in average only five doses of insemination can be obtained from an ejaculate and the fertility rates reported are very variable. The use of in vitro fertilization could be an alternative to generate many offspring from selected males through in vitro embryo production and transfer. The in vitro embryo production in alpacas is in its very infancy, to date very few successful in vitro embryo production in alpacas has been reported being our work (Gamarra et al., 2009) the first reported successful in vitro embryo production up to blastocyst stage in alpacas.

It is great news that there is still so much to do and develop that will keep busy to the next generation of researchers and practitioners.

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OVIDUCTAL MECHANISMS CONTRIBUTING TO THE SUCCESS OF FERTILIZATION IN MAMMALS

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Why is the oviduct becoming the focus of attention for researchers working in ARTs?

The oviductal physiology is becoming more and more studied in the last years because, together with the classic functions of the oviduct, new mechanisms are being described that could contribute to the success of fertilization if they were included into the laboratory protocols.

In mammals, the oviduct provides not only the physical but also the chemical microenvironment necessary for the encounter of the sperm and oocyte. So, the anatomical shaping of the infundibulum is designed to capture, with a very low margin of error, the unique or the diverse oocytes, depending on the species, shed from the ovarian follicles. Once in the oviduct, and during their short travel throughout the ampullar conduit, these oocytes are exposed to the fluid bathing the tube and to the changes that the components of such fluid can induce in them. But, which are these components? How important are each of them for the final ability of the oocyte to develop into a newborn individual?

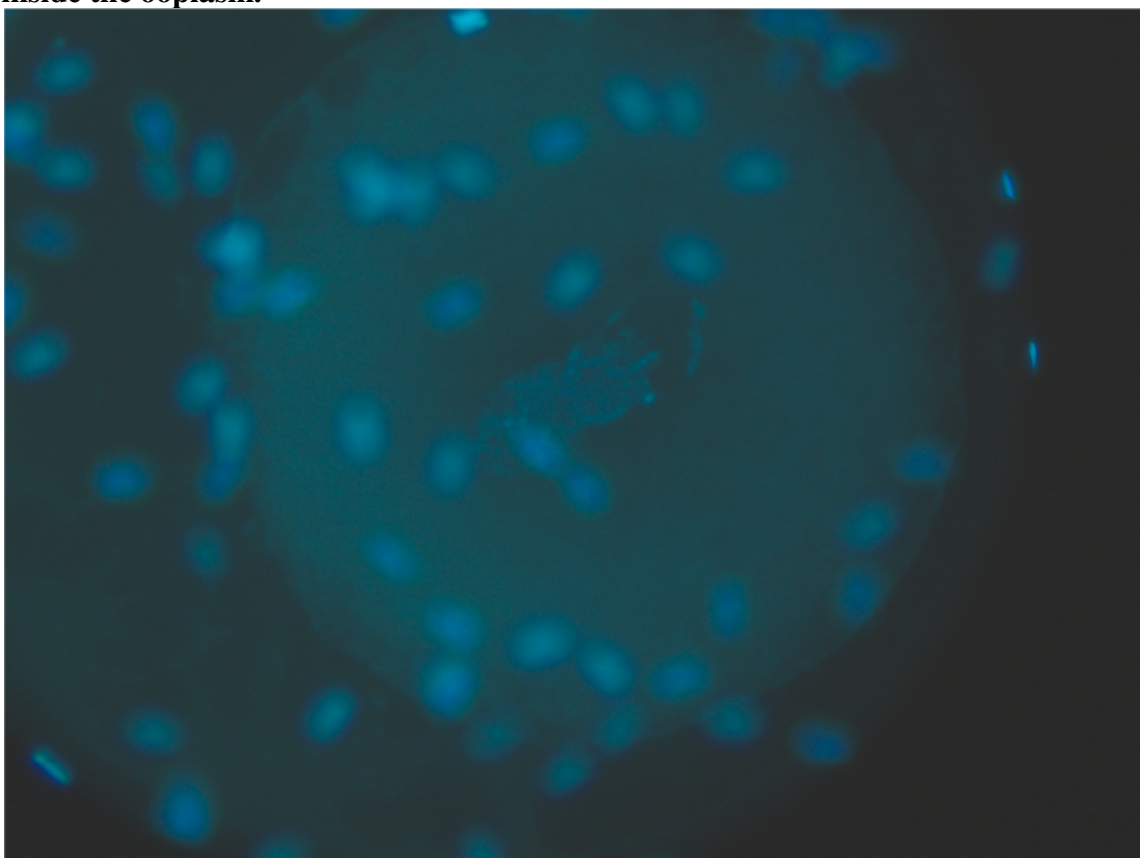
Proteomics and functional genomics are providing names of a number of proteins and genes up or down-regulated in the oviduct during the fertilization period. Analysis of gene expression in bovine oviductal epithelial cells have shown 37 up-regulated genes at estrus and 40 at diestrus (Bauersachs *et al.* 2004). Proteins in oviductal fluid such as osteopontin (Gabler *et al.* 2003), glycodelin (Chiu *et al.* 2007), OVGP1 (Coy *et al.* 2008), HSPA8 (Lloyd *et al.* 2009), plasmin (Mondéjar *et al.* 2012), lactoferrin (Zumoffen *et al.* 2013) or DMBT1 (Teijeiro & Marini 2012), among others, have a demonstrated role on sperm-oocyte interaction. Consequently, their future use in the ARTs laboratories, either human or animal, as regular components of the culture media, is anticipated. Here, we are going to focus on the role of two of these proteins, named OVGP1 and plasmin, and in the implications they might have, depending on the species of study, for the fertilization process either *in vivo* or *in vitro*.

Pre-fertilization zona pellucida (ZP) hardening

According to the original concept, "zona hardening" is defined as an increased resistance of the zona pellucida to dissolution with various proteases or chemical reagents. This physiological change in the zona pellucida occurs subsequent to dehiscence of the cortical granules during fertilization" (Gulyas & Yuan 1985). Even though "zona hardening" can be referred today to "physical hardening" (Papi *et al.* 2010), which is a different concept, the chemical hardening of the original concept from Gulyas and Yuan comes from observations in mouse and later in rat (Zhang *et al.* 1992). However, experiments with bovine or porcine oocytes showed that the ZP did not increase its chemical resistance after IVF (Coy *et al.* 2002, Coy *et al.* 2005), what raised the question about the possible lack of hardening for *in vitro* fertilized oocytes. If that were the case, one could consider the failure in cortical granule exocytosis after IVF as the possible reason. But this was not the answer. The answer was that, in contrast to mouse, pig and cow ZP is already hardened before fertilization if it is in contact with oviductal fluid ("oviductal zona maturation", Coy & Avilés 2010) and that oviductal specific glycoprotein OVGP1 and heparin-like glycosaminoglycans contained in the fluid are responsible for this effect (Coy *et al.* 2008). Moreover, the binding of OVGP1 to ZP makes it more resistant to sperm binding and decreases the risk of polyspermy. Consequently, one can wonder: are the rodents different to large animals in their fertilization mechanisms or does this concept need to be revised and newly defined for every species?

Let us consider a second concept related to the sperm-ZP binding. Since years ago, it has been accepted that cortical granule (CG) content renders the (golden hamster´s) oocyte refractory to i) additional sperm binding and ii) penetration (Barros & Yanagimachi 1971). However, the former observation (i) cannot be generalized when studies with large animal models are developed: as an example, porcine images of zygotes and early embryos collected *in vivo* showed a steady increase in the number of sperm bound to ZP what indicated that they were not resistant to sperm binding after fertilization (Hunter 1974). In our laboratory we have also obtained zygotes from the porcine oviduct with a huge number of sperm attached to the ZP but still monospermic (Figure 1). Coming back to the mouse, interesting studies at the molecular level with transgenic mice demonstrated that the ZP2 cleavage seems to be responsible for the lack of sperm binding to the ZP after fertilization but this was independent on cortical granule exocytosis and fertilization since both procedures occurred in mice with or without ZP2 cleavage (Gahlay *et al.* 2010). Then, what is the meaning of ZP2 cleavage in mouse? Is it just a mechanism to avoid sperm binding to ZP after fertilization? Why does this mechanism not exist in the pig if ZP changes have been also demonstrated after fertilization (Hedrick *et al.* 1987)? And a last but not least question, what happen in humans? Do human oocytes follow the mouse model, the pig model or their own model regarding ZP binding of sperm post-fertilization? We will try to answer these questions in the second part of this review.

Figure 1
Zygote recovered from the oviduct of a sow after artificial insemination showing a big number of spermatozoa attached to the zona pellucida (out of focus) and two pronuclei in singamy inside the ooplasm.



Meanwhile, from the above considerations, it can be concluded that the different mechanisms participating in the sperm-ZP binding and in the regulation of polyspermy (or in the fertilization process in general) must be studied species by species. In the case of the pre-fertilization ZP hardening, a series of experiments with oocytes from nine species and oviductal fluids from five species were conducted (Mondéjar *et al.* 2013), yielding the following results:

1) The rabbit oviductal fluid was the one producing a stronger effect on ZP resistance to digestion in mouse, rat, hamster, human, cow, pig, ewe and goat oocytes as well as in its own oocytes. Mouse oocytes, which did not undergo hardening in their own oviductal fluid (Coy *et al.* 2008), showed a hardened ZP after incubation in rabbit and goat oviductal fluids. Even the rat, where OVGP1 is a pseudogene and could be thought that is insensitive to the fluid effect, experienced a ZP hardening after contacting with rabbit oviductal fluid.

2) The human ZP, either isolated or surrounding oocytes, did not become hardened after treatment with any fluid, including the human one.

3) All the five major animal species analyzed (pig, cow, goat, rabbit and sheep) showed a change in their ZP resistance to digestion after a 30 min period of incubation in their own oviductal fluids.

In summary, the pre-fertilization ZP hardening seems to be an evolutionarily conserved mechanism which could have been lost in primates. As demonstrated in the pig and cow, the biological meaning of this event can be related with the decrease in the number of sperm bound to the ZP and with the regulation of polyspermy (Coy *et al.* 2008). In addition, the binding of different oviductal proteins to the ZP could exert a protective effect on the zygote or early embryo, avoiding premature ZP degradation and attachment to the oviductal wall (Kouba *et al.* 2000). Since humans lack of this mechanism and it is the only species where tubal ectopic pregnancy has been demonstrated, a possible relationship between both events should be investigated.

Breakage of binding between "supernumerary" sperm and ZP

If, as above explained, nature has designed a mechanism to avoid sperm binding to the ZP once the fertilization has taken place in mouse, the possibility of a similar counterpart in other mammals should be considered. Let us imagine that the dialog between the mouse zygote or early embryo and the oviductal epithelia can be disturbed by the presence of excessive spermatozoa attached to the ZP. If the porcine or bovine species do not have this mechanism, could this fact mean a disadvantage?

A series of experiments in our laboratory have showed that the plasminogen-plasmin system play a role in the detachment of the supernumerary spermatozoa from the ZP after fertilization (Coy *et al.* 2012). Whether this mechanism responds to the need of "cleaning" the ZP to facilitate the correct signaling pathways between the mother and the embryo, or to the observations in mouse showing that some spermatozoa initially attached to the ZP are removed and substituted by new spermatozoa (Gahlay *et al.* 2010), remains to be elucidated.

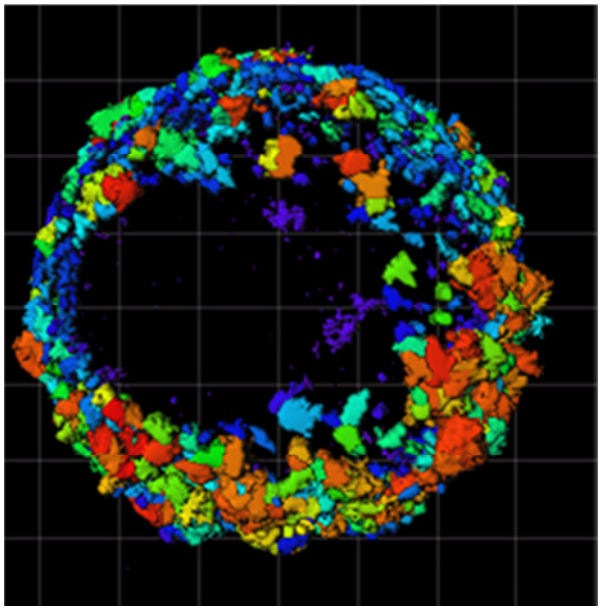
What we have seen (and quantified) is that plasminogen is present in the oviductal fluid of the pig and cow. According to the model we propose, oocytes, once in the oviduct, bind plasminogen to their ZP and oolemma. Since plasminogen is a zymogen, nothing happens until the arriving of spermatozoa. It is then when the contact of the first spermatozoa with the oolemma induces the releasing of plasminogen activators (tPA and uPA) from the oocyte cortex, which bind to plasminogen and convert it into the active protease plasmin. By using live cell imaging methods, we

could observe how the PAs were released from the oocyte a few minutes after sperm contact (Figure 2). We could also quantify the production of plasmin in the culture media where oocytes and spermatozoa met and, finally, we recorded a videoclip showing how the addition of plasmin near the ZP modified the strength of the sperm-ZP adhesion: in absence of plasmin, the adhesion was firm and impossible to break with a micropipette, whereas in the presence of plasmin this adhesion turned weak and, after a light touch with the micropipette, became broken.

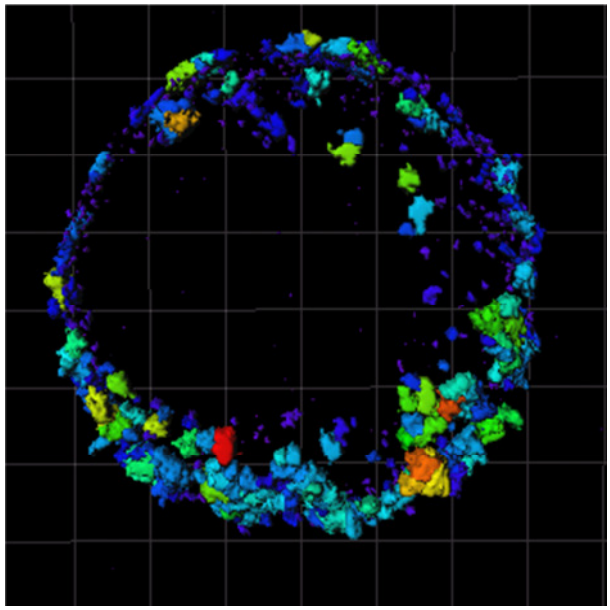
What is the physiological meaning of these observations? Is this an additional mechanism to avoid polyspermy independent on cortical granules? In a recent publication with human gametes it has been observed, by time-lapse cinematography, that just ten seconds after the contact of the first spermatozoon with the oolemma, the remaining ones attached to the ZP were arrested from further penetration (Mio *et al.* 2012). These results match to those from our laboratory and reinforce the idea of an additional mechanism to control polyspermy whose importance must be assessed in the different species.

Figure 2.
Three-dimensional reconstructions (25–35 2D images) showing high (red color) and low (green color) fluorescence intensities in ZP-free *in vitro* matured and fertilized oocytes incubated with uPA antibody. The fluorescence signal started decreasing considerably just 2 min after the sperm–ooplasm contact (a) and continued decreasing during the incubation time (6–18 min) (Modified from Coy *et al.*, 2012, *Human Reproduction* 27(7):1985-1993).

2 min after sperm contact



18 min after sperm contact



In conclusion, we have investigated and only partially clarified the mechanisms by which two oviductal proteins (OVGP1 and plasmin) affect the fertilization process. As above indicated, there are many other factors under study and the coming future predicts the discovery of additional ones which will complete piece by piece the complex puzzle of the beginning of life.

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BIOLOGICAL SENSORING OF THE CONCEPTUS BY THE MATERNAL ENVIRONMENT

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Introduction

In mammals, the birth of a viable and healthy progeny involves a continuum of complex biological processes and several checkpoints (or hurdles) that have to be passed successfully. These hurdles include production and quality of the gametes (oocytes, sperm), fertilization, luteal function rescue, early development of the embryo, implantation, development of the foeto-placental unit until term and parturition. Each of these steps is crucial for a successful pregnancy and their onset or progression can be influenced by environmental change. In term of contribution to pregnancy (onset, progress and issue), the male differs from the female. Indeed, whereas the father contributes with the production of fertile sperm, the mother not only produces the gametes (oocyte) but she also hosts the whole gestation in a reproductive tract until term. Acute challenges, short or long periods of perturbations related to nutrition, stress, infections or endocrine disruptors have been identified as factors that affect gamete quality and fertilization, journey of the early embryo through the oviduct, cellular interactions between endometrium and hatched blastocyst or conceptus, foeto-placental development or parturition (Fleming *et al*. 2004; Leroy *et al*. 2008). In addition, biotechnologies of reproduction (or assisted reproductive technologies; ART) associated with embryo transfer have been shown to alter the biological properties of the embryo with a subsequent impact on later stages of pregnancy (Sinclair 2008). Therefore an inadequate maternal compartment and/or suboptimal quality of the embryo may impact the two way communication between mother and embryo, precluding completion of successful pregnancy and affecting long term health status of the offspring (Douglas 2010).

Although the oviduct from unstimulated animals appears to be the optimal environment for early embryonic nursing (Besenfelder *et al*. 2010; Gad *et al.* 2011), *in vitro* embryo production and embryo transfer have demonstrated the oviduct to be a dispensable organ for supporting progression of pregnancy to term. Until now, despite several attempts to maintain early and late foetal life outside the uterus (Bulletti *et al*. 2011), no surrogate biological or artificial system has been derived for the uterus. In normal physiological conditions, the uterus and its internal part referred as the endometrium constitute the maternal site for embryo implantation. Implantation process has been recognized as a critical step of pregnancy associated to a high rate of embryo loss in all mammalian species studied so far. As an illustration, whereas the calving rate of pregnancy has been estimated to 40% in Holstein-Friesan dairy cows, more than 70% of pregnancy failure has been associated to embryo death occurring during the early and late pre-implantation period, (Diskin and Morris, 2008). Obviously this poor reproductive performance represents a major cause of economic loss that prompts the need for a thorough investigation of the events leading to embryo mortality during the establishment of pregnancy. External events (as described in the first paragraph) or intrinsic maternal features may affect biological functions of maternal organs or tissues that will in turn impact endometrial physiology. As the ultimate and unique biological layer facing the implanting embryo in normal early pregnancy, the endometrium drives the development of the embryonic disk and extra-embryonic tissues (Hue *et al*. 2007). Complementary to this driver feature, recent data based on *in vitro* embryo manipulations have unveiled an unexpected biosensoring property of the endometrium (Sandra *et al*. 2012). Various publications have also underlined the modifications of gene expression in extra-uterine tissues of pregnant females compared to cyclic females. This short review aims to present data that have explored how the maternal environment exhibits global and

subtle reactions to the nature of the conceptus (including the production by ART) during early pregnancy, at the local and systemic levels.

Response of the endometrium to the conceptus during early pregnancy

Ruminants make ideal models for investigating the biological properties of the endometrium before placentation occurs. Of note is the long pre-implantation phase (10 to 15 days) allowing the dissection of the first cellular contacts and molecular events occurring between the conceptus trophoblast and the endometrium. In addition, the lesser invasive activity of the synepitheliochorial placenta makes it relatively easy to separate the maternal from the fetal component, there is abundant endometrial tissues and accessible uterine fluids allowing easy sampling, and high-throughput genomic tools dedicated to ruminants are now available.

In ruminants, the bicornuate uterus is covered with the endometrium displaying two specific areas, namely the caruncles and the intercaruncular areas (Chavatte-Palmer and Guillomot 2007). The caruncles represent aglandular structures of limited size and are distributed over the endometrial surface. The intercaruncular areas are large and contain the endometrial glands that produce histotroph, a collection of numerous and diverse factors including cytokines and growth factors (Spencer and Bazer 2004). Upon oocyte fertilization and after hatching, the extra-embryonic tissue of the ruminant conceptus undergoes a progressive and critical elongation phase (Degrelle *et al.* 2005) before implanting at day 15-16 post-oestrus in the sheep and the goat gestation (gestation period: 5 months) or day 19-20 post-oestrus in cattle (gestation period: 9 months). Although a decidual-like process (related to hemochorial implantation) has been reported in sheep (Johnson et al. 2003), ruminant implantation is characterized by the apposition then the adhesion between the trophectoderm and the uterine luminal epithelium ultimately forming a synepitheliochorial placenta (Bazer *et al.* 2009).

The anatomy of the ovine uterus represents an advantage for identifying the local endometrial reaction triggered by embryo-related factors *in vivo*. In the ovine model of unilateral pregnancy, the conceptus is confined to the uterine horn ipsilateral to the corpus luteum by placing a ligature proximal to the uterine body (Bazer *et al*. 1979). By comparing the gravid and the non-gravid horns, the paracrine impact of conceptus-secreted factors on the endometrium can be dissociated from their endocrine actions. In this model, dynamic changes of endometrial T lymphocyte populations were reported to be independent from embryo secretions (Majewski *et al*. 2001) whereas the localization of macrophages was affected by the presence of the conceptus (Tekin and Hansen 2004). At the molecular level, Sandra et al. (2005) reported the paracrine influence of the conceptus on the endometrial expression of SOCS genes, a family of intracellular factors displaying major functions in the negative control of cytokine signalling pathways.

Whereas the first steps of uterine remodelling and the timing of the window of implantation are programmed by maternal hormones independently from the presence of the embryo, successful pregnancy will require embryo recognition by the maternal organism with a critical contribution of the uterine reaction. This reaction is promoted by the production of embryonic factors that are indispensable for initiating the implantation process through the establishment of permanent cellular interactions between the trophectoderm and the endometrium (Guillomot 1995). Embryo derived signals vary according to mammalian species and they have been abundantly reviewed, particularly in ruminants for which the major signal of pregnancy recognition has been identified as a type I interferon (Spencer et al. 2008; Dorniak *et al*. 2013). The production of this interferon (interferon-tau; IFNT) is unique since it is secreted by trophectoderm cells during the elongation phase then stops when trophectoderm cells appose onto the luminal endometrial epithelium. IFNT is crucial for maternal recognition through the inhibition of endometrial prostaglandin-F2 alpha secretion thus preventing luteolysis, an indispensable step for maintaining P4 luteal secretion (Martal *et al*. 1997; Roberts *et al*. 2008). Nevertheless IFNT is not the only factor of embryo origin affecting uterine

physiology and other molecules have been identified. Indeed, at the conceptus-endometrial interface of ruminants, two main categories of lipid mediators have been detected, namely the cyclooxygenated derivatives from arachidonic (prostaglandins) and the lysophospholipides.

Numerous cyclooxygenated derivatives from arachidonic acid have been described in the uterine milieu (Charpigny *et al.* 1999; Ulbrich *et al.* 2009) and in the supernatant of cultured endometrial cells (Asselin *et al.* 1997; Arosh *et al.* 2002). In the ovine species, the endometrium is able to synthesize large amounts of prostaglandins in non pregnant and pregnant females in keeping with the similar expression levels reported for the main prostaglandin generating enzyme (PTGS2, formerly COX-2; Charpigny *et al.* 1997). A recurrent and poorly resolved question relates to the role of these high levels of prostaglandins in the uterine milieu at the time of conceptus implantation. An elegant study has demonstrated the mechanisms by which IFNT inhibits the transport of endometrial prostaglandins (Banu *et al.* 2010). Additional reports have suggested that conceptus-derived prostaglandins would regulate the endometrial function in sheep. Using intrauterine infusion of various prostaglandins or an inhibitor of cyclooxygenase activity, Bazer and Spencer's group has demonstrated that prostaglandins increased the expression of genes involved in the migration and the attachment of trophectoderm cells (Dorniak *et al.* 2012). Moreover, in coordination with IFN or cortisol, PTGS2-derived prostaglandins have been found to regulate endometrial functions and genes which are crucial for conceptus elongation (Dorniak *et al.* 2012; Dorniak *et al.* 2013)

Lysophosphatidic acid (LPA) is a naturally occurring small phospholipid. LPA has been reported to be produced by both the endometrium (Woclawek-Potocka *et al*. 2009) and the conceptus (Liszewska *et al*. 2012). The significant impact of the LPA pathway in the conceptus-endometrium interactions has been strengthened by the detection of LPA receptors whose expression levels are regulated in the pregnant endometrium of bovine (Woclawek-Potocka *et al*. 2009) or ovine (Liszewska *et al*. 2012) origin as well as in the ovine conceptus (Liszewska et al. 2009). As shown in various cell models, LPA is known to modulate prostaglandin release. In the bovine species, the intrauterine administration of LPA increased PGE2 release (Woclawek-Potocka *et al*. 2010). In endometrial cultured cells, LPA was shown to stimulate the transcriptional level of PGE-synthase (Woclawek-Potocka *et al.* 2010). In addition, LPA increased the mitotic index of ovine trophoblastic cells in parallel with prostaglandin release (Liszewska *et al*. 2009). In addition to LPA, the activation of Sphingosine-1-phosphate pathway has also been demonstrated in the early pregnant ewe (Dunlap *et al*. 2010). The authors have suggested that this lysolipid mediator generated from ceramides could be necessary for conceptus implantation and may be cirtical for the angiogenesis of the uterus and placenta. Collectively, all these published data support the major biological roles played by the lipid mediators at the conceptus-endometrium interface during the establishment of pregnancy (Vilella *et al*. 2013).

In ruminants, global endometrial reaction to the presence of the conceptus has been recently investigated using large-scale analyses (Spencer *et al*. 2008). In cattle and to a lesser extend in sheep, analyses of molecular and cellular events at the endometrial level have first benefited from the development of microarray platforms (Evans *et al*. 2008) before the coming of Next Generation Sequencing (NGS) that allows the comprehensive analyses of RNA profiles based on RNA sequencing technologies (Forde *et al.* 2012; Mamo *et al.* 2012). Nevertheless, there still remains some limitation in data mining since the current free or priced softwares are built and dedicated to human and rodent data and they lack records of factors interactions specifically associated to pregnancy. Large changes in endometrial gene expression -including innate immune response and associate factors- has been shown to be significantly altered during the early and late pre-implantation period compared to the equivalent day of the oestrous cycle (d*po*) in sheep (Gray *et al*. 2006; Satterfiled *et al.* 2009) and cattle (5 to 16 d*po*: Forde *et al.* 2011; day 18: **Bauersachs and Wolf. 2012**). Very interestingly, some of the identified genes appeared to be preferentially regulated

in bovine caruncles or in intercaruncular areas (17 d*po*: Walker *et al*. 2010; 20 d*po*: Mansouri-Attia *et al*. 2009a), suggesting specific functions related the development of the placentomes in the caruncles and to the production of histotroph in the intercaruncular areas. Regarding the immune function, variations in endometrial genes expression coincides with changes in immune cells populations. As in other mammals (Lea and Sandra, 2007), the local immune system in the endometrium of ruminants protects the uterus against infection and it has to be modulated to prevent embryo from being rejected from the maternal organism (Hansen, 2010). In cattle, mid- and late pregnancy has been shown to affects the proportion of the various populations of endometrial and circulating leucocytes identified during the oestrus cycle including CD4 (T helper cells), CD8 (T cytotoxic cells), CD5 (B-cells), macrophages, dentritic cells and NK cells (Cobb and Watson 1995; Oliveira and Hansen 2008, 2009; Mansouri-Attia *et al.* 2012; Oliveira *et al.* 2012). These reports strongly suggest that the regulation of the immune function during pregnancy is as complex in ruminants as in other mammals with an invasive type of implantation. Nevertheless, deciphering the functions of immune cells and genes in ruminants will require an extensive investigation that has just been initiated during the peri-implantation period (Mansouri-Attia *et al*. 2012; Oliveira *et al.* 2012).

Collectively, these high-throughput data have demonstrated an important global reaction of the endometrium –quantitatively and qualitatively- while facing the conceptus. They have offered unique opportunities to identify novel factors that are involved in the physiology of the endometrium, including transcription factors that control endometrial gene networks during the onset of uterine receptivity or during the peri-implantation period (*EPAS1*, Bauersachs *et al*. 2006, Mansouri-Attia *et al*. 2009b; *FOXL2*, Eozenou *et al*. 2012; *NR2F2*, Bauersachs *et al*. 2009; *STAT1*, Bauersachs *et al*., 2006, Walker *et al*. 2010; Vitorino-Carvalho *et al*. 2012; *MSX1*, Mansouri-Attia *et al*., 2009a). In cattle, a major impact of IFNT on the global endometrial response has also been highlighted with numerous classical IFN-stimulated genes being part of the differentially expressed genes. A combination of an *in vivo* bovine model (a 2H infusion of recombinant IFNT into the uterus of 14 days post-estrus cyclic cows) and cultured endometrial cells of glandular epithelial or stroma origin treated with IFNT for 2H or 24H was used as a strategy to confirm the regulation of classical type I IFN-stimulated genes (ISG) in cattle including *IFIT3, IFIT5, IRF9, ISG15, MX1, MX2, OAS1, OAS2, RSAD2, SOCS1, SOCS3, STAT1*. In addition, new non-classical ISG were identified (*CXCL12, CXCR7, DDX58, HER6, IL12B, MCP1, MCP2, MGC127874, PARP12, PLAC8, PTX3, RNF213, TNFA, ZNFX1*) whose diversity underlines the pleiotropic biological effects of IFNT during early pregnancy (Mansouri-Attia *et al*. 2009a; Forde *et al*. 2012; Mansouri-Attia *et al*. 2012).

Peripheral reaction of the endometrium to the presence of the conceptus

Beyond the local reaction of the uterus to the presence of the conceptus, recent publications have also demonstrated the reaction of extra-uterine tissues to the conceptus, including circulating blood cells and the corpus luteum whose progesterone secretion is indispensable for the establishment and early maintenance of pregnancy in large animal species. Up to now, the systemic effect of the conceptus has been mainly investigated with regard to IFNT and the expression of ISG on peripheric blood leucocytes (PBL; Oliveira *et al*. 2008; Ott *et al*. 2010)..

During early pregnancy, gene expression of various ISGs (*MX1, MX2, OAS1, ISG15*) is altered in PBL collected from ewes (Yankey *et al*., 2001) or from cows (Han *et al*., 2006; Gifford *et al*., 2007; Green *et al*., 2010a, 2010b). Based on the mRNA levels of these genes, diagnosing early pregnancy has been proposed in cattle with a double objective (i) managing cows that failed to be pregnant on day 18 allowing a second insemination when the next oestrus takes place (ii) reducing the interval from calving to conception and thus the economic losses associated to repeated intervention . Han *et al*. (2006) determined *ISG15* mRNA level on blood cells and plasma P4

concentration on day 18 after AI (day 0) in dairy cows whose pregnancy status was confirmed at day 32. Detection of pregnancy using *ISG15* mRNA level in PBC or P4 blood level revealed that *ISG15* mRNA level was less accurate than P4 concentration to detect non pregnant cows at day 18. Nevertheless, measuring *ISG15* mRNA levels in serial collection of PBC (day 17 to 25) could represent an accurate indicator of non pregnant cows although it appears tedious to be carried out in commercial herds. In another study, Holstein heifers were classified as pregnant and non pregnant based on the fold change in *MX2* mRNA from day 0 (oestrus) to day 18 (Stevenson *et al.* 2007). Compared with P4 concentration on day 21 or ultrasonography on day 30 respectively, the authors concluded that this biomarker was not a reliable method for the diagnosis of pregnancy at 18 days after AI. In Holstein lactating cows at either day 17 or day 18 post-AI, pregnancy diagnosis based on the expression of *OAS1* and *MX2* transcripts appeared inappropriate although a reliable cut-off could be determined for heifers (Green *et al*. 2010a, 2010b). In the same study, a microarray approach based on PBL of primiparous lactating and pregnant Holstein cows at day 15 and day 18 post-AI identified 17 genes displaying with a change in gene expression was greater than or equal to 2-fold, including OAS1, ISG15, MX1 and MX2. Eventually, in PBL of multiparous pregnant cows of mixed breed (Japanese Black x Holstein), expression levels of *ISG15, MX1, MX2* and *OAS1* were evaluated in three subpopulations of leucocytes (granulocytes, monocytes, lymphocytes) collected from pregnant or cyclic females (Kizaki *et al*. 2013). All four genes were significantly up-regulated in the granulocyte fraction of pregnant cows at day 14 post-insemination compared to the non-pregnant cows. Altogether, these data suggest that (i) subpopulations of immune circulating cells display distinct reactions to IFNT and likely to the presence of the conceptus (ii) ISGs could represent a reliable source of early pregnancy biomarkers for heifers but not for multiparous females. These data prompt the need for additional studies that could help in determining the pregnancy status using ISG (Forde *et al*. 2012) or interferon-independent genes in females regardless of their parity.

Conceptus gene expression and biosensor properties of the endometrium

During the free-life period specific of implantation in ruminants, three biological processes concomitantly take place in the conceptus: the elongation of the extra-embryonic tissues, the gastrulation of the embryonic disk and the cross-talk with the uterus. Most studies report the development of the embryo based on days post-insemination (dpi) or days post-coitum (d*pc*). However the elongation process is rarely synchronised after fertilization and it appears highly advisable to combine criteria associating dpi, conceptus length (Degrelle *et al*. 2005) and developmental staging (Degrelle *et al*. 2011) to appropriately generate and interpret data related to genes driving the elongation and gastrulation processes (Hue *et al.* 2012). Indeed, as reported in pig, ovoid and filamentous conceptuses may simultaneously develop at a given d*pc* within the same cohort and exhibit gene expression differences related to their size (Blomberg *et al*. 2010). This matters as well in the understanding of the elongation and gastrulation processes, that most frequently appear coordinated after AI (Degrelle *et al.* 2012; Valour *et al.* submitted), but are too rarely evaluated in experimental situations including somatic cell nuclear transfer (SCNT; Betsha *et al*. 2013; Rodriguez-Alvarez *et al.* 2010) or in physiological conditions such as under-nutrition, low P4 circulating level, post-partum or immunological conditions such as post-partum subclinical infections. Indeed, the embryonic disk does not bear any amnion for a few days (day 14 to day 17 post-insemination) and, based on recent proteomic data on in vitro cultured mouse epiblast cells (Fröhlich *et al*. 2013), could also signal to the extra-embryonic tissues or the uterus. Last but not least correct staging of the conceptus also matters for the cross-talk with the endometrium when potential asynchronies occur e. g. when the conceptus lags so much behind the "uterus-tempo" that the conceptus either reflects troubleshooting in early uterine signalling (or "nursing") or signals itself inappropriately to the uterus, or both.

Among the sources of gene variation in embryos, the transcriptional differences resulting from different sex chromosome complements have been unveiled as early as the blastocyst stage (Bermejo-Alvarez et al. 2010). Based on a precise staging of the conceptuses, a high-throughput transcriptome analyse did not underline any subsequent sex-specific difference in a late pre-implantation stage of development (day 18 post-insemination; Degrelle *et al.* 2012) although a selection of candidate genes (*DNMT3A, IFNT2, GATA4, WT1*) showed a sex-specific transcriptional regulation at day 14 of pregnancy (Bermejo-Alvarez *et al*. 2011). Upon transfer of male or female *in vitro* produced embryos, an impact of the sexual dimorphism has been described in the uterine fluid of recipients after a 3 days *in vivo* development (Gomez *et al*. 2013). The authors have suggested that the uterine response induced by males, but not by females, would lead to a favorable uterine environment in accordance with sex selection mechanisms operating through males.

In term of contribution to pregnancy issue, the embryo transfer procedure allows to separate the effects of the oocyte and/or embryo quality from the impact of the genital tract. *In vitro* maturation, *in vitro* fertilization (IVF) and subsequent *in vitro* embryo culture have been reported to significantly alter gene expression patterns in blastocysts and elongating embryos when compared to their *in vivo* derived counterparts (Clemente *et al*. 2011; Gad *et al.* 2012; Marjani *et al*. 2009; Mermillod *et al*. 2010;). Although the rate of success is still very low, SCNT can lead to term-development of cloned embryos when correct nuclear reprogramming takes place (Heymann 2005; Niemann *et al*. 2008; Khan *et al*., 2012). Nevertheless, severe or fatal consequences on embryo and foetal-placental development have also been reported (Chavatte-Palmer et al. 2012) and shown to considerably vary according to the origin of somatic cell lines and across laboratories (Yang *et al*. 2007). These different types of embryos were postulated to elicit different endometrial gene patterns that could account for the final outcome of a pregnancy.

By comparing endometrial transcriptomes of cows recipient for IVF-derived embryos or SCNT embryos generated with various somatic cells lines, several endometrial genes have been shown to be associated with the nuclear transfer procedure at day 18 post-oestrus (Bauersachs *et al*. 2009). Interestingly, in the uterine lumen of pregnant heifers carrying SCNT versus IVF conceptuses, abundances of 10 amino acids (AA) were reduced. In this study, the mRNA level of the *SLC7A8* AA transporter solute carrier gene was also lower in the endometrium of SCNT conceptuses carrying recipients (Groebner *et al*. 2010). In these studies as in an other report (Mansouri-Attia *et al.* 2009b), the variation of gene expression in the endometrium was unlikely to be attributable to differences in IFNT production since the expression of this factor was found to be similar in SCNT and IVF pregnancies. The decreased supply of numerous AA and derivatives in SCNT conceptuses carrying females might account for the placental abnormalities that have been reported in later stages of pregnancy.

Our research group (Mansouri-Attia *et al*. 2009b) compared the impact of *in vivo* generated embryos on the endometrium to that of embryos generated by IVF-embryo transfer (IVF-ET) and SCNT. SCNT embryos were produced using a unique somatic cell line of fibroblast origin (5538). Reprogramming efficiency of the 5538 cell line was high and was characterised across various stages of embryo development and pregnancy until term (implantation rate: 80%; calving rate: 13%; Degrelle *et al.* 2012). Transcriptomes profiles by array revealed different patterns of gene expression between the 3 groups and thus a differential response of the endometrium depending on the developmental trajectory of the embryo (Mansouri-Attia *et al.* 2009b). Interestingly, the SCNT embryos introduced more marked changes in the endometrium than the IVF-ET embryos did, consistent with the more severe alterations of extra-embryonic gene patterns displayed by the SCNT conceptuses produced with this 5538 somatic cell line (Degrelle *et al*. 2012). Although the mechanistic bases of the epigenetic perturbations are unknown, these data are indicative of altered epigenetic programming of embryo and endometrium. Data mining showed immune response and metabolism as the two most affected biological functions and differential expression of candidate

genes was confirmed, including *C11ORF34*, *FABP3, FBP1, GJA1*, *MX2* and *SOCS3,* a major regulator of the JAK-STAT signalling pathway (Mansouri-Attia *et al.* 2009b; A. Vittorino-Carvalho *et al.* unpublished data). Our data have led us to propose the original concept defining the endometrium as an early biosensor of embryo quality (Mansouri-Attia *et al*. 2009b). Very interestingly, recent human data have proposed that decidualizing endometrial stromal cells can sense embryo quality and would be able to eliminate compromised embryos (Salker *et al*. 2010; Teklenburg *et al*. 2010). Therefore the endometrium as a biosensor of embryos displaying divergent developmental potencies appears to be a feature valid across mammals, despite structural differences in implantation and placentation.

Conclusion

In mammalian species, placentation and foetal development represent the major part of pregnancy and last much longer than the pre- and peri-implantation periods. Detrimental events taking place in the uterine environment beyond implantation have been shown to dramatically affect the outcome of pregnancy by altering placenta functions and foetus development (Burton *et al*. 2010). Nevertheless, current data demonstrate that congenital anomalies, acquired diseases or perturbations of adult maternal physiology before and during reproductive life (e. g. stress, nutrition; endocrine disruptors, infection) can affect endometrial function in a permanent or transient manner. Distinct endometrial responses can also be elicited by embryos presenting different post-implantation fates, making endometrium an early biosensor of embryo developmental potential, useful for the prediction of pregnancy issues (http://www.nature.com/stemcells/2009/0904/090430/full/stemcells.2009.67.html). Hence mammalian endometrium appears as a dynamic and reactive tissue whose compromised or suboptimal physiology can deeply or subtly affect embryo development before implantation with visible and sometimes severe consequences on placentation process, foetal development and pregnancy outcome. Consequently, although term pregnancy issues incontestably relies on the quality of the embryo (the seed; Patrizio *et al*. 2007), it is obvious that endometrium (the soil; Salamonsen *et al*. 2009) has to be considered as a critical epigenetic contributor for the embryo trajectory from the very earliest stages of pregnancy. Two major issues can emerge from a comprehensive investigation of the endometrium:

- The careful analysis of *in vivo* situations in which embryos can overcome the control exerted by the endometrium, as illustrated by the defect in nature's quality control proposed by Aplin and collaborators (Quenby *et al*. 2002). In other words, embryos that fail to develop to term would be able to implant. In this situation, the unusual properties of these embryos (e. g. producing an excess of embryo-derived signals) as well as the quality of the endometrial milieu will have to be clearly defined.

- The development of diagnosis and prognosis tools based on endometrial as well as systemic factors would be valuable to improving fertility and the proportion of term pregnancy in cattle. First insights have been provided that aim to predict pregnancy success based on transcriptional profiles established from the pre-conceptional endometrium and pre-implanting blastocysts (Salilew-Wondim *et al.* 2012**).** Very interestingly, if the altered endometrial quality can be determined, it paves the way for the design of compensatory strategies (e. g. by optimizing maternal nutrition; Al-Gubory *et al*. 2013) aiming to restore the compromised uterine function to a level compatible with term pregnancy and the birth of a healthy calf.

In conclusion, determining the limits of endometrial plasticity at the onset of pregnancy represents difficult tasks but essential challenges for providing new insights on the contribution of the maternal environment to embryo epigenetic shaping in link with success, alterations or failure of pregnancy in mammals, including farm species.

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SHORT COMMUNICATIONS

THE EFFECT OF OSMOTIC STRESS PRIOR TO VITRIFICATION ON THE METAPHASE II SPINDLE OF *IN VITRO* MATURED BOVINE OOCYTES

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It has been previously described that a simple treatment with medium containing elevated NaCl concentrations increases the cryotolerance and developmental competence of *in vitro*-matured porcine oocytes after vitrification and parthenogenetic activation (Lin *et al*. Reprod. Biomed. Online 18: 360-366. 2009). This work was designed to investigate whether the exposure to increased concentrations of NaCl prior to vitrification improved cryotolerance of *in vitro*-matured bovine oocytes. In Experiment 1, bovine *in vitro*-matured oocytes were exposed to seven NaCl concentrations (0% -control-, 0.25%, 0.50%, 1%, 1.50%, 2%, 3% and 4%), incubated for 1 h at 38.5°C and fixed to analyze microtubule and chromosome distributions. In Experiment 2 and according to the results obtained, oocytes were exposed to 375 mOsm NaCl (0.25%), allowed to recover for a further 1 h, vitrified, warmed and fixed to analyze microtubule and chromosome configuration. Data were analyzed using ANOVA. After exposure to 375 mOsmol NaCl, similar percentages of oocytes showing normal spindle patterns were obtained compared to control group (375 mOsmol NaCl: 83.4%; Control: 85.0%; $p>0.05$). Groups treated with higher (443-1517 mOsmol) NaCl concentrations triggered significantly lower proportions of normal spindles, showing higher percentages of oocytes with dispersed or decondensed chromosomes or microtubules. When oocytes were vitrified/warmed, no significant differences in terms of percentages of normal microtubule configuration were observed either after 1 h exposure (41.9%) or not (40.2%) to 375 mOsm NaCl, although both treatments differed significantly from control (79.1%). Furthermore, no significant differences were observed between non-vitrified oocytes and those treated with NaCl prior to vitrification/warming procedure when normal chromosome organization was analyzed ($p>0.05$). In conclusion, this study showed that a 375 mOsm NaCl pre-treatment of bovine oocytes previous to vitrification does not have a deleterious effect on the organization of the meiotic spindle of vitrified/warmed bovine oocytes. Further experiments are required to investigate if *in vitro*-matured oocytes subjected to this osmotic stress can improve their development competence after being vitrified/warmed.

Notes

APPLICATION OF POLYMERASE CHAIN REACTION FOR FETAL GENDER DETERMINATION USING CERVICAL MUCOUS SECRETIONS IN THE SHEEP

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In the current study we aimed to use PCR to investigate the presence of fetal DNA in the ovine cervical secretions and to assess the effectiveness of this method in fetal gender determination.

Pregnant uteri samples were collected from 20 sheep in a local abattoir. Overall, 13 male and 8 female fetuses were included in the study. Cervical mucus was sampled at the laboratory. After DNA extraction, the PCR amplified a 280 bp fragment from the X-chromosome and a 217 bp fragment from the Y –chromosome based on a sex-related polymorphism in the amelogenin locus. The presence of fetal Y-chromosome was confirmed in seven out of 13 cervical mucus samples collected from sheep with male fetuses. Overall test sensitivity for correct sex determination based on PCR assay on cervical samples was equal to 71.4 ± 2 %. In contrast, no fetal Y-chromosome DNA was detected in maternal serum samples from sheep with male fetuses.

This is the first report on validating the presence of fetal DNA material in the ovine cervical mucus and its potential usefulness for fetal sexing. Further investigations are needed to maximize the accuracy and evaluate the practicality of this approach.

Keywords : sheep . Cervical Mucus . Fetal sexing . PCR

Notes

OVULATION RATES AND SITES, EMBRYO YIELD OF THE PROLIFIC PUREBREED AND G1 ROMANOV DONOR EWES

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Multiple ovulation and embryo transfer (MOET) has the potential to increase the rate of genetic gain through the female line. However, the full realization of this potential depends upon maximizing the number of progeny born from high merit females. A large proportion of the variability in superovulation response, embryo development and quality was attributed to the donor animal. The superovulatory response, ovulation sites, embryo yields were studied in prolific purebred Romanov (R; n=7) and 3/4 Romanov x ¼ Native Turkish Breed (G1; n=7) ewes in breeding season. Estrous synchronization was carried out with the aid of intravaginal sponges containing 40mg fluorogeston acetate (FGA; Chronogest®, Intervet Laboratories, Boxmeer, Holland) for a period of 14 days. Donors were superovulated using FSH-p (total of 20 mg) (Folltropin-V; Vetrepharm, Canada) applied in eight decreasing doses of 2.4, 2.4, 1.8, 1.8, 1.6, 1.2, 1, 1 mg i.m. at 12 h intervals, starting 60 h before sponge withdrawal. Donors sedated with anesthetic cocktail containing 100 mg ketamine (Vetalar, Boehringer Ingelheim Vetmedica, Inc) and 0.12 mg xylazine (Romphun, Bayer) were undergone intrauterine insemination with fresh diluted semen (a minimum of 50 x 10^6 motile sperm/each uterine horn) 40h after sponge removal. The success of superovulation program was found similar in R (16.4 ± 2.54) and G1 (12.3 ± 3.89) donors. Ovulation site was similar between right and left ovaries in R (Right; 8.6 ± 1.45; Left; 7.6 ± 1.61) and G1 (Right; 5.7 ± 2.22; Left; 6.7±2.46) donors. Transferable embryos were found relatively but not significantly higher in R (9.3 ± 2.79) donors than G1 (5.7 ± 4.27) donors. Embryo yields were recorded 57% for R donors which were 11% higher than G1 donors. These results show that two times more recipients should be calculated for R donors than for G1 donors.

Notes

EFFECT OF OVIDUCTAL FLUID ON THE PORCINE *IN VITRO* FERTILIZATION WITH FROZEN-THAWED SPERM

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Fertilization is a physiological phenomenon that involves the binding and fusion of a capacitated spermatozoa with a mature oocyte to develop a zygote which will lead to a new diploid individual. It takes place in the oviduct, where the fluid secreted by the epithelial cells provides a suitable environment for the maintenance of the functions of the sperm until the oocyte arrives after ovulation. The effect of this fluid on the in vitro fertilization outcome could differ depending on the source of sperm used (fresh or frozen). We hypothesize that, in frozen samples, where there are changes at the sperm membrane level and the motility decreases due to the cryopreservation process, the fluid provides a protective effect. Our goal in this work was to perform porcine in vitro fertilization cycles (IVF) with frozen-thawed sperm in the presence and absence of oviductal fluid to compare the efficiency of the system.

The oocytes, matured in vitro and decumulated, were incubated in porcine oviductal fluid before incubation with boar frozen-thawed sperm (at 38.5°C and 5% CO_2 in air). At the time of IVF, 1% of fluid was added. A control group without oviductal fluid was used. After 22 hours, various parameters were evaluated (monospermy and penetration rates) with an epifluorescence light microscope, after fixing (1% glutaraldehyde) and staining (Hoechst 33342) of the potential zygotes.

In the presence of oviductal fluid, the penetration rate was similar to the control group (100 and 90.98±2.49, respectively), while the monospermy significantly decreased (23.97 ± 3.90a and 2.94 ± 1.68b, to $p<0.05$). These results show that there is an increase in the of number of sperm that are able to penetrate the oocytes in the presence of oviductal fluid due, probably, to the maintenance of the sperm vitality, acrosomal integrity and motility that the oviductal fluid offers (Abe et al., 1995; Boquest et al., 1999; Iman et al., 2008, 2010; Kumaresan et al., 2005, 2012). More studies are needed to find out what are the factors or proteins that produce that effect upon sperm.

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Notes

OXYGEN CONCENTRATION DURING IN VITRO MATURATION AFFECTS GLOBAL DNA METHYLATION IN IN VITRO PRODUCED BOVINE ZYGOTES

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In vitro maturation (IVM) of bovine COC has commonly been performed using an oxygen concentration which is around 20% (atmospheric oxygen concentration), whereas the oxygen concentration in follicular fluid ranges between 3-13%. Thus, the gaseous environment to which the oocyte is exposed varies considerably between in vitro and in vivo conditions.

Epigenetic modifications, including DNA methylation, regulate gene expression without alterations of the DNA sequence and thus play a crucial role in gametogenesis and early development. Oxygen concentration during the IVM culture period can contribute to the extent and velocity of oxidative stress by reactive oxygen species (ROS) which can be associated with various types of DNA damage, including hyper- and hypo-methylation of DNA.

Therefore, the aim of the present study was to investigate the effect of different oxygen tensions during in vitro maturation of bovine oocytes on global DNA methylation of in vitro produced zygotes.

Bovine zygotes were generated employing a standard protocol as described recently (Stinshoff et al. 2011). COC were matured under 5% or 20% oxygen tension. After IVF, presumptive zygotes were denuded and fixed for immunocytochemistry. In total, quantitative analysis of fluorescence signals was assessed in 1261 zygotes from 9 IVP sessions. Global DNA methylation was determined using an anti-5MeC antibody. The specificity of the DNA methylation antibody was confirmed by omitting the primary antibody and resulted in all cases in the lack of staining. DNA was stained with Hoechst 33342. The fluorescence signal of DNA was used for normalization of 5-methylcytosine fluorescence signal. Total fluorescence intensity of the female and male nuclei was measured by using NIH ImageJ software and data were analysed by ANOVA.

Normalized 5MeC signals were significantly higher in paternal compared to those of maternal pronuclei of zygotes stemming from oocytes matured under 5% oxygen. No differences were detectable in both parental pronuclei of zygotes from oocytes matured under 20% oxygen. Following IVM under 20% oxygen, maternal pronuclei of zygotes displayed a significant higher fluorescence signal compared to those stemming from oocytes matured under 5% oxygen. Maturation at 5% or 20% oxygen did not cause an effect on normalized fluorescence signals of paternal pronuclei.

Results indicate a reduction of the global DNA methylation level of the maternal genome in in vitro produced bovine zygotes derived from oocytes matured under 5% oxygen. These data suggest that the oxygen tension during IVM affects the global DNA methylation pattern in the female pronucleus, which may affect fertilization and further development.

Notes

DEVELOPMENT AND DISTRIBUTION OF PORCINE FETUSES AFTER LONG OR SHORT TIME PERIOVULATORY UNILATERAL OVARIECTOMY (ULO)

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Corpus luteum (CL) activity is closely linked with initiation and maintenance of pregnancy and with fetal development. Experimental intervention by unilateral ovariectomy (ULO) to reduce the number of CL has been used to study the impact of progesterone on fetal survival (Athorn et al. 2011). The present study was aimed to analyze the impact of unilateral ovarian withdrawal on fetal distribution and development. ULO was performed in gilts long time, i.e. 20 days before subsequent ovulation (group ULO-LT, n=16), short time, i.e. one day before ovulation (group ULO-ST, n=16) or ovaries remained intact (group INTACT, n=14). Gilts were inseminated by single fixed-time laparoscopic intrauterine insemination (LIUI). Animals were slaughtered on day 30 of gestation and the ovarian features were measured and the number, weight and distribution of fetuses recorded. Ovarian and fetal parameters are presented in Table 1.

Table 1. Mean (± SE) ovarian and fetal parameters of control and ULO gilts

PARAMETER	GROUP		
	ULO-LT	ULO-ST	INTACT-CONTROL
Pregnancy rate (%)	10/16 (62.5)	11/16(68.8)	10/14 (71.4)
No. CL	15.9 ± 1.3[a]	11.2 ± 1.1[b]	19.1 ± 1.0[a]
Total CL weight (g)	9.69 ± 0.92[a]	6.00 ± 0.43[b]	10.05 ± 0.68[a]
Weight per CL (g)	0.63 ± 0.05	0.57 ± 0.04	0.51 ± 0.04
No. Fetuses	14.2 ± 1.2[a]	8.7 ± 1.1[b]	15.3 ± 1.2[a]
CL-Weight per Fetus (g)	1.26 ± 0.53	0.75 ± 0.06	0.67 ± 0.04

[a,b] P<0.01

Differences (P<0.01; t-test) were obtained in the number of CL and of fetuses. Although the total CL weight was different (P<0.01) in ULO-LT and INTACT compared to ULO-ST gilts, the weight per CL and the CL weight per fetus did not differ.

Fetuses were balanced distributed in both uterine horns of INTACT gilts (7.3 and 8.0; SE±0.7). In gilts of the ULO-groups, the number of fetuses in the ipsilateral horn was higher compared to the contralateral one, i.e. without ovary (ULO-LT: 8.0 *vs*. 6.2; ULO-ST: 5.0 *vs*. 3.7; SE±0.7). Additionally, the number of fetuses in the respective uterine horns was different (P<0.01) between ULO-LT and ULO-ST gilts. However, only in the ULO-ST group the fetuses were different distributed between the ipsi- and contralateral uterine horns (59.9 *vs*. 40.1%; SE±0.9, P<0.05). The weight of fetuses in the ipsilateral horn of ULO-LT, ULO-ST and INTACT gilts was similar (1.45, 1.40, 1.41 g; SE±0.03); in the contralateral horn the respective weights were 1.48, 1.36 and 1.39 g (SE ± 0.03; ULO-LT *vs*. ULO-ST P<0.01). Embryo mortality was lower in ULO-LT (9±5%, P<0.05) compared to ULO-ST and INTACT gilts (22±4 and 20±3%).

In summary, the ovarian function and the number of fetuses were, compared to INTACT animals, compensated only in long time ULO gilts. Both, the total number of CL and of fetuses, and the distribution of fetuses in the uterine horns were affected by ULO immediately before ovulation. However, ULO did not influence CL and fetal weights. Therefore, the time window but not the ULO, which probably reduces the local supply of progesterone, has an impact on fetal development and survival.

Notes

SUPEROVULATION IN COWS SYNCHRONIZED WITH TWO DIFFERENT PROGESTERONE+OESTRADIOL PROTOCOLS

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A total of 26 Brown Swiss cows were used to compare the synchronization and superovulatory response of follicle stimulating hormone (FSH) treated cows that were synchronized with progesterone+oestradiol valerate or benzoate.

Control cows (n=8) were superstimulated with FSH (Folltropin-V, total: 400 mg NIH-FSH-P1, Bioniche Animal Health Inc., Ontario, CANADA) using twice daily injections with decreasing doses (4,4,3,3,2,2,1,1 ml) from day 10 to 13 after determined reference oestrus. Cows in treatment groups received either an ear implant (n=9) containing norgestomet+oestradiol valerate or progesterone releasing intravaginal device (PRID) (n=9) containing progesterone+oestradiol benzoate, at random stage of the oestrus cycle, for 9 days. Seven days after the implant and PRID insertion, FSH was injected as described in the Control group. Observation of oestrus was initiated following the last FSH injection and, all cows were artificially inseminated twice 12 and 24 h after the onset of oestrus. Superovulatory response was assessed by rectal palpation and ultrasonography, 7 d after the first insemination, with regard to the number of corpora lutea in the ovaries.

All cows used in this trial showed signs of oestrus within 24 h of the last FSH injection. There was no significant difference between the groups in any of the criterions that were evaluated (last FSH injection-oestrus intervals: 18.0 h, 16.67 h and 18.0 h; recovery rates: 70%, 69% and 84%; the mean numbers of corpora lutea: 8.4, 9.1 and 7.3; total ova and embryos: 5.9, 6.3 and 6.1; grade 1 embryos:2.9, 2.1 and 2.9; grade 2 embryos: 1.1, 1.4 and 1.6; grade 3 embryos: 0.6, 0.9 and 0.3; transferable embryos: 4.6, 4.4 and 4.8; degenerated embryos: 0.6, 1.4 and 0.8; unfertilized ova: 0.6, 0.4 and 0.6 in CONTROL, IMPLANT and PRID groups, respectively).

In conclusion, both of the progesterone+oestradiol treatments synchronized the oestrus cycle in FSH superstimulated cows in this study. Superstimulation started 7 days after synchronization of follicular wave emergence by progesterone+oestradiol valerate or benzoate treatment has resulted in comparable superovulatory response with conventional protocol which is started on d 10 after reference oestrus. That is, any of the progesterone+oestradiol valerate or benzoate protocols can be used for synchronization prior to superstimulation according to the results of this study. Findings of this research suggest that, exogenous control of oestrus cycle by using progesterone+oestradiol valerate or benzoate combination as an ear implant or PRID for 9 days, and gonadotrophin injection over a 4-d period initiated on the 7th day of the implant or PRID application, may offer the advantage of initiating superstimulatory treatments at a time that is optimal for follicle recruitment in Brown Swiss cows.

Notes

HEPATOMA-DERIVED GROWTH FACTOR (HDGF) PROMOTES BOVINE EMBRYO DEVELOPMENT AND QUALITY IN VITRO

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Media containing defined compounds will help normalize and lead to more efficient procedures for in vitro culture (IVC) of mammalian embryos. HDGF was identified in the uterine fluid (UF) on Day-8 as the first growth factor up-regulated in response to early cattle embryos (Muñoz et al, J Proteome Res, 2012, 11:751-66). In this work, recombinant (r) HDGF produced in a HEK293 transfected cell line, was tested during IVC by analyzing blastocyst development and differential cell counts. In vitro matured bovine slaughterhouse oocytes were fertilized and cultured in SOFaaci + 6 mg/mL BSA. On Day-6, morulae were selected and cultured in groups (N=20-30) in 50 µL drops of SOFaaci + 0.5 mg/mL polyvinyl-alcohol (PVA) under mineral oil. rHDGF was added at concentrations of 0, 1 and 100 ng/mL (5 replicates). Data were analyzed by GLM procedure and REGWQ test. Results were recorded on Day-7 and Day-8. Blastocyst development rates were not altered on Day-7. In contrast, rHDGF at 100 ng/mL increased Day-8 blastocysts and expanded blastocyst rates vs. 0 ng/mL (70.9±3.4 and 54.2±3.0 vs. 54.7±3.3 and 39.0±2.9, respectively; $P<0.05$), while 1 ng/mL rHDGF had no effect. Trophectoderm (TE) cells increased in response to 100 ng/mL HDGF (142.1±6.2 vs. 117.3±5.7 and 120.7±6.2, for 1 and 0 ng/mL, respectively); no changes were recorded in the inner cell mass (ICM) and ICM/Total cells ratio. In a second experiment, Day-6 morulae were individually cultured in droplets of SOFaaci containing PVA (0.5 mg/mL) or BSA (6 mg/mL), each with 0 or 100 ng/mL rHDGF in 7 replicates (20 embryos/group). On Day-7, addition of rHDGF to SOFaaci containing PVA improved % blastocysts (48.6±3.9 vs. 33.6±3.9; $P=0.03$). However, in the presence of BSA, rHDGF had not effect. Differences in development recorded on Day-7 were suppressed on Day-8. Within cell counts, 100 ng/mL rHDGF tended to increase ($P=0.07$) values in the ICM, both with PVA (21.1±2.4 vs. 15.4±2.7) and BSA (21.5±2.3 vs.17.2±2.9). A similar trend was also observed in the TE cells for rHDGF with PVA (128.6±7.0) vs. PVA alone (108.5±7.8), BSA with rHDGF (104.3±6.8) and BSA alone (106.9±8.7). These changes in the ICM and TE led embryos cultured in PVA+rHDGF to contain more total cells ($P<0.01$) than the other three groups without significant alteration in the ICM/TE ratio (not shown).

rHDGF improves embryo development and quality in defined conditions (i.e. PVA) both in single and group culture. However, the same dose of rHDGF (i.e., 100 ng/mL) has no effect in the presence of BSA in single culture. As BSA shows binding properties different to PVA, probably a specific dose-response assay for rHDGF in culture with BSA is required. Our study demonstrates that identification of compounds that overexpress in the bovine UF in response to developing embryos is a useful strategy to improve in vitro embryo culture media.

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Notes

EFFECT OF THE YEAR SEASON ON *IN VITRO* FERTILIZATION OF PREPUBERTAL GOATS USING FRESH SEMEN.

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In our laboratory we performed in vitro fertilization (IVF) of prepubertal goats for several years. During these years we have noticed that the embryo production rates are different according with the season year. The aim of this study was to compare outcome IVF using fresh semen in the 4 year seasons: autumn (21 September to 20 December), winter (21 December to 20 March), spring (21 March to 20 June) and summer (21 June to 20 September).

Ovaries from 2 months old goats were obtained from a local abattoir and transported to laboratory in sterile dulbecco's (PBS) held at 34–37 8C. COCs were recovered by slicing the surface of the ovary in Hepes-buffered TCM-199 medium with 0.5 g/L BSA. Oocytes with two or more complete layers of compact cumulus cells and homogeneous cytoplasm were used for in vitro maturation (IVM). Groups of 25-30 COCs were in vitro matured in 100 µL microdroplets of conventional TCM199 medium with hormones and foetal bovine serum (FBS) for 24 h at 38.5ºC in a humidified air atmosphere with 5%. Subsequently 15-20 matured oocytes were transferred to 100 µL microdrops of modified Tyrode's medium (TALP) and co-cultured with fresh goat sperm (4×10^6 motile sperm/mL) at 38.5°C and 5% CO_2 in humidified air. At 18 h post-insemination (pi), groups of 10–15 presumptive zygotes were placed into 10 µL droplets of synthetic oviductal fluid medium (SOF) supplemented with 10% FBS under mineral oil in a humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2 for 8 days. Cleavages rates were evaluated at 48 h pi, and blastocyst rates were recorded at day 8 *pi* (Table 1).

Table 1 Blastocyst production in different season of prepubertal goat oocytes at day 8 post insemination.

season	months	Total Oocytes	CVL	CLV(%)	BL	BL/n(%):	BL/CLV(%):
summer	21/6-20/9	115	89	77^{a}	15	$(13\%)^{a}$	$(17\%)^{a}$
autumn	21/9–20/12	123	46	37^{b}	3	$(2\%)^{b}$	$(7\%)^{a}$
Winter	21/12-20/3	99	51	51^{c}	17	$(17\%)^{a,c}$	$(33\%)^{b}$
Spring	21/3-20/6	62	49	79^{a}	16	$(25\%)^{c}$	$(33\%)^{b}$

CVL: cleavage; CVL (%) percentage of cleaved oocytes; BL: blastocyst; BL/n (%): percentage blastocyst/cleavage embryos; BL/CLV (%): percentage blastocyst/cleavage embryos. Different letters (^{a,b}) are significantly different (χ^2, PROC FREQ SAS9.52; $P<0.05$).

Results in table 1 show a significant difference on the in vitro production of blastocysts depending on the season year IVF was performed. The blastocyst production is significantly increased in the increasing photoperiod while in the decreasing photoperiod the blastocyst production is diminished.

In our laboratory we have been working with prepubertal animals but using frozen semen in both sheep (Catala et al., Reproduction 2011: 142(4)517) and cows (Morato et al Reprod. Fertil. & Dev. 2010: 22(7)1141) and we have not observed this difference in the production of blastocysts as those presented in this work. We hypothesize that fresh semen is the one being affected by seasonality increasing or reducing *in vitro* results.

Notes

CULTURE OF BOVINE EMBYOS PER DONOR USING THE CORRAL DISH DOES NOT IMPROVE BLASTOCYST DEVELOPMENT

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Since embryo identity plays an important role during commercial in vitro embryo production, bovine oocytes and embryos have to be cultured strictly per donor. Due to the rather low yield of oocytes per individual cow, culture takes place in small groups, which is often associated with an inferior embryo development. Because of the large variation between donors in blastocyst formation, we wanted to evaluate the effect of small embryo numbers cultured per donor in the Corral dish. The central wells of the Corral dish are divided into four quadrants by a semi-permeable wall, allowing embryos from each donor cow to stay together in one quadrant while medium and autocrine growth factors can diffuse and influence embryos of the other three donors.

Bovine ovaries (4 replicates) were collected at the slaughterhouse. Oocytes were matured in three groups: from sixteen individual cows, ten oocytes were matured grouped per cow. Oocytes from different cows were matured either in groups of ten or in groups of 60. The oocytes were matured in 500 µl TCM-199 supplemented with 20 ng/ml epidermal growth factor (EGF). After in vitro fertilization, zygotes were further cultured in synthetic oviductal fluid (SOF) supplemented with 0.4% bovine serum albumin (BSA), 5 µg/ml insulin, 5 µg/ml transferrin and 5ng/ml selenium (ITS). From oocytes matured in groups of ten, only eight presumed zygotes were transferred to culture medium, from the large group (60) two groups of 25 zygotes were made. From the individual cows, 8 zygotes were allocated to either a 30 µl droplet of medium (IND8) or to a quadrant of the Corral dish (COR8) (4x8/120 µl). The mixed group containing eight randomized zygotes was cultured in 30 µl medium (MIX8). The groups with 25 randomized zygotes were cultured in 50 µl medium (GR). The three groups (IND8, COR8, MIX8) were compared with the control, group culture (GR). Blastocyst development was evaluated at 8dpi. Subsequently blastocysts were fixed for a Hoechst staining to evaluate the total cell number (TCN). Developmental data were analyzed using a binary logistic regression model and data concerning the blastocyst quality using a linear mixed model analysis.

At 8dpi, a significant difference was found concerning blastocyst development for the embryos cultured per individual cow compared to the control group (GR: 40.8%; IND8: 24.8%, COR8: 26.9%). The control group had the tendency to produce more blastocysts compared to the MIX8 group (32.0%) (p=0.053). No differences were found in the TCN of the blastocysts collected at day 8 for the embryos cultured in small groups compared with the group culture.

In this study, we demonstrated that the use of the Corral dish for culturing small embryo numbers per individual cow could not raise the blastocyst yield compared to culture in individual droplets. A difference in embryo density (GR: 1/2 vs Corral: (4x8)/120 (1/4) can be a possible explanation for the lower blastocyst development. Nevertheless the blastocysts obtained in the small groups were of similar quality when compared to those obtained in the group culture.

Notes

BOVINE IN VITRO PRODUCED EMBRYOS INDUCE MODIFICATIONS OF GENE EXPRESSION PROFILE OF BOVINE OVIDUCT EPITHELIAL CELLS *IN VITRO*

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In vivo, the oviduct provides the optimal environment to allow the successful development of the early mammalian embryo. A molecular dialogue may take place between the embryo and its maternal milieu, modulating the embryo microenvironment during its migration towards the implantation site. *In vitro* co-culture with bovine oviduct epithelial cells (BOEC) has been widely used to mimic this maternal environment and to study its effect on embryo development. The exact mechanisms of BOEC action on embryo development have not been fully elucidated yet. Therefore, the purpose of this research was to evaluate the BOEC *in vitro* responsiveness to embryos, according to the regional origin of the oviduct cells (isthmus vs. ampulla). Oviducts ipsilateral to ovaries with sign of recent ovulation were brought to the laboratory. Exp. 1: Ampulla and isthmus regions were dissected, washed thoroughly in TCM199 and epithelial cells were scrapped out using a sterile slide. BOEC from Ampulla (A-BOEC) or Isthmus (I-BOEC) were seeded separately in 4 well NUNC plates and cultured to confluence (7 days) to be used for *in vitro* embryo development (IVD). Immature cumulus oocyte complexes were aspirated from slaughterhouse ovaries. Zygotes produced by *in vitro* maturation and fertilization were cultured in SOF medium supplemented with 10 % FCS in the presence of A-BOEC, I-BOEC or without cells (control). Some A- and I-BOEC wells were cultured without embryos. At Day 8 p.i., RNA were extracted (Trizol). Exp. 2) Confluent BOEC in 4 well NUNC plates were stimulated by synthetic INFtau (0, 1, 10 and 100 ng/mL) for 6 or 24 hours and RNA were extracted (Trizol). All RNA samples were treated with DNAse and RT was performed (MMLV RT kit). The level of expression of some known oviduct expressed genes (GPX4, C3, OVGP), as well as some genes related to IFN signaling (STAT1, IFIT5, ISG15, OAS1, IFITM1, MX1, OAS1, USP18), were evaluated by RT-qPCR. Data were analyzed by Mann Whitney non parametric test using PRISM 5 software. The relative abundance of all IFNt related genes was significantly upregulated when epithelial cells (either A-BOEC or I-BOEC) were exposed to embryos during 8 days *in vitro*. The IFNt stimulation reproduced this embryo effect, whatever the concentration and duration of treatment. Furthermore, a regional difference in expression level was found for OVGP (higher in I-BOEC, $p<0.05$) and C3 (higher in A-BOEC, $p<0.05$), without effect of the presence of embryos. GPX4 mRNA abundance was not significantly different among the culture conditions tested. In conclusion, these data confirm the specialization of oviduct regions and show the ability of oviduct cells to respond to embryo signaling, at least through IFN-like pathway in our *in vitro* model, by modulating gene expression profile, thus supporting the existence of a real dialog between early embryo and oviduct.

Notes

EFFECT OF RETINOIC ACID ON IN VITRO MATURATION OF OVINE IMMATURE OOCYTES CONTAINING CUMULUS CELLS

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Abstract

This study was conducted to investigate the effect of all-trans Retinoic Acid (t-RA) on in vitro maturation of ovine immature oocytes containing cumulus cells. The Oocytes with cumulus cells were recovered from follicles (2-6 mm in diameter). Different concentrations of t-RA (0, 1, 1.5 and 2 µmol) were added in the maturation medium. For oocyte maturation, all groups were located in the CO2 incubator for 24 hours. The meiosis and nuclear maturation status of the oocytes in each experimental group were assessed using an invert microscope and the data was analyzed with GLM procedure. There were not any oocytes in GV stage. The percentage of oocytes in GVBD stage at control group in comparison with 1 µmol and 2 µmol treatments was significant ($P<0.05$). The rate of oocyte maturation in 2 µmol retinoic acid treatments was significantly higher than other in groups ($P<0.05$). In conclusion, the use of 2 µmol Retinoic Acid increased the ovine oocyte maturation significantly in the commercial TCM199 medium.

Keywords: In vitro maturation, Retinoic Acid, GV, GVBD, Ovine oocyte

Notes

SURVIVAL TO CRYOPRESERVATION OF BOVINE BLASTOCYSTS THAT DIFFER IN DEVELOPMENT KINETICS AT EARLY CLEAVAGE STAGES

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Selecting embryos with good developmental competence is essential for pregnancy success after embryo transfer both as fresh and after cryopreservation. Embryos that cleave early have classically been associated to increased performance. In this work we analyzed in vitro development and cryoresistence of blastocysts as derived from development stages found on Day-2 (D2) of culture (Day-0: IVF onset).

Blastocysts were produced *in vitro* as reported (Trigal *et al*., 2012 Theriogenology 10.1016/j.theriogenology.2012.06.018). On D2, embryos were separately cultured by development stages as follows: 1) not cleaved, 2) 2-cells; 3) 3-4 cells and 4) >5 cells. Excellent and good quality expanded blastocysts were frozen (n=199) or vitrified (n=186) in 6 replicates. Freezing/thawing and vitrification/warming procedures (fibreplugs) were previously described (Trigal *et al.*, 2013 Reprod. Fert. Dev. 25(1):189; and Trigal *et al*., 2013 Reprod. Dom. Anim. 2013 (48):200-6), respectively). Thawed and warmed embryos were subsequently cultured in mSOFaaci + 6 g/L BSA + 10% FCS for 48 h. Re-expansion (RE) (at 2, 24 and 48h) and hatching rates (HR) (at 24 and 48h) were recorded. Blastocysts that hatched at 24 and 48 h were subjected to differential cell counts (Thouas *et al.,* 2001 RBMOnline 3:25-29). Data were analyzed by GLM and REGWQ test for means, and are shown as LSM±SE.

Embryos that had one cell on D2 did not produce blastocysts, while embryos with 3-4 cells and >5 cells produced similar Day-8 blastocyst rates (43.54±1.9 and 52.87±2.2; respectively). These rates were higher ($p<0.001$) than those from 2 cell-stage embryos on D2 (8.21±2.3; $p<0.001$).

Two hours after cryopreservation, RE rates were similar between vitrified and frozen embryos (data not shown). However, at 24h, HR of vitrified blastocysts derived from 3-4 cell-embryos on D2 tended to be higher than HR of frozen blastocysts that were >5 cell-embryos on D2 (37.56±4.7 *vs* 16.98±5.4, respectively; $p=0.09$). At 48h, HR after vitrification were higher than after freezing (data not shown); interestingly, after vitrification, blastocysts obtained from 3-4 cell-embryos had HR (82.64±6.9) higher ($p<0.01$) than those that developed faster (>5 cells) (62.44±7.9). Differential staining showed that vitrified embryos had more trophectoderm and total cells than frozen embryos, with no changes in the inner cell mass. These results show that, interestingly, a faster embryo development is not necessarily related to improved survival to cryopreservation, which can condition decisions to either transfer as fresh or to cryopreserve embryos.

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Notes

DAILY SUPPLEMENTATION WITH GHRELIN IMPROVES IN VITRO BOVINE BLASTOCYSTS FORMATION RATE AND ALTERS GENE EXPRESSION RELATED TO EMBRYO QUALITY

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Ghrelin is a gastric peptide having regulatory role in the reproductive system functionality acting mainly at central level. As the expression of ghrelin system (ghrelin and its receptor) has been detected in the bovine ovary, the objectives of the present study were to investigate whether ghrelin can affect the developmental potential of in vitro produced embryos, and to test their quality in terms of relative abundance of mRNA of various genes related to metabolism, apoptosis and oxidation.

Presumptive zygotes were produced by in vitro maturation and fertilization (day 0) of oocytes derived from abattoir material. In the first experiment zygotes were cultured in the absence (C control) and in the presence of three different concentrations of acylated bovine ghrelin (200 pg/ml, Ghr200; 800pg/ml, Ghr800; and 2000pg/ml, Ghr2000), and blastocysts formation rate was examined on days 7, 8 and 9. In the second experiment only the 800pg/ml dose of ghrelin was used. Zygotes were in vitro produced as in experiment 1 and 24 hours post insemination (hpi) they were divided into 4 groups; in two groups (control C, without ghrelin; Ghr800 with inclusion of ghrelin) embryos were culture without medium replacement, and in the remaining two groups (Control N and GhrN) the culture medium was daily renewed. From each group, a pool of day 7 blastocysts were snap frozen for relative mRNA abundance of various genes related to metabolism, oxidation implantation and apoptosis (*IGF2R, GPX1, MNSOD, SLC2A1, G6PD, DNMT3A*).

In experiment 1 no differences were detected between C, Ghr200 and Ghr2000, while significantly fewer blastocysts were produced in the Ghr800 in comparison to C. In experiment 2 the lowest blastocysts yield was found in Ghr800, while daily renewal of ghrelin (Ghr800) resulted in an increased blastocyst formation rate, which on day 7 was the highest among groups ($p<0.05$). Significant differences were detected in various relative mRNA abundance, giving an overall final notion that embryos produced in the presence of ghrelin are of better quality compared to the control group.

Our results imply for a specific role of ghrelin in early embryonic development; its role at periods of reduced fertility such as negative energy balance of cattle or metabolic syndromes in humans should be further investigated.

Notes

IMMUNOLOCALIZATION OF HA-SYNTHASES AND HYALURONIDASES IN CAMEL OVIDUCT

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Hyaloronan (HA) is present in the extracellular matrix of the expanded COCs after maturation, and is also present in the oviductal and uterine fluids. HA is synthesised by HA synthases (HASs). The most abundant HASs are HAS2 which produce HA of largest size (>2x10^6Da) and HAS3 which produce smaller HA (0.1-1x10^6Da). HA binds to its membrane receptors CD44. Membrane bound HA can be depolymerised by Hyaluronidase-2 (HYAL2) into 25,000 Da fragments which can be internalised for further degradation by lysosomal HYAL1. The biological functions of HA are determined by its molecular weight, therefore the differential expression of HASs and HYALs defines the role of HA in different organs and tissue. The aim of this study was to determine the protein expression of HASs and HYALs in the isthmus and ampulla of the camel oviduct. Oviducts were collected immediately after slaughter and fixed in formalin. Paraffin embedded sections were immune-stained by using antibodies specific to HAS2, HAS3, CD44, HYAL1 and HYAL2. Luminal epithelium (LE) of the ampulla and isthmus showed positive staining for HAS2, HAS3, CD44 and HYAL2, while HYAL1 was faintly detected. The expression of HAS3 in the LE of the isthmus was high in contrast to the ampulla where it was weakly stained. In contrast, HAS2 expression was higher in the peripheral LE folds adjacent to the muscles in the ampulla compared to secondary folds located more central and to the isthmus. CD44 and HYAL2 were equally expressed in the LE of both ampulla and isthmus. These results suggest that HAS2-produced larger HA is required in the ampulla probably for the process of capacitation and fertilization whereas smaller HA produced by HAS3 might be required to facilitate sperm transport to the site of fertilization and for later stage of embryo development. Since HYAL2 was expressed in both regions this suggest a continuous turnover of HA that may be required to support early embryo development.

Notes

EFFECT OF LASER IRRADIATION ON *IN VITRO* MATURATION RATE AND GENE EXPRESSION OF BUFFALO OOCYTES

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Although IVF techniques in buffalo have been established since decades, maturation rate is still low compared to other species. Several studies have been done to improve maturation rate using different types of media and/or different incubation times. The objective of this study was to develop more sensitive and less costly laser irradiation technique to improve maturation rate of buffalo oocyte with studying the laser effects at molecular level. Cumulus oocytes complexes (COCs) were recovered from ovaries by aspiration and good grade were allocated into six different groups (each has three biological replicates) with a total number 75 oocyte/group. A green light of wavelength 532 nm from a Diode Pumped Solid State (DPSS) laser [LSR-PS-II] with a total output power of 1 mW has been used to irradiate oocytes with irradiation spot area of 1.5 cm^2. Oocyte groups were exposed to laser light for 0 (control), 2, 4, 6, 8 and 10 minutes with irradiance of $6.5x10^{-4}$ W/cm^2. Afterwards, oocytes were matured in TCM-199 medium at 38.5 ^oC and 5% CO_2 in humidified air for 24h. Maturation rate was calculated based on excursion of the first polar body and statistically analyzed by X^2-test.

Total RNA was isolated from mature oocytes using PicoPureTM RNA isolation kit, and then converted into cDNA via reverse transcription kit. Quantitative Real-Time PCR was performed using specific primers for a set of selected candidate genes regulating cell cycle (CCNB1, PTTG), polyamine biosynthetic (ODC1), signal transduction and activation of transcription (STAT3), transcription factor activity (OCT4), growth and differentiation factor (BMP4), protein binding (KRT8), ATP binding (ERK, DNAJC5) and C-8 sterol isomerase activity (SREBF2). Maturation rate showed an increased pattern with time of exposure starting from 2 min until 8 min and decreased at 10 min. Oocyte group exposed to laser beam for 8 minutes significantly represents the highest maturation rate (59,3%, $p<0.05$) compared to control group (33.4%). However, other groups showed no significant differences. The same trend was found in gene expression analysis, where quantitative real-time PCR results showed a higher expression of all the selected genes in treated group compared to the control one. These results indicate the possibility of adopting laser irradiation as an easy and straight forward technique for improving *in vitro* maturation of buffalo oocytes.

Notes

EVALUATION OF SPERM SUBPOPULATION STRUCTURE IN RELATION TO *IN VITRO* SPERM-OOCYTE INTERACTION AND FIELD FERTILITY OF FROZEN-THAWED SEMEN FROM HOLSTEIN BULLS

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An experiment was designed to study the interaction between frozen-thawed bull sperm with different motile sperm subpopulations structure on sperm-oocyte binding ability and subsequent fertility after artificial insemination. In a first experiment, fifteen ejaculates from 5 separate bulls were collected and cryopreserved through a standard protocol. Post-thaw sperm motility was analyzed by using a CASA system and evaluated to identify sperm subpopulations as described by Muiño et al. (Ani. Repr. Sci. 109; 27-39, 2008). Adhesion to zona pellucida of post-thawed samples with low, medium and high percentages of sperm included in the subpopulation with the fastest and most progressive subpopulation (SP4) were tested through a zona binding assay (ZBA). For this purpose, in vitro maturated cow oocytes were denuded and transferred in groups of 10 to a 45µL drop of fertilization medium. A 5µL aliquot of sperm suspension ($10x10^6$ spz/mL) was added to each drop. After 20 hpi, the oocytes were fixed and stained with DAPI. The number of sperm bound to the ZP was counted. In a second experiment, six sperm frozen/thawed samples for two different Holstein bulls containing high, medium or low rates of SP4 were used to the assess pregnancy rates after artificial insemination. Pregnancy was confirmed by transrectal ultrasonography at day 45 after insemination. The statistical analysis was performed using the SPSS Statistics program, differences between means were analyzed by Tukey's test and correlations by Spearman rank correlation.

The mean number of spermatozoa bound to the ZP ranged between 2.1 to 14.7. A significant difference ($p < 0.05$) was found between the groups with high and low rates of sperm from SP4, except for one of the five bulls, in which no difference was found. A significant correlation ($r = 0.79$, $p < 0.01$) was found between the ZBA and the SP4. Furthermore, a significant correlation between this sperm subpopulation and *in vivo* fertility ($r=0.89$, $p < 0.05$) was also observed. So, results suggest that this specific high motile and progressive subpopulation is positively and significantly correlated with the ability of a thawed bull semen sample in binding to the ZP and with *in vivo* bull fertility.

Notes

SUPEROVULATION AND EMBRYO RECOVERY BY TRANSCERVICAL TECHNIQUE IN TOGGENBURG GOATS DURING SEASONAL ANESTRUS

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Superovulation is a less predictable event in the production of embryos. Its success is dependent upon the stage of follicular development in the ovary at the start of the administration of FSH. The aim of this study was to evaluate two estrus synchronization protocols based on superovulation programs in Toggenburg goats during seasonal anestrus. Twenty-eight goats were randomly assigned in 2x2 experimental groups, consisting in two protocols of estrus synchronization with 6 (G6) or 17 (G17) days of intravaginal device permanence (CIDR®, Pfizer Animal Health, São Paulo, Brazil) and two sources of FSH including 133mg (A; Folltropim®, Bioniche, Canada) or 250UI (B; Pluset®, Hertape Calier) both diluted into 20mL saline solution and administered in six decreasing doses (5-5-3-3-2-2 mL) 12 h apart. G6 goats received 37.5µg d-cloprostenol (Prolise®, Tecnopec Ltda, São Paulo, Brazil) at device insertion. The same dose was also given to animals 12h before the first dose of FSH in G17 goats and simultaneously to the fifth FSH dose in G6 animals. All animals were natural mated at 12h interval and received three doses of 1.5 mL flunixin-meglumine (Banamine®, Sheringh Plough, São Paulo, Brazil) 84h after the onset of estrus. Embryos were collected 6 to 7 days after the onset of estrus by nonsurgical transcervical technique. Estrous response was observed in 100% (7/7) G6-A, 71.4% (5/7) G6-B, 57.2% (4/7) G17-A, and 100% (7/7) G17-B goats. Embryo collection was done in all goats (82.1% - 23/28) that showed estrus. The total number of structures, viable embryos, un-fertilized oocytes and degenerated structures were 5.7±5.79, 3.7±3.8, 1.7±2.4 and 0.3±0.5 for G6-A; 6.5±4.6, 0.5±1.0, 5.2±5.1 and 0.7±1.5 for G6-B, 3.2±3.0, 1.0±1.1, 0.0±0.0 and 2.2±3.3 for G17-A; and 4.2±6.2, 1.0±2.0, 0.0±0.0 and 3.2±6.5 for G17-B animals, respectively. Both protocols need to be adjusted to superovulate Toggenburg goats. The choice of the protocol must consider other parameters such as the FSH dose and the ovarian follicular status at the beginning of the FSH treatment.

Keyword: Follicular dynamics, ovulation, superovulation and goats.
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Notes

MELATONIN IMPROVES BLASTOCYST RATE AND AFFECTS EXPRESSION OF EMBRYO QUALITY AND ANTIOXIDANT RELATED GENES IN FRESH AND VITRIFIED RABBIT EMBRYOS

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Melatonin plays a fundamental role as an antioxidant, it can directly scavenge hydroxyl free radicals or stimulate the activities of enzymes involved in antioxidative defense. In the present study, we investigated the protective effect of melatonin on blastocyst rate and its influences on expression of embryo quality and antioxidant related genes in fresh and vitrified rabbit embryos.

Twenty five nulliparous does from local (Egyptian) colored rabbit breeds were synchronized, artificially inseminated (AI) and immediately induced for ovulation. Does were slaughtered 72 hr after AI and reproductive tracts were flushed out. A total of 183 normal embryos (Morula stage) were recovered and cultured in vitro in TCM199 media supplemented with 0 (Cont) or 10^{-3} M of melatonin (Mel) for 2 hr. Afterwards, embryos of each group were either transferred to fresh culture media for 48 hr and collected as fresh blastocysts (ContF and MelF groups) or directly vitrified, thawed and cultured in vitro for 48 hr until blastocyst stage (ContV and MelV groups). Blastocyst rate was calculated as a percentage from cultured embryos and statistically analyzed using X^2-test. Expressions of 6 different transcripts (GJA1, Oct4, Nanog, NEF2L2, SOD1, and GPX1) were analyzed using real-time PCR and fold changes were calculated using ΔΔCt method.

Compared with control groups, melatonin supplementation significantly improved blastocyst rate in both fresh (MelF: 93% *vs.* ContF: 82%) and vitrified embryos (MelV: 95% *vs.* ContV: 75%). Gene expression analysis revealed that supplementation of culture media with melatonin up-regulate the expression of genes related to embryonic quality (GJA1, Oct4, Nanog) and antioxidant response genes (NEF2L2, SOD1, GPX1) either in fresh or vitrified embryos, compared to its corresponding control groups. These results indicated that melatonin, as an antioxidant, improves embryonic development and quality of embryos by increasing the expression of antioxidant related genes which can protect embryonic cells from impaired culture conditions.

Notes

INCUBATION WITH LENTIVIRUS DID NOT AFFECT BULL SPERM FUNCTIONALITY

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Transgenic domestic animals would be of high relevance for biomedical and agricultural research (Gadea & Garcia-Vazquez ITEA 2010). However up to now the efficiency and safety of the techniques is not good enough for a wide use of transgenic farm animals. In one hand, lentiviruses have been used for the generation of transgenic farm animals mainly by the injection in the perivitelline space of early zygotes or oocytes (reviewed by Pfeifer Transgenic Research 2004). On the other hand Sperm Mediated Gene Transfer (SMGT) has been used with different performances (García-Vazquez et al. Reproduction 2010). An alternative methodology could be the use of lentivirus to infect spermatozoa that later could be applied by AI or other assisted reproductive techniques as recently reported (Zhang et al, PlosOne 2012). In this way the first objective is to establish the experimental conditions to optimize the infection of the spermatozoa with the lentivirus and minimize the possible deleterious effect on the sperm functionality.

In this study we incubated the frozen-thawed bull spermatozoa in a sperm Talp media (Parrish et al., Biol Reprod 1988) in presence or absence 10^6 viral particles/mL of lentivirus (MISSION TurboGFP Control Transduction Particles, Catalog Number SHC003V, Sigma) that are produced from the lentiviral backbone vector, pLKO.1-puro, containing a gene encoding TurboGFP, driven by the CMV promoter. After incubation at 37ºC, sperm functionality were evaluated at 0, 30, 60 and 90 min of incubation in terms of motility and motion parameters measured by CASA (ISAS, Proiser, Valencia, Spain) and simultaneously viability and acrosome status by flow cytometry. Viability and acrosome status were evaluated by flow cytometry after staining with Propidium Iodide and PSA-FITC lectin (Gadea et al., Int J Androl. 2008) at 0, 30, 60, 90 and 180 min. No differences were found for the parameters evaluated among experimental groups (control vs. lentivirus). Viability, acrosome status, motility and motion parameters were affected by time of incubation.Later in vitro penetration capacity was evaluated in an IVF system, spermatozoa incubated in presence of lentivirus showed a similar penetration rate (control=58.3 vs. lentivirus=50,9%, $p>0.05$) and number of sperm per penetrated oocyte (control=1.05 vs. lentivirus=1, $p>0.05$).

In summary, we can conclude that the title of lentivirus used in the present experiment has not effect on sperm functionality. So, these samples could be used to be applied in different reproductive processes (AI, IVF, ICSI) to attempt to produce transgenic embryos or animals.

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Notes

EFFECT OF DIFFERENT DOSE OF PROLACTIN ADMINISTERED *PER OS* ON THE VIABILITY AND BODY WEIGHT OF PIGLETS

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Prolactin (PRL) is a protein hormone synthesized in and secreted predominantly by lactotroph cells of the anterior pituitary gland. This hormone has been found to stimulate the immune system in animals. Because prolactin secreted in milk by the mother's body is often insufficient, the administration of exogenous prolactin may significantly contribute to improving the health and growth of piglets. The aim of the study was to determine the dose prolactin administered *per os* to newborn piglets to improve their health and growth rate. The study was carried out at the Experimental Station of the National Research Institute of Animal Production in Żerniki Wielkie. The prolactin ("Biolactin", Biochefa, Poland) was isolated from freeze-dried pig hypophyses. The preparation was administered *per os* to newborn piglets at a dose of 0.1 (group exp. 1; n=660), 0.5 (group exp. 2; n=686) and 1.0 mg (group exp. 3; n=555) prolactin/kg body weight. Piglets in the control group 1 (n=304), 2 (n=252) and 3 (n=243) received *per os* NaCl physiological saline at a dose 0.1, 0.5 and 1.0 ml/ kg body weight, respectively. The number of piglets born alive, litter weight at birth and on days 21, 28 (weaning) and 70, as well as piglet mortality to 21, 28 and 70 days of age were recorded. The results were analysed statistically with the T and the Chi-square tests. The results are presented in Table 1.

Table 1. Mean body weight of piglets, mean increments and percentage of mortality following administration of 0.1 (exp.1), 0.5 (exp. 2) and 1.0 mg (exp. 3) PRL/ kg body weight

Group	Mean body weight (kg)				Mean increments (g)			Mortality (%)		
	at birth	21 day	28 day	70 day	0-21 day	0-28 day	0-70 day	0-21 day	0-28 day	0-70 day
Exp. 1	1.42	5.31	6.70	23.02	188	192	312	10.5	10.5	12.6[g]
Contr. 1	1.41	5.22	6.61	23.36	184	187	315	13.2	15.5	17.8[h]
Exp. 2	1.44	5.38[a]	6.89[c]	24.78	195[i]	198[k]	333	8.5	11.1	12.2
Contr. 2	1.42	5.15[b]	6.62[d]	24.26	179[j]	185[l]	325	11.9	13.1	14.7
Exp. 3	1.38[e]	5.08	6.49	23.57	184	189	323	11.5	13.5	15.7
Contr. 3	1.45[f]	5.09	6.50	23.99	176	182	322	13.6	15.6	17.3

a,b; c,d; e,f ;g,h - P<0.05 i,j; k,l - P<0.01

In conclusion, prolactin administered to newborn piglets *per os* a dose of 0.5 mg/kg body weight, may have beneficial effect on their postweaning growth. Moreover, the experiment showed a positive effect a dose of 0.1 mg PRL on reducing piglets' mortality.

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Notes

IN VITRO FERTILIZATION IN BUFFALO USING SEXED SEMEN: EFFECT OF DIFFERENT SPERM PREPARATION PROCEDURES AND CALCIUM-HEPARIN SUPPLEMENTATION IN IVF MEDIUM

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The production of buffalo embryo in vitro represents an important option for exploiting superior genotypes, given the low efficiency of embryo collection by super-ovulation in this species. However, the quality of frozen buffalo semen is generally lower than bovine semen, which affects the fertilization rates that can be achieved in vitro. Data generated in our laboratory on 8 buffalo bulls tested for penetration rate using bovine oocytes fixed 18h post IVF indicate an average rate of 94% (116/124) for non sexed semen and 34% (64/186) for sexed semen ($p<0.01$). When the best performing buffalo bull was used for IVF the cleavage rate was 59% (3213/5369) for non sexed semen and 34% (254/748) for sexed semen, with 9.26% and 3.88% of the embryos reaching developmental stage suitable for freezing for non sexed semen and sexed semen respectively ($p<0.05$). In this study we investigated whether fertilization rate of sexed semen could be improved by (1) changing sperm preparation protocol and (2) optimising the calcium and heparin concentrations in TALP-IVF. The conventional sperm preparation procedure using a Redigrad gradient was compared with "swim-up". Preliminary tests indicated that the efficiency of motile sperm recovery was much lower with swim-up than Redigrad gradient and in order to recover a sufficient number of sperm it was necessary to use 4-4.5 semen straws for swim-up versus 1-1.5 for Redigrad gradient (3 replicates). The cleavage and development rates were not different between the 2 sperm preparation procedures, as shown in Table 1.

Tab. 1. Effect of sperm preparation procedure on cleavage and embryo development using buffalo sexed semen

Sperm preparation procedure	N. of straws	million sperm recovered	N. per straw	sperm conc. In IVF	N. of oocytes	N. of cleaved	% cleavage	N. of blastocysts	% over cleaved	% over oocytes
Redigrad gradient	3.5a	2.84	0.81a	3×10^6	252	88	34.92	8	9.09	3.17
swim-up	12.5b	1.49	0.12b	3×10^6	250	89	35.60	12	13.48	4.80

Control IVF conditions using TALP-IVF medium containing conventional calcium (39mg/ml, 2.65mM) and heparin concentration (1microg/ml) was compared with TALP-IVF supplemented with either higher calcium (89mg/ml, 6.05mM) or heparin (10microg/ml) concentrations. Cleavage and development to blastocyst were not different between control and higher calcium or higher heparin (cleavage rate: 37.14, 31.85, 33.33; development rate: 5.00, 4.38, 5.19 respectively). In conclusion, our data indicate that the efficiency of buffalo IVF with sexed semen is not improved through sperm separation using swim-up vs Redigrad gradients or increased calcium/heparin concentrations.

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Notes

SUPEROVULATORY RESPONSE AND OOCYTE RECOVERY AFTER OVUM PICK UP IN FEED RESTRICTED HEIFERS WITH TWO PROFILES OF AMH

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Anti–Müllerian hormone (AMH) was found to be a reliable endocrine marker of the population of small antral gonadotropin-responsive follicles in the cow. The measurement of circulating AMH concentrations can help predicting the follicular and ovulatory responses to gonadotrophin treatment in cows (Rico et al., 2009, Rico et al., 2012). The aim of this study was to determine the superovulatory response and the number and quality of oocytes recovered by Ovum Pick Up (OPU) in feed restricted heifers (LWG: 600 g/d) with two profiles of AMH. Sixteen Holstein heifers (15.8 ± 1.2 months old; LW: 370±41.2 kg) were grouped according to AMH concentrations: low (L = 1-80 $pgmL^{-1}$; n=7) or high (H: >150 $pgmL^{-1}$; n=9). Plasma concentrations of AMH were determined using AMH GENII ELISA kit (Beckman Coulter France, Roissy CDG, France), as described previously (Monniaux et al., 2008). OPU was performed during two periods (P1 and P2) at an interval of 6 weeks. Two OPUs were performed in each period. Before each OPU, estrous cycles were synchronized with subcutaneous 3mg Norgestomet implants (Crestar; MSD, Angers, France) inserted under the convex surface of the ear for 9 days. On the day of implant insertion, heifers received an i.m. injection of GnRH (Receptal®, Intervet, Angers, France). Two days before implant removal, 500 µg cloprostenol (Estrumate®; Schering-Plough, Levallois-Perret, France) were injected. On day 2.5 of the synchronized estrous cycle (day 0 was the day of estrus), heifers were superovulated with a total dose of 300 µg follicle-stimulating hormone (pFSH; Stimufol; Reprobiol, Belgium) divided into 5 i.m. injections given 12 h apart, at decreasing doses. Cumulus–oocyte complexes were collected by OPU 12 h after the last FSH injection (day 5 of the cycle). Oocytes were graded for quality as, 1, 2, 3 and 4, according to Marquant-Le Guienne (1998). Before OPU all follicles (diameter 3-12 mm) were counted. The statistical analyses (Student's T test and data correlation) were performed using GraphPadprism. There was a high correlation in both periods 1 and 2 between AMH concentrations and the number of follicles aspirated (P1: r=0.86, P<0.0001; P2: r=0.74, P<0.001), the number of oocytes collected (P1: r= 0.83, P< 0.0001; P2: r=0.81, P< 0.0001) and the number of quality 1, 2 and 3 oocytes (P1: r=0.84, P<0.0001; P2: r=0.82, P<0.0001). Animals with AMH H had significantly higher numbers of aspirated follicles, total oocytes and 1, 2 and 3 quality oocytes than AMH L animals (Table 1). The percentage of 1, 2 and 3 quality oocytes and the percentage of follicles aspirated were similar between AMH H and AMH L. There was no significantly difference between periods. The number of oocytes aspirated was not altered by multiple stimulations.

Table 1: Number of aspirated follicles, total number of oocytes collected, number of oocytes (quality 1, 2 and 3; Q1-3) and aspiration rate.

Parameters	PERIOD 1				PERIOD 2			
	AMH H		AMH L		AMH H		AMH L	
	Lsmean	S.E.M.	Lsmean	S.E.M.	Lsmean	S.E.M.	Lsmean	S.E.M.
Aspirated follicles	40.5[a]	3.6	16.5[b]	2.0	32.4[a]	3.9	5.1[b]	1.7
Total Oocytes	25.7[a]	2.4	10.6[b]	1.5	21.1[a]	2.8	9.0[b]	1.4
Oocyte Q1-3	22.6[a]	2.2	7.9[b]	1.1	19.1[a]	2.4	7.9[b]	1.3
% Oocytes Q1-3	88.3		74.5		90.5		87.7	
Aspiration rate (%)	63.5		64.1		65.0		59.4	

[a,b] P<0.0001

This study shows that oocytes collection by OPU is higher in heifers selected for high circulating AMH concentrations.

Notes

THE EFFECTS OF OMEGA-3 AND OMEGA-6 POLYUNSATURATED FATTY ACIDS ON LAMB EMBRYO DEVELOPMENT AND QUALITY IN ENTIRELY SERUM-FREE MEDIA

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Polyunsaturated fatty acids (PUFAs) such as omega 3 (α-linolenic acid, ALA) and omega 6 (linoleic acid, LA) are known to affect reproductive performances including oocytes and embryos quality and development in mammals. The aim of this study was to assess the effect of ALA and LA supplementation to serum free IVM medium on in vitro fertilization and embryo development of lamb oocytes. The PUFAs were added at increasing concentration of 0 (control), 50, 100 and 200 µM to TCM199 based medium and the oocytes were matured for 24 h and then fertilized using swim up method and 2-2.5 million sperm per millilitre. After 20 h of fertilization in serum-free media, a group of presumptive zygotes were fixed in 4% Paraformaldehyde (PFA) for 30 min and stained with DAPI to analyse pronuclear (2PN) formation. The remainder were transferred to SOF medium and cultured for 8 days to assess development to blastocyst stage. The number of blastocysts were counted and the embryos were fixed in 4% PFA containing 30 µg/ml Hoechst for 10 min that was prepared in 2 X PBS. Afterwards, they were permeabilized in 0.1% Triton-100 in SOF-BSA media for 5 min and stained by TUNEL using a FITC conjugated in situ cell death detection kit to assess the apoptosis. Data were analysed using SPSS version 20. The results showed that ALA increased the two pro-nucleus (2PN) zygotes according their concentrations (46.34%, 51.11%, 60.00%, respectively, compared to control group 47.82, $P \leq 0.05$). Similarly, LA regardless of its concentration increased the percentage of 2PN zygotes as compared to control group (57.89%, 47.50%, 50.50%, 45.45%, respectively, $P \leq 0.05$). However, no differences were found in the fertilization rate with ALA (77.69%, 70.62%, 81.87%, and 74.82%, respectively) and LA treatments (84.95%, 83.78%, 81.98%, and 87.80%, respectively). At day 8, the percentage of blastocyst development was not affected by ALA treatments (15.74%, 6.93%, 11.47%, and 14.01%, respectively) and LA treatments (15.62%, 13.97%, 13.18%, and 11.11%, respectively). ALA treatments improved embryos quality with increasing the total cell numbers and decreasing the apoptotic cell numbers significantly ($P \leq 0.05$) but for LA treatments were different from control group. In conclusion, supplementation of ALA and LA to lamb oocytes IVM media increased their fertilization rate but did not affect developmental competence to blastocyst stage.

Notes

A DIET ENRICHED IN LINOLEIC ACID COMPROMISES THE CRYOTOLERANCE OF EMBRYOS FROM SUPEROVULATED BEEF HEIFERS

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Dietary rumen-protected fat rich in linoleic acid may affect the superovulatory response and embryo yield; however, its effects on in vivo embryo cryotolerance are unknown in zebu cattle. The present study evaluated the production and cryotolerance after freezing or vitrification of embryos from Nelore heifers supplemented with rumen-protected polyunsaturated fatty acids (PUFA).

Forty heifers kept in pasture were randomly distributed into two groups according to the type of feed supplement (F, supplement with rumen-protected PUFA, predominantly linoleic; C, control fat-free supplement with additional corn). Supplements were formulated to be isocaloric and isonitrogenous. Each heifer underwent both treatments in a crossover design with 70 days between replicates. After 50 days feeding, heifers were superovulated. Embryos were evaluated morphologically and vitrified or frozen. After thawing or warming, embryo development was evaluated in vitro.

There was no difference between the F and C groups ($P > 0.10$) in terms of embryo production. Regardless of the cryopreservation method used, Group C embryos had a greater hatching rate after 72 h in vitro culture than Group F embryos (44.3 ± 4.2% (n - 148) vs 30.9 ± 4.0% (n = 137), respectively; $P = 0.04$). Moreover, vitrified and frozen embryos had similar hatching rates ($P > 0.10$).

In conclusion, dietary rumen-protected PUFA rich in linoleic acid did not improve embryo production and compromised the cryotolerance of conventionally frozen or vitrified embryos from Nelore heifers.

Notes

EFFECT OF THE MODIFIED SUPEROVULATION PROTOCOL ON THE EMBRYO DEVELOPMENT IN CATTLE

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Superovulatory treatment protocols have been constantly modified with the objectives of recovery of higher numbers of viable embryos and simultaneously lower variability in superovulatory responses. Traditional superovulatory protocols use the pituitary extract containing FSH with variable ratio of LH. The aim of this study was to adapt the superovulatory protocol to physiological conditions of growing follicles by the use of pure FSH during the initial phase of superstimulation.

A total of 20 Simmental heifers were pre-synchronised by 2 injections of $PGF2_{\alpha}$ and at day 8 of oestrous cycle the dominant follicle was punctured. The superstimulation was started at day 10 of oestrous cycle. Animals were superstimulated by 8 i.m. injections decreasing doses of FSH during 4 days. Half of the animals (control-group) were treated with Stimufol (Reprobiol SPRL, Belgium, in total 450 µg p FSH and 90 µg pLH). The other half of the animals (FSH-group) the pure FSH was administrated for first five injections (in total 355 µg pFSH) followed with three injections of the Stimufol (Reprobiol SPRL, Belgium, in total 95 µg p FSH and 19 µg pLH). The $PGF2_{\alpha}$ was administrated simultaneously with the sixth and seventh FSH injection. Heifers were inseminated 48 h after the $PGF2_{\alpha}$ injection using fresh semen and simultaneously GnRH was administrated. In half of the animals of each group embryos were recovered at day 2 by combined flushing of oviducts and uterus and further cultured in vitro. In the other half of the animals embryos were flushed from oviducts and uterine horns at day 7. The superstimulation was repeated in the same animals using the complementary treatment protocol, respectively.

At day 2 there were not differences between the numbers of CL´s, recovered embryos and 4-8 cells stage embryos (14.3, 15.5; 10.9, 11.6 and 5.5, 7 respectively) between the FSH-group and control-group. The number of day 7 embryos developed in vitro to blastocysts and morulae was not different between the FSH-group and control-group (6.5 and 7 respectively). On the contrary despite similar numbers of CL´s between FSH-group and control-group (17.2 and 16.7 respectively) the number of total recovered embryos and number of recovered morulae and blastocyst after the flushing at day 7 was lower in FSH-group in comparison with the control-group (8.2, 11 and 3.4, 5.7 respectively).

In conclusion it was shown that the modified superovulation protocol had no negative effect on the number of ovulated follicles or early embryo development. However, the results demonstrate that embryo development between day 2 and day 7 of the oestrous cycle is negatively affected by the hormonally guided oviduct/uterus environment.

Notes

IN VITRO AGEING OF BOVINE OOCYTES PRIOR TO FERTILIZATION INFLUENCES GENE SPECIFIC METHYLATION AND IMPAIRS EMBRYONIC DEVELOPMENT

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In order to ensure optimal embryonic development bovine oocytes are usually fertilized *in vitro* 22-24 h after onset of maturation. A delay leads to a time dependent deterioration regarding the oocyte-cumulus interactions, spindle formation, the accumulation of reactive oxygen species and is associated with assisted reproductive failures.

In this study, we extended the *in vitro* maturation period of bovine oocytes from 24 h to 48 h in order to investigate the effects of delayed fertilization on developmental potential, spindle formation, transcript abundance and gene specific methylation marks in bovine oocytes.

Bovine oocytes were collected from slaughterhouse ovaries and subjected to *in vitro* maturation using TCM199 supplemented with Suigonan® for either 24 or 48 h followed by standard *in vitro* fertilization and *in vitro* culture. Maturation rates (79±3 vs. 64.2±7%) did not differ dramatically and first results indicate no difference in the incidence of spindle aberrations between the two groups. Morphologically, oocytes after prolonged maturation showed distinct detachment of cumulus cells. Fertilization rates, however, did not differ significantly (47.5±9 vs. 52.8±7%). Cleavage (53.7±9 vs. 41±14%) rates were reduced by prolonged maturation and development to the blastocyst stage was severely impaired in the group matured for 48 h compared to 24 h (1.3±1 compared to 22.4±6%). Analysis of transcript abundance on single oocytes for a panel of genes indicative of oxidative stress, imprinting, methylation processes and maternal effect genes (*PRDX1, IGF2R, PEG3, SNRPN, DNMT1B, DNMT3A, HSF1, NLRP9* and *ZAR1*) did not reveal significant differences between the two groups. Methylation analysis by Limiting Dilution of *DNMT3Lo* showed a significant increase of aberrantly methylated alleles in the 48h-IVM group ($p=0.03$), other investigated genes (*H19*, *SNRPN, OCT4 and DNMT3A*) however were not influenced. Results so far indicate that *in vitro* oocyte ageing highly impairs the development to the blastocyst stage. The reduced developmental competence seems not to be reflected in the progression to metaphase II and transcript abundance of the investigated genes, but could be indicated in the aberrant epigenetic marks found for DNMT3Lo.

Notes

CULTURE ENVIRONMENT BUT NOT ORIGIN OF EARLY EMBRYOS AFFECTS CRYOPRESERVATION, LIPID CONTENT AND EXPRESSION OF MITOCHONDRIAL RELATED GENES IN BOVINE BLASTOCYSTS

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It is well accepted that in vitro culture conditions have negative impact on bovine blastocysts in terms of quantity and quality, implicating high lipid accumulations which in turn enhance the sensitivity to cryopreservation. The lipid content of bovine embryos has been reported to be strongly influenced by the culture environment, especially by the presence of serum. A causative link between serum supplementation to culture media and lipid accumulation in bovine embryos, however, is still under research. To investigate the effect of altered preimplantation environments in terms of developmental competence, lipid metabolism, survival after cryopreservation and global gene expression profiles, four groups of blastocysts were generated, namely I. completely in vitro produced blastocysts, II. in vitro derived 2-Cell stage embryos, endoscopically transferred to the oviduct and flushed back at blastocyst stage, III. in vivo derived 2-Cell stage embryos endoscopically flushed from the oviduct and cultured in vitro until blastocyst stage, IV. fully in vivo derived blastocysts. As a first result, our experiments showed that developmental quantity is already determined in the 2-Cell stage embryo. The complete in vitro group as well as embryos transferred at 2-Cell stage into the oviduct yielded blastocyst rates around 42% whereas the complete in vivo group as well as 2-Cell embryos flushed from the oviduct followed by in vitro culture yielded blastocyst rates around 80%. In contrast, culture environment but not embryo origin affected lipid content as well as embryos survival rates after cryopreservation. Embryos cultured in vitro showed 1.7 fold higher lipid contents compared to their in vivo cultured counterparts, irrespectively of their origin. Likewise, embryos cultured in vitro showed a significant lower survival rates after cryopreservation compared to embryos cultured in vivo within the oviduct. Global gene expression analysis identified a total 315 differentially expressed genes due to culture environments (FC 1.5; FDR 0.05%). Among these, the abundance of two candidate genes which are known to play a crucial roles in lipid metabolism were further analysed by quantitative real time RT PCR. For PGC1α (Peroxisome proliferator-activated receptor y coactivator 1α), which has been reported to regulate the amount of mitochondria, as well as for CPT1 (carnitin palmitoyltransferase 1), which regulates the activity of mitochondria in terms of ß-oxidation, a significant higher transcript abundances were detected for embryos cultured in vivo irrespectively of their origin. Taken together, our results show that the culture environment affect the expression of genes related to mitochondrial ß-oxidation which could be causative for lipid accumulation as well as bovine embryo survival after cryopreservation, due to invalid mitochondrial metabolism whereas developmental rate to the blastocyst stage is already fixed at 2-Cell stage oocyte.

Notes

HYDROCHLORIDE OF VETROBUTIN (MONZAL®) IMPROVES THE APPLICATION OF POST-CERVICAL ARTIFICIAL INSEMINATION IN GILTS

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The application of post-cervical insemination (post-CAI) in pigs is nowadays in a clearly expansion. The benefits of this technique have been demonstrated in sows getting the same or better results than with the use of cervical insemination (Hernández-Caravaca *et al*. 2012). However, the use of this methodology in gilts is not as spread as in sows due to the physical impossibility which avoid the penetration of the post-cervical inner catheter in this type of females. Monzal® (hidroclorhide of vetrobutin, Boehringer Ingelheim Spain, S.A.) is a medicament routinely used to relax the uterine muscle during the farrowing. Taking into account the antispasmodic action of this product the objective of this study was to evaluate the application of post-CAI method after i.m. Monzal® injection in the gilts where previously post-CAI could not be applied.

Eighty gilts (7-9 months old) (Landrace × Large White) were used. Oestrus detection was performed twice daily and gilts were inseminated 24 h after oestrus detection. Post-cervical AI (post-CAI) was performed with a combined catheter-cannula kit (Soft & Quick®, Import-vet, SA, Barcelona, Spain). Doses of $1.5x10^9$ sperm in 40 ml were used. Post-cervical insemination was considered well applied when the inner catheter penetrated the cervix without difficult and/or any backflow was observed inside cervical catheter at the moment of dose application.

Only in 25% (n=20) of the total gilts used in this experiment the post-CAI technique could be directly applied. A dose of Monzal® (2-4 ml; 100 mg/ml) was i.m. injected in the rest of gilts. After 28.07±2.50 min of the injection a second attempt were tested and in 34.21% of the gilts post-CAI could be carried out. No differences were found between groups (post-CAI vs. post-CAI+Monzal®) in terms of pregnancy (72.22±0.11% vs. 84.62±0.10%, respectively) and total born litter size (12.92±0.82 vs. 13.50±0.69, respectively).

In conclusion, the use of Monzal® can be considering a new tool to improve the success rate in the application of post-cervical artificial insemination in gilts.

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Notes

ENERGY STATUS OF PORCINE OOCYTES WITH DIFFERENT MEIOTIC COMPETENCE BEFORE AND AFTER THEIR IN VITRO MATURATION

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Meiotic and cytoplasmic competences of porcine oocytes closely correlate with the size of follicles from which the oocytes have been recovered. Important markers of cytoplasmic maturation of oocytes are mitochondrial activity, lipid droplets and ATP content. Some relationships between developmental competence of porcine oocytes and energy status have been described. The aim of this study was to characterize porcine oocytes with different meiotic competence in terms of mitochondrial reorganization, lipid and ATP content before (0 hours – GV stage) and after (44 hours – MII stage) maturation. Cyclic sows, examined for ovarian status, were used as oocyte donors. The meiotically highly competent (MHC) and less competent (MLC) oocytes were isolated from medium (6-9 mm) and small follicles (<5 mm) by aspiration and slicing, respectively. Only morphologically healthy oocytes were matured for 44 h, using a standard protocol. Mitochondria were stained with MitoTracker Orange and detected by confocal microscopy. For visualization of lipid droplets, we used the molecular probe Nile Red and confocal microscopy. The ATP content (pmol per oocyte) was determined, using FL-ASC assay kit.

In porcine oocytes, active mitochondria encircled lipid droplets in the so-called metabolic units. A significantly higher ($P<0.05$) proportion of oocytes with metabolic units was detected in MHC than in MLC oocytes before (44.7% vs. 15.5%) and after (48.8% vs. 31.7%) maturation. The proportion of oocytes with metabolic units increased from 44.7% to 48.8% in MHC- and from 15.5% to 31.7% in MLC-oocytes between stages GV and MII. The total area covered with lipid droplets in immature oocytes was similar in MLC and MHC oocytes (29.2% vs. 30.4%), but the total number of lipid droplets in immature porcine oocytes was significantly higher ($P<0.05$) in MHC (381±95 lipid droplets/oocyte) in comparison to MLC oocytes (338±82 lipid droplets/oocyte). After maturation, the total area covered with lipid droplets was similar in MHC and MLC (28.2% vs. 29.8%) and also the total number of lipid droplets was very similar in both categories (MHC 296±109 and MLC 278±97 lipid droplets/oocyte). During the process of maturation, the total number of lipid droplets dramatically decreased in MHC oocytes. The ATP content was higher ($P<0.05$) in MHC oocytes compared to MLC oocytes before (2.6 pmol vs. 2.0 pmol) and after maturation (1.9 pmol vs. 1.0 pmol).

In conclusion, the levels of metabolic units and ATP contents are higher in oocytes with higher meiotic competence before and after *in vitro* maturation. Before maturation, oocytes with higher meiotic competence have a much higher number of lipid droplets, but not total contents of lipid granules in comparison with oocytes with lower meiotic competence. These results indicate that small lipid droplets are important energy source during in vitro maturation of oocytes with higher meiotic competence.

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Notes

INTRAFOLLICULAR OOCYTE TRANSFER (IFOT) – A NEW METHOD TO PROVIDE IN VIVO CULTURE CONDITIONS FOR IN VITRO MATURED BOVINE OOCYTES

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Although the in vitro production (IVP) of bovine embryos is a well-established technique for about 20 years, there are still major differences of IVP-derived blastocysts compared to their in vivo derived counterparts. One major aberration of IVP-derived embryos is high accumulation of lipid droplets within the cytoplasm suggested to cause low survival rates following cryopreservation. To circumvent the negative impacts due to in vitro culture conditions, a new method, the so called intrafollicular oocyte transfer (IFOT), was established in the present study. Using a modified Ovum-Pick-Up (OPU) equipment, in vitro matured oocytes derived from slaughterhouse ovaries were injected preovulatorily into the dominant follicle of synchronised Simmental heifers enabling subsequent in vivo fertilization as well as in vivo culture. A total of 4175 in vitro matured oocytes were transferred into 71 heifers. Subsequently, 1240 embryos (29.7 %) were recovered after flushing of the uteri at day 7. Of these, 23.3 % developed to the blastocyst stage which did not differ compared to completely in vitro derived embryos. In contrast, lipid content of IFOT-derived blastocysts was optically and quantitatively comparable to that of fully in vivo produced bovine embryos but differed significantly from complete in vitro derived blastocysts. IFOT blastocysts showed significantly higher survival rates after cryopreservation compared to complete IVP-derived embryos (72 % vs. 9 %), which may be due to a reduced lipid accumulation. Thus, our results indicate that the ability to develop to the blastocyst stage is already determined in the matured oocyte whereas the quality in terms of lipid content and survival rate after cryopreservation is affected by the environment thereafter. In addition, transfer of cryopreserved IFOT-derived blastocysts resulted in higher pregnancy rates compared to completely in vitro derived blastocysts (46.2 % vs. 13.8 %) and gave rise to the birth of healthy calves proving that IFOT offers a new innovative method to circumvent harmful effects of in vitro culture conditions for in vitro matured bovine oocytes.

Notes

REAL-TIME PROLIFERATION OF PORCINE CUMULUS CELLS IS RELATED TO THE PROTEIN LEVELS AND CELLULAR DISTRIBUTION OF CDK4 AND CX43

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The proper maturation of cumulus somatic cells (CCs) depends on bidirectional communication between the oocyte and the surrounding CCs. The aim of this study was (i) to investigate maturation markers, such as Cx43 and Cdk4 protein levels, and (ii) to analyze the distribution of these two proteins in CCs cultured for 44 h, 88 h, 132 h and 164 h in both separated and cumulus-enclosed oocyte cultures.

CCs were isolated from porcine ovarian follicles following the treatment of the recovered COCs with collagenase. Then, the separated CCs were cultured in TCM199 for 0-164 h, using a real-time cellular analyzer (RTCA), however, the immunostaining was performed only after 44h, 88h, and 132h. The protein levels and distribution were analyzed using confocal microscopy.

After the CCs underwent IVC for 25 h, a logarithmically increasing normalized proliferation index was found throughout the entire 164 h cultivation time. The Cx43 and Cdk4 proteins were observed at higher levels after 44 h of culture than before IVC. After 88 h and 132 h of IVC, no significant alterations in either mRNA or protein levels of Cx43 and Cdk4 were found. Cx43 and Cdk4 were localized in the cell nucleus before IVC, whereas after 44 h, 88 h, and 132 h of IVC both proteins translocated to the cytoplasm. In cumulus-enclosed oocyte cultures, Cdk4 was localized both in the nucleus and cytoplasm, whereas Cx43 was only in the cytoplasm. Additionally, only low levels of the cumulus expansion markers MIS and SNAT3 were observed.

In summary, we could demonstrate that the *in vitro* cultivation of CCs was associated with cell proliferation and that Cx43 and Cdk4 gene expression was upregulated after IVC, resulting in significantly higher protein levels. Moreover, the two proteins translocated from the nucleus to the cytoplasm of the CCs during IVC. The protein distribution is presumably related to different protein functions during bidirectional communication via gap junction communication.

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Notes

CORRELATION BETWEEN THE PROTEIN EXPRESSION AND CELLULAR DISTRIBUTION OF ESTROGEN AND PROGESTERONE RECEPTORS AND THE REAL-TIME PROLIFERATION OF PORCINE CUMULUS CELLS

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Cumulus cells (CCs) influence the maturation of cumulus-oocyte complexes (COCs) primarily via gap-junction connections. Steroid hormones play a specific role in the development of follicles and oocytes including COC maturation and CC expansion. However, the cultivation of separated CCs in relation to real-time proliferation *in vitro* and the protein expression or cellular distribution of progesterone and estrogen receptors has not yet been investigated.

In this study, a model of separated CC was used to analyze the cell proliferation index and the expression of progesterone receptor (PGR), PGR membrane component 1 (PGRMC1) and of estrogen-related receptors (ERRγ and ERRβ/γ) during a 96-h cultivation *in vitro* (IVC). Using confocal microscopic observation, we found that PGR protein expression was increased before IVC, compared with PGR protein expression after 96 h of IVC ($P<0.001$). The expression of PGRMC1, ERRγ and ERRβ/γ was unchanged. However, we observed that before IVC, PGR and PGRMC1 proteins were localized in the cell nucleus, whereas after 96 h of IVC these proteins were distributed in the cell cytoplasm. ERRγ was always localized in the cell nucleus, and ERRβ/γ was distributed only in the cell cytoplasm. We suppose that the differential expression of the PGR protein before and after IVC is related to a time-dependent down-regulation, which may activate a negative feedback.

The distribution of PGR, PGRMC1 proteins may be linked with the translocation of receptors to the cytoplasm after the membrane binding of respective agonists and intra-cytoplasmic signal transduction.

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Notes

INCLUSION OF TISSUE TYPE PLASMINOGEN ACTIVATOR (T-PA) IN IVF MEDIUM INDUCES ALTERATIONS IN GENE EXPRESSION AND AFFECTS BLASTOCYST FORMATION RATE IN BOVINE IVP

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The presence or activity, of the proteolytic plasminogen activators/ plasmin system (PAA/PAI/PL) has been detected in various cell types of the reproductive system, while its involvement into oocyte maturation, fertilization, embryo development and implantation has been shown in many studies. However, these factors are practically absent from the standard culture media used in IVF labs.

Here, through a modification of IVF medium, we investigated the effects of tissue type plasminogen activator (t-PA) on fertilizing capacity and subsequent embryo development. Bovine cumulus oocytes complexes (COCs) were collected from abattoir material and matured for 24 hours into TCM199 enriched with 10ng/ml EGF and 10% FCS at 39°C under 5% CO_2 in air and max humidity. Matured oocytes were inseminated with frozen- thawed swim-up separated bull sperm and gametes were co-incubated in standard IVF medium (controls n=488), in modified medium containing 50IU/0.1 ml t-PA (n=583), and in medium containing t-PA and its inhibitor (ε-aminicaproic acid, final concentration 10mM, n=259). Presumptive zygotes were cultured in SOF culture medium at 39°C under an atmosphere of 5% CO_2, 5% O_2 in air, max humidity. Blastocyst formation rate was assessed in eight replicates. From each experiment, a pool of 10 day-8 blastocysts was snap frozen into liquid nitrogen for gene analysis. To evaluate the quality of produced embryos, real time PCR was carried out for nine gene transcripts (*PLAC8, AKR1B1, BIRC5, BBC3, PGHS2, BCL2L1, SLC2A5, MnSOD, PLG*) controlling apoptosis, metabolism, implantation and oxidation pathways. Three housekeeping genes were used for normalization of gene expression data (*18srRNA, H2a.z* and *GAPDH*).

Differences in embryo development (cleavage and blastocysts formation rates) and relative mRNA abundance were analyzed by one-way repeated measures ANOVA.

Under our in vitro culture conditions, modification of IVF medium with tPA resulted in a significant decrease both in cleavage and blastocyst formation rates in comparison to controls (85% vs 66% cleavage; 30% vs 13% Day 7; and 35% vs 16% Day 8, for control vs treated, respectively). When combined ε-ACA and tPA was used, no difference was noted between treated and controls (85% vs 84.3% cleavage; 30% vs 28% day 7; and 35% vs 30%, day8, respectively). Gene expression analysis showed that *Bcl2L1* antiapoptotic gene was present in greater relative concentrations in control than in tPA treated embryos ($P<0.05$). Relative changes in transcript levels for *SLC2A5* (*GLUT5)* between all groups indicate an increase in metabolism in tPA embryos which was reversible when ε-ACA was added to medium ($P>0.05$). We infer that under our lab's culture conditions tPA addition in fertilization medium seriously affects embryonic development and reduces the blastocyst yield that may be a sequel of downregulation of antiapoptotic genes. Further research is warranted to elucidate possible roles of this enzymatic system in the compromised fertility of dairy cows.

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Notes

REPRODUCTIVE CHARACTERISTICS OF TURKISH NATIVE SHEEP BREEDS TO BE USED AS RECIPIENT IN EMBRYO TRANSFER

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Pregnancy rates from embryo transfer (ET) vary widely. Variation in recipient suitability and breed, the number of corpora lutea is thought largely responsible for variation in observed success. The reproductive characteristics of fat (Morkaraman, n= 14 and Kangal; n=27) and thin tailed ewes (Daglic; n=72 and Kivircik; 27) for the use as recipients in embryo transfer programs were evaluated in spring season. Estrus of recipient ewes was synchronized with vaginal sponges containing 30 mg FGA for 12 days and ewes received an i.m. injection of 400 I.U. PMSG at sponge removal. Estrus and ovulation rates, time to onset of estrus and percentage of recipient ewes assessed as suitable for receiving embryos by evaluation corpus luteum and uterine tone were determined. The success of synthetic progestagen to establish a fertile estrus in and out of breeding season (May) was found 64.3%, 74.1%, 58.3% and 66.7% for Morkaraman, Kangal, Daglic and Kivircik, respectively. Ovulation rates were found significantly higher ($P<0.05$) in Morkaraman ewes (1.6±0.25) than Kangal (0.95±0.17), Daglic (1.07±0.12) and Kivircik (0.72±0.18) ewes. The suitability for the transfer in terms of observation of corpus luteum and uterine tone, Morkaraman breed was found the best with 100% acceptable rates and, Daglic (85.4%), Kangal (73.75) and Kivircik (61.1%) ewes followed respectively. Time to onset of estrus differed significantly among the four breeds of recipients. Kangal ewes were recorded latest for the onset of estrus with 74.04h after sponge removal while the other three breeds of recipients (Daglic: 58.8h, Morkaraman: 64.3h and Kivircik: 66.7h) had similar results. These preliminary results show that, in the Morkaraman breed a higher number of animals could be selected for embryo transfer including higher ovulation rates which make these animals appear to be better recipient candidates in embryo transfer programs in and out of season compared to other breeds.

Notes

RELATIONSHIP BETWEEN PLASMA AMH AT PREPUBERTY AND ADULTHOOD IN SHEEP

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The high between-animal variability in the ovarian response to exogenous stimulation treatments is one of the main reasons responsible for the low efficiency of LOPU-IVP in sheep. At present, Anti-Müllerian Hormone (AMH), which is produced by granulosa cells of preantral and small antral follicles, is a great predictive endocrine marker of the number of available follicles in response to ovarian stimulatory treatments in many species. Recently, we also extended its ability to adult sheep (Reprod. Domest. Anim. 2012, 47(s4):492). While the use of AMH in adulthood is of great interest, it would be even more interesting if it could be determined earlier in life, allowing for a precocious selection of the best future donors for embryo biotechnologies. Therefore, our objective was to define plasma AMH before puberty and to relate it with AMH levels and ovarian responses to FSH in the adulthood in the same animals.

With this purpose, ten Rasa Aragonesa ewe lambs were blood sampled when aged 3, 4.5 and 6 months, and later at the adult age (19 months), at the first session of four repeated LOPU sessions. Lithium heparinized tubes were used and plasma was stored at -20 ºC until assayed for AMH (GenII ELISA kit; Beckman Coulter, France). Before puberty, plasma AMH concentrations increased from 3 to 4.5 months, and then declined at 6 months up to levels similar to those observed later in adults: 370.4 ± 77.7, 519.2 ± 153.3 and 171.1 ± 29.7 pg/ml, respectively (Mean ± SEM; $P<0.1$). At these early ages, strong between-animal variability in AMH was found, with concentrations ranging from 20.4 to 1370.8 pg/ml. Within-animal repeatability in AMH at 3, 4.5 and 6 months was found to be very low (0.21; NS). At the adult age, mean plasma AMH concentration was 201.9 ± 24.1 pg/ml. Within-animal repeatability of AMH at 3, 4.5 or 6 months with AMH at 19 months was close to zero. Relationships between AMH before puberty and AMH or follicle yield after FSH stimulation at LOPUs in the adult age were not significant.

In conclusion, plasma AMH before puberty showed a great variability between individuals and between ages, apparently reflecting chronological differences between animals on ovarian maturity further related with sexual precocity, as we proposed in a previous work. Therefore, a direct relationship with AMH levels at the adult age seems to be uncertain and could not be established for now, as it seems to reflect different physiological situations which require more studies. While in adult sheep circulating AMH determination by a single blood sample prior to FSH treatment was a suitable method for selecting the best oocyte donors, the precocious selection of such animals by AMH sampling at their prepubertal age would not be feasible.

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Notes

EFFECT OF THE *FecX*^{R} ALLELE ON FOLLICLE SIZE AND OOCYTE COMPETENCE ASSESSED BY LOPU AND IVP OF SHEEP EMBRYOS

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In several sheep breeds, different mutations in the *BMP15* gene which lead to increased ovulation rate in heterozygous ewes when compared with wild-type animals have been found. One of these polymorphisms is the *FecX*^{R} allele in the Rasa Aragonesa breed. Although the exact mechanism by which these mutations increase ovulation rate is not fully known yet, the reduced activity of the BMP signalling system would lead to smaller antral follicles with fewer granulosa cells and altered sensitivity to gonadotropins, leading to higher ovulation rates. As the proportion of competent oocytes is widely accepted to increase along with follicular size, in the present study we aimed to investigate the effect of the *FecX*^{R} allele on follicle number and size, as well as its potential effect on oocyte competence.

With these aims, 8 heterozygous (R+) and 8 wild-type (++) ewes were subjected to 2 laparoscopic ovum pick-up (LOPU) trials (4 sessions per trial; 2 with and 2 without FSH). Collected COCs were subjected to in vitro maturation, fertilisation and culture. Day 7 and 8 blastocysts were fresh transferred to assess their viability by their ability to sustain pregnancy to term.

A total of 1673 follicles were aspirated, yielding 995 oocytes (mean collection rate, 59.5%). Genotype did not affect the total number of aspirated follicles per ewe and session (10.4 and 10.2 in R+ and ++ untreated ewes, 17.4 and 14.3 in R+ and ++ FSH-treated ewes, respectively), but mean follicular size was significantly reduced in R+ ewes (4.1 and 4.3 in R+ and ++ untreated ewes, 4.3 and 5.1 in R+ and ++ FSH-treated ewes, respectively; $P<0.01$). Cleavage rate ranged from 84.0% to 87.8%, with no differences between genotypes. No significant differences were observed in the rate of day 7 blastocysts between R+ and ++ groups (27.0% and 21.4% in untreated ewes, and 31.3% and 33.7% in FSH-treated ewes, respectively), or in day 8 blastocysts (32.5% and 25.6% in untreated ewes, and 37.7% and 36.8% in FSH-treated ewes, respectively). Fertility of the recipients and survival rate of transferred embryos were similar in both genotypes. Overall fertility was 77.1% at 25 days post transfer determined by PAG, 66.7% at 30 days by ultrasonography, and 56.3% at lambing. Overall in vivo survival rate of the transferred embryos was 38.5% (37/96).

In conclusion, the presence of the *FecX*^{R} allele led to reduced follicular size without affecting the number of punctured follicles per ewe and session. However, despite oocytes from R+ ewes came from smaller follicles, they demonstrated to be as competent as those from wild-type ewes for embryo IVP, with no differences between genotypes in survival rate at birth of the transferred embryos.

Notes

SEX AND K-CASEIN MULTIPLEX ANALYSIS FROM BIOPSIED BOVINE EMBRYOS

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Genomic tools are now available for most livestock species and are used routinely for Genomic Selection (GS) in cattle. Recently, multiple markers detection on pre-implantation stage embryos after Whole Genome Amplification (WGA) of genomic DNA from embryo biopsies has been achieved thus allowing to select embryos before transfer (Le Bourhis *et al*. 2011 Reprod. Fertl. Dev.23 (1), 197 abst). This strategy may also provide the opportunity to estimate some traits of particular interest and/or the presence of genetic abnormalities in breeds which GS is not yet available. The present work aimed to test this genotyping possibility of Simmental embryos for sex and k-casein simultaneously before embryo transfer.

Eighteen embryos were collected by conventional techniques from two Simmental donor cows inseminated twice on observed oestrus following a standard superovulation treatment and collected on day 7. Biopsy from 5 to 10 cells was realized on grade 1 (IETS morphological criteria) embryos with a steel blade and biopsied embryos were frozen using a standard protocol until the genotyping results. Biopsies were then transferred individually as dry samples in tubes and sent frozen to the genotyping laboratory. The genomic DNA of each biopsy was amplified using a whole genome amplification (WGA) kit according to the manufacturer instructions (WGA; QIAGEN REPLI-g Mini Kit). After WGA, DNA were diluted 1/10 in AE buffer before genotyping by multiplex PCR-RFLP of the Y-specific and the k-casein loci as an internal amplification control of DNA quality. The final volume of the mixture was 40 µl. One µl of diluted pre amplified DNA was used as a template. The PCR mixture further contained 1X PCR buffer, 1.5 mM MgCl2, 100 µM dNTPs, 0.25 µM primers specific for k-casein and Y-specific locus and 3U of Taq polymerase (Qiagen-France). After the first denaturation step at 95°C for 5 min, samples were subjected to 35 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 45 sec followed by a 5 min extension in the last cycle. PCR products were then digested by 5 U of HindIII restriction enzyme for 2 h at 37°C. Digested fragments were separated electrophoretically using 2% agarose gels containing 0.5 µg/ml ethidium bromide and visualized by UV transilluminator. Y specific primers amplified a 550pb sequence and k-casein primers amplified a 443 pb for AA genotype and 348, 95pb for BB genotype.

Analyses of k-casein and sex genotypes were successful in 16 embryos (89.0%). Eight embryos were sexed as male. Eight embryos were determined to present an AA k-casein genotype, 7 embryos an AB genotype and one a BB genotype.

Coupled with DNA-WGA, the determination of traits of interest such as sex and k-casein genotypes is possible by using a multiplex PCR-RFLP method before transfer of biopsied embryos. Moreover, depending on the result for these specific traits, genotyping using high throughput can be ordered.

Notes

APPLICABILITY OF DAY 0 SUPEROVULATION PROTOCOL IN BOER GOATS

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This study evaluated whether a Day 0 superovulation protocol could be utilised in Boer goats by comparing it to a traditional multiple follicle stimulating hormone (FSH) protocol. Twenty Boer goat does were allocated into two groups of 10 animals per treatment. In Day 0 protocol group, the oestrous cycles of does were synchronised for 9 days with controlled internal drug release dispensers (CIDR) and injected with PGF2α at CIDR insertion. At CIDR withdrawal does were injected with eCG. One dose of GnRH analogue was given 36 hours after CIDR withdrawal. Does were then superovulated with porcine follicle stimulating hormone (pFSH) administered in 7 dosages given at 12 hour intervals, starting 88 hours after CIDR removal. Does were injected with PGF2α concurrently with the fifth and sixth pFSH treatment. For the traditional multiple FSH protocol, does oestrous cycles were synchronised with CIDRs , inserted for 9 days. The superovulatory treatment with pFSH was similar to the Day 0 protocol but initiated 48 hours prior to CIDR removal. Timed cervical inseminations with fresh undiluted semen were performed at 24 and 36 hours following the last PGF2α injection for Day 0 protocol and 24 and 36 hours following CIDR withdrawal for the traditional multiple FSH protocol. Ultrasonographic evaluation of the ovaries was performed at the initiation of a superovulation treatment in both protocols. Embryos were surgically flushed on day 6 following the second artificial insemination for both groups. The total number of corpora lutea, structures (embryos and unfertilised ova), unfertilised ova, embryos, degenerated embryos and transferable embryos flushed did not differ significantly between treatments. The number of follicles 2-3 mm, 4-5 mm and total number of follicles at the beginning of a superovulation treatment was positively correlated to the total number of structures and embryos recovered. The number of follicles with a diameter ≥ 6mm at the beginning of superovulation treatment was positively correlated with the number of degenerated embryos. The two superovulation protocols led to similar response to superovulation and quality of embryos recovered therefore, a Day 0 protocol could be used in Boer goats. The results of this study suggest that superovulation protocols used had limited effect on the response to superovulation and quality of embryos recovered. Therefore, more efforts should be directed on creating favourable environment at the initiation of a superovulation treatment, as the response and quality of embryos recovered are more associated with the size and number of follicles at the beginning of a superovulation treatment.

Notes

EXTRACELLULAR VESICLES SECRETED BY BOVINE OVIDUCTAL EPITHELIAL CELLS INCREASE THE QUALITY OF IN VITRO PRODUCED BOVINE EMBRYOS

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Bovine oviductal epithelial cells (BOEC) have been used extensively in in vitro culture for improving embryo quality; however, they can be used also as a model studying embryo-maternal interactions. Extracellular vesicles (EV) released from cells constitute are recently unveiled mode of intracellular communication. The aim of the present study was to evaluate the developmental capacity of bovine zygotes and the quality of the produced embryos when cultured in vitro with previously purified EV secreted by BOEC monolayers. Presumptive zygotes were produced by in vitro maturation and fertilization of oocytes derived from ovaries of slaughtered heifers and cultured in groups of 20-25 in droplets of 25µL under specific conditions. Frozen line of BOECs was cultured in DMEM till cell confluence. Media were replaced with SOF+5% FCS for further 48h culture and then EV were purified from conditioned media and suspended in SOF with or without serum and used for embryo culture. EVs were quantified with the use of Nanosight and their integrity and size assessed by electron microscopy. In the first experiment, presumptive zygotes were cultured with fresh or frozen EV of different concentrations: SOF+5% FCS (C^+); C^+ with $3x10^5$ EV/mL; C^+ with $1.5x10^5$ EV/mL; and C^+ with $7.5x10^4$ EV/mL. In the second experiment, presumptive zygotes were cultured with frozen EV in the absence of FCS: SOF+5% FCS (C^+); SOF (C^-); and C^- with 3 x 10^5 EV/mL. Cleavage rate was assessed on day 2 and blastocyst development on day 7, 8, and 9 (D0: day of fertilization). Representative number of blastocysts on days 7/8 was used for quality evaluation through differential cell count and survival after vitrification/warming. In the first experiment, no differences were found between groups for cleavage rate or blastocyst yield on Day 7-9 (range: 87.4±1.0-89.6±0.9 and 40.7±1.9-45.7±3.1 for cleavage rate and blastocyst yield on day 9 respectively). Total cell number was significantly higher in embryos derived from fresh or frozen EV (range: 175.3±8.0 - 191.3±8.6) when compared to C^+ (160.8±7.3), and this was due to a greater number of Trophectoderm Cells (TE) between EV groups (range: 127.4±5.8-131.7±5.6) and C^+ (111.5±5.4). No differences were found between groups in terms of Inner Cells Mass (ICM) (range: 47.9±2.8 - 59.3±3.7). After vitrification/warming, significantly more embryos survived at 24 hours for EV groups (range: 67.2±3.4 - 75.5±2.9), compared to C^+ (44.1±4.4). Similarly, with increasing time post warming, at 72 hours, embryos from EV groups survived significantly higher (range: 48.6±3.2-56.3±3.7) than the C^+ (22.3±4.2). In the second experiment, no differences were found between groups for cleavage rate, or blastocyst yield on Day 7-9 (range: 84.5±0.9- 86.3±1.4 and 26.7±2.1-28.9±1.2 for cleavage rate and blastosyst yield on day 9 respectively). After vitrification/warming, significantly more embryos survived at 24 hours for C^- and EV groups (range 54.4±7.0 - 67.2±4.3), compared to C^+ (42.2±6.78). Similarly, with increasing time post warming, at 72 hours embryos from C^- and EV groups survived significantly higher (range: 35.6±7.7-37.0±4.8) than the C^+ (16.4±5.2). The size of EV was confirmed to 220 µm by Nanosight and electron microscope. In conclusion, the use of extracelullar vesicles secreted from BOEC in *in vitro* culture has a positive effect on the quality of *in vitro* produced bovine embryos particularly on the absence of FCS. In addition, these data reveal that extracellular vesicles have functional communication between the oviduct and the embryo in the early stages of development.

Notes

EFFECT OF VITAMIN C AND PSAMMAPLIN A ON MOUSE SOMATIC CELL NUCLEAR TRANSFER EMBRYOS

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Nuclear reprogramming of differentiated cells towards an embryonic totipotent state through somatic cell nuclear transfer (SCNT) is still an inefficient process. Recent studies suggest that vitamin C (VitC) could have a positive role in nuclear reprogramming as it improves induced pluripotent stem cells generation and the development of pig cloned embryos, and acts as an epigenetic modifier. The aim of the present work was to investigate the effect of VitC on mouse SCNT and whether this effect is additive with that of psammaplin A (PsA), an histone deacetylase and DNA methyltransferase inhibitor that increases SCNT efficiency.

Enucleated oocytes were reconstructed with a cumulus cell nucleus, parthenogenetically activated and cultured *in vitro*. The resulting blastocysts were stained to analyse the number of trophectoderm and inner cell mass (ICM) cells and also to quantify the expression of Cdx2, Oct4 and Nanog.

In a first set of experiments, embryos were exposed to VitC 100 µM during increasing periods of time (8 h, 16 h, 24 h and 120 h post-activation). We found that the addition of VitC to embryo culture medium for at least 16 h post-activation significantly increased blastocyst rates (ranging from 42.6 to 45.9 %) and the mean cell number of ICM (ranging from 14.3 to 15.3) in SCNT embryos compared with the control group (26.7 % of blastocysts and 10.7 cells, respectively). In a second set of experiments, we compared the effect of VitC 100 µM and PsA 10 µM during 16 h and we combined both treatments. We found that the combination of VitC and PsA did not significantly improve blastocyst rate and quality when compared with treatments with only VitC (56.6 % and 14.2 ICM cells *vs*. 52.1 % and 9.6 ICM cells, respectively), but it improved blastocyst rate when compared with treatments with only PsA (41.3 %). Moreover, the combined treatment with VitC and PsA significantly increased Oct4 expression in the cloned blastocysts, similar to the treatment with PsA only, whereas VitC treated blastocysts showed no differences in Oct4 staining but an increased Cdx2 expression was found.

In conclusion, the combined VitC-PsA treatment improves blastocyst rate, quality and pluripotency compared with non-treated SCNT embryos. Studies are currently being performed to determine whether this improvement in blastocyst rates by VitC and the combination of VitC and PsA treatments correlates with an increased development to term.

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Notes

HYALURONAN DEGRADATION IS REQUIRED FOR OPTIMAL EMBRYO DEVELOPMENT IN SHEEP

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Hyaluronic acid, an important component of the extracellular matrix, exists in a wide range of molecular sizes (from tetrasaccharides up to 10,000 kDa) depending on its synthesis and degradation. The biological functions of HA are highly dependent on its size. We have recently shown that HA synthase enzymes and HA receptor CD44 are expressed in the *in vitro*-produced cleaved embryos until the blastocyst stage whereas Hyaluronidase-2 (HYAL2; which degrade HA into ~25 kDa fragments) is expressed in the oviduct but not in the embryos until the Morula stage. In the present study we examined the effect of HYAL2 supplementation on development of *in vitro* produced sheep embryos to blastocyst stage and its quality. We also examined the effect of Hyalovet® (HA ~500 kDa) alone or pre-treated with HYAL2. Our results show that significantly higher ($P<0.05$) percentage of embryos supplemented with HYAL2 reached blastocyst stage compared to control. HYAL2 did not affect hatching rates. In contrast, Hyalovet had no effect on development to blastocyst but completely blocked hatching ($P<0.05$). Pre-treatment of Hyalovet with HYAL2 numerically increased blastocyst rate ($P>0.05$) and abrogated the inhibitory effect on hatching to become similar to control ($P>0.05$). Total cell numbers tended to be higher in HYAL2 treated embryos ($P>0.05$) and resulted in significantly lower apoptosis ($P<0.05$). These results are in accordance with our previous observation that infusion with HYAL2 in sheep oviducts of superovulated ewes on day 2 of pregnancy resulted in more blastocysts of better quality. These on-going experiments provide strong evidence that HYAL2 but not larger size HA is required for optimal early embryo development and can be considered as a valuable supplement for embryo culture media.

Notes

NEW THECNOLOGY FOR THE VITRIFICATION, FIELD-WARMING AND DIRECT TRANSFER OF IN VITRO PRODUCED BOVINE EMBRYOS

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The introduction of the vitrification technology into current practice would require that vitrified embryos could be warmed in-straw dilution in order to be directly transferred to the uterus, at the same level of difficulty as one artificial insemination. Herein we developed a new device (designated VitTrans) for embryo vitrification that provides microscope-free warming and direct embryo transfer. The advantages of this method are that warming is easily and rapidly achieved and embryo transfer is directly performed without the need for optical equipment and using a 0.5 mL french straw as the protective cover, being optimal for its use in field conditions. This study was performed to test the use of the VitTrans for the vitrification of bovine embryos in terms of its effects on embryo survival and apoptosis after warming. *In vitro* produced 7-day-old blastocysts were vitrified in a mixture of 15% ethylene glycol + 15% DMSO + 0.5M sucrose using the cryotop or the VitTrans as devices. Embryos were warmed using a 0.5M sucrose solution and transferred to the SOF culture medium. Survival rates were determined as the rates of re-expanded, hatching and hatched blastocysts at 24 h post-warming. The TUNEL method was used to quantify total cell numbers and apoptosis in each blastocyst. When the VitTrans was used for vitrification, 61.5% (n=40) of blastocysts survived at 24 h post-warming whereas a significantly higher percentage of embryo survival was recorded when the cryotop was used as a device (89.1%; n=57). However, the two cryodevices yielded similar proportions of embryos developing to the hatching/hatched blastocyst stages after 24 h of culture (Cryotop: 33%; VitTrans: 35%). Moreover, no significant differences were observed in total cell numbers (Cryotop: 173.0 ± 9.61; VitTrans: 146.3 ± 10.4) and apoptosis indices (Cryotop: 8.21% ± 1.96; VitTrans: 12.5% ± 1.77) calculated for each blastocyst when comparing both groups of embryos. Although embryo survival rates were lower than those obtained using the cryotop as the vitrification device, this study provides useful data on which to base future studies designed to optimize this new technique and test its performance in field conditions.

Notes

DEVELOPMENT OF A SERUM AND BSA FREE MEDIUM FOR THE IN VITRO CULTURE OF BOVINE EMBRYOS

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The presence of fetal calf serum (FCS) in the culture medium has been shown to have a negative effect on embryo quality; the use of animal-derived proteins in culture media increases the risks of disease transmission through in vitro embryo production. The aim of this study was to develop an in vitro embryo culture medium that is free from FCS and bovine serum albumin (BSA), but with the addition of various growth factors and cytokines (GF-CYK) (IGF-I, IGF-II, bFGF, LIF, TGF-β1 and GM-CSF) 50ng/ml, supplemented with polyvinilpirrolidone (PVP) and recombinant albumin (RA), or hyaluronan (HA) and recombinant albumin (RA). Bovine oocytes (n=1255, 5 replicates) from abattoir ovaries were matured in TCM 199 medium supplemented with 60µg/ml penicillin, 60µg/ml streptomycin, and 10ng/ml EGF for 24h at 39°C and 5% CO_2 in humidified air. After maturation, the oocytes were fertilized in IVF-TALP medium supplemented with 6mg/ml fatty acid-free BSA and 1.7 IU/ml heparin for 18h under the same conditions. After fertilization, the cumulus cells were removed from the presumptive zygotes by vortex. Presumptive zygotes were divided into three groups and cultured in 30 µl droplets of synthetic oviductal fluid (SOF) supplemented with: 1) 0.4% BSA + 5µg/ml insulin, 5µg/ml transferrin, and 5ng/ml selenium (ITS) as a control (CONT); 2) GF-CYK + 0.4% PVP + 0.15% RA (M1), or 3) GF-CYK + 0.5mg/ml HA + 0.15% RA (M2). Droplets were preserved under mineral oil in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 39°C. Blastocyst development was observed at 7 and 8 days post fertilisation (dpf). At 7 dpf, the percentages of blastocysts were not significantly different between the control: 33.5% ± 4.0 and M2 medium: 31.3% ± 3.4 ($P > 0.05$); however a significantly lower blastocyst rate was observed for the M1 medium: 17.9% ± 1.5 ($P < 0.05$). At 8 dpf no significant difference was observed between the control and M2 medium (40.8% ± 4.4 and 38.1% ± 3.2 respectively); the blastocyst rate was significantly lower with the M1 medium: 29.8% ± 2.6. In conclusion, the FCS and BSA free medium with GF-CYK, HA, and RA (M2) shows a comparable development rate to the control medium; as an entirely synthetic method of embryo culture, it presents a valuable tool for reducing the risks of disease transmission via embryo transfer.

Notes

THE EFFECT OF DIFFERENT SUPEROVULATION PROTOCOLS ON MOET SUCCESS OF DORPER EWES

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Unpredictable variability in the superovulatory response is the most critical step in sheep embryo production programs. This is attributed to a number of endogenous (genetics, nutritional status, follicular status, season of the year) and exogenous (superovulatory treatment, nature and possible 'contamination' of the gonadotrophin) factors (Baril et al., 1993). The superovulatory response and embryo yields were studied in Dorper ewes raised in southeast of Anatolia. Three years old primiparous donor ewes weighing of 60- 65kg on average received a CIDR for 14 days. The ewes were divided into four superovulation treatment groups such as; Exp. I: 12ml Folltopin + 200 I.U. eCG (n=25); Exp. II: 10ml Folltropin + 200 I.U. eCG (n=20); Exp. III: 10 ml Folltropin + 600 I.U. eCG and Exp. IV: 10ml Folltropin + 750 I.U. eCG with single shot of FSH at CIDR removal. Donors were superovulated using FSH-p (total of 20 mg) (Folltropin-V; Vetrepharm, Canada) applied in six decreasing doses of 3, 2.5, 2, 2, 1,5, 1 ml i.m. starting 60h before CIDR withdrawal. Donors were sedated with anesthetic cocktail containing 100 mg ketamine (Vetalar, Boehringer Ingelheim Vetmedica, Inc) and 0.12mg xylazine (Romphun, Bayer) were subjected to intrauterine insemination with fresh diluted semen (a minimum of 50 x 10^6 motile sperm/each uterine horn) 40h after sponge removal. Embryos were collected 6 days after insemination. The success of the superovulation program was found similar within all treatment groups with similar total number of CL (I: 11.7±1.18; II: 9.7±1.31; III: 8.4±1.76 and IV: 9.9±1.63). Superovulation response (CL>3) was found relatively but not significantly higher in the groups I (92%) and II (95%) treated with a lower dose of eCG compared to III (76%) and IV (77%). Transferable embryos were found slightly higher (P: 0.333) in donors in exp. III with 7.8 ±1.2 than those recorded for donors in exp. I (4.7±1.12), II (6.3±1.24) and IV (6.3±1.7). The only significant difference was observed in embryo recovery rates. Donors in exp. II and III were recorded with 63% embryo recovery rates while donors in exp I (38%) and IV (43%) resulted with significantly lower ($P<0.05$) recovery rates. In conclusion, it was found that Dorper ewes treated with different superovulation protocols out of season (May) yielded on average in 6 transferrable embryos and a single FSH injection can replace decreasing doses of multiple FSH injections.

Notes

EFFECT OF HYALURONAN ON IN VITRO MATURATION OF BOVINE OOCYTES ON DEVELOPMENTAL COMPETENCE AND QUALITY OF OOCYTES AND OBTAINED BLASTOCYSTS

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The objective of the present study was to evaluate the effect of hyaluronan (HA) during IVM on meiotic maturation, embryonic development, quality of oocytes, granulose cells and obtained blastocysts. To our knowledge, this is the first attempt that has been made to mature oocytes *in vitro* in the presence of exogenous hyaluronan. COCs were matured in vitro in three types of medium: control, medium with addition of 75µl and 150µl of HA (final concentration of 0.035% and 0.07% respectively).

The meiotic maturity did not differ between analyzed groups. The number of obtained blastocysts was the highest when 150µl of HA was used, however there was no significant difference between this group of oocytes and the Controls. The quality of blastocysts developed from oocytes matured with 150µl HA was the highest- the significant difference for DCI was noted between these blastocysts and the Controls ($P<0.001$). To better analyze the impact of HA we estimated the relative BAX and BCL2 transcript levels in granulose cells (GC) which served as co-culture. We found significantly higher Bax mRNA expression in GC cultured with 150µl HA compared to Controls ($P<0.001$) and GC cultured with 75µl HA ($P<0.05$). No significant differences were noted in Bcl-2 relative expression between analyzed groups of GC.

Our results suggest that addition of 150µl HA per 2ml of IVM increases the number of developed blastocysts and decreases the level of DNA fragmentation. The mechanism of this action needs further research.

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Notes

THE EXPRESSION LEVEL OF SELECTED PROTEINS IN UNDIFFERENTIATED PORCINE MESENCHYMAL STEM CELLS AND STEMS CELLS SUBJECTED TO DIFFERENTIATION[1]

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Mesenchymal stem cells (MSC) isolated from adult tissue, and cultured under appropriate conditions have the ability to differentiate in the bone, cartilage, adipose tissue and skeletal muscle (Caplan, 1991). The application of MSC created high expectations in regenerative medicine due to participation in the process of damaged tissues or organ repair. In some of these treatments the non-differentiated MSC are used while in others before administrating the cells into the patient they must be differentiated towards the required cell type. Unfortunately, the *in vitro* culture conditions as well as differentiation can adversely affect the quality of cells. Moreover, the efficacy of cells subjected to differentiation to undergo desired direction of transformation is low (Cai et al., 2011). The situation is complicated by the fact that there are differences in the expression of typical MSCs markers between the species as well as within the same species between the lines (Bieback et al., 2012). These differences are most probably caused by different protocols for MSC isolation and expansion as well as *in vitro* culture (Bieback et al., 2012). Taking into consideration all the above-mentioned reasons, it is justified to search for genes involved in differentiation and to monitor MSCs quality in varied points of their *in vitro* culture.

The aim of the research was i) to estimate cell quality by evaluating the proapoptotic BAD protein expression in undifferentiated and subjected to differentiation mesenchymal stem cells ii) to estimate if the matrix metalloproteizanze-2 (MMP2) protein is involved in adipogenic and/or osteogenic differentiation.

MSCs isolated from the pig bone marrow were cultured in vitro for five weeks and then a portion of cells was subjected to differentiation towards the osteocytes and adipocytes. In all groups of cells the protein expression was estimated by western blotting.

The significant differences in BAD expression were noted between cells differentiated towards the adipocytes and the osteocytes ($P < 0.05$). No significant differences were observed between the analyzed groups in MMP2 protein expression.

Summarizing the results, we conclude that BAD protein can be used to assess the quality of mesenchymal stem cells. Interestingly, the level of BAD expression in differentiated cells was lower than in undifferentiated cells demonstrating that the culture conditions including medium composition does not adversely affect the quality of MSC. Moreover, the MMP2 gene does not show commitment to the differentiation of stem cells into the appropriate cell line.

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

Notes

IMPROVING TECHNOLOGY EFFICIENCIES IN ARTIFICIAL INSEMINATION, EMBRYO RECOVERY AND EMBRYO TRANSFER IN DORPER SHEEP AND DOMASCUS GOATS

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Abstract

A commercial trial using 100 Dorper ewes and 30 Domascus goats as donors and 2,000 Merino ewes as recipients in a MOET program at Zirve University was devised to demonstrate innovative technologies that improve the efficiency of laparoscopic artificial insemination, embryo flush and embryo transfer.

Efficiencies in laparoscopic artificial insemination are improved with the use of an innovative designed AI gun (Minitube) used for direct uterine insemination in set time AI programs. Throughput of AI for animals is increased which reduces stress to the ewe and assists with fertility.

A new technique has been developed to flush embryos from superovulated sheep & goats using flushing techniques that drains into a Minitube MiniFlush embryo filter dish for embryo collection. The technique allows for quicker flushing and embryo recovery without detriment to the numbers of embryos retrieved.

Embryos are transferred into programmed recipients using the Minitube AI gun. Recovered fresh embryos are loaded into straws and injected directly into a uterine horn that has an associated corpus luteum(s) on its ovary, a similar technique to laparoscopic AI. The technique does away with exteriorising a uterine horn and injecting the embryo into that horn, potentially improving numbers of pregnancies by reducing possible uterine damage and trauma and adhesions. The transfer technique improves the numbers of transfers that are able to be performed per day. There is potential to use frozen direct transfer embryos or vitrified embryos for direct transfer.

These techniques simplify the artificial breeding technologies around laparoscopic artificial insemination and embryo flush and transfer. The techniques are readily adaptable to commercial use and are ideal for assisting to improving sheep numbers in regions developing their small ruminant industries.

Key words: embryo transfer, artificial insemination, new technologies,

Notes

EFFECT OF EXOGENOUSLY SUPPLIED PROGESTERONE ON THE BOVINE CORPUS LUTEUM DURING EARLY PREGNANCY

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Exogenous progesterone (P4) supplementation around the time of conception supposedly improves pregnancy rates. Aim of the present study was to assess the effects of such a supplementation on the bovine corpus luteum (CL) in sixty Holstein-Frisian cows. The animals were randomly allocated to one of three groups: (1) *CIDR*: CIDR® intravaginal device between day 4 and 10 post insemination (p.i.); (2) *CIDR Placebo*: CIDR® placebo (without P4) between day 4 and 10 p.i.; (3) *Zero*: Untreated control animals.

Animals were estrous synchronized and inseminated artificially (d0). Blood samples (for P4 determination) were obtained in the mornings of days -10, 0, 4, 6, 9, 12, 16, 20 and 42 as well as on the evening of day 4. Furthermore, luteal size and blood flow were concurrently analyzed via transvaginal ultrasonography from d4 onwards. Only animals with a serum P4 concentration of <1 ng/ml on day 0 were further included in the study.

Transvaginal ultrasound-guided CL biopsies were obtained on days 6, 9, 12, 16 and 42 for luteal P4 determination. The biopsies obtained on days 6, 12 and 42 were analyzed regarding the relative mRNA abundance of genes associated with P4 and prostaglandin metabolism as well as angiogenesis (STAR, CYP11A1, HSD3B1, VEGF, PGR, LHCGR, PTGS2, PTGFR). Pregnancy rates were determined by ultrasonography on d30 and d42 allowing a further subdivision of the groups: *CIDR pregnant*: n=6, *CIDR non pregnant*: n=7, *CIDR Placebo pregnant*: n=8; *CIDR Placebo non pregnant*: n=6, *Zero pregnant*: n=6; *Zero non pregnant*: n=8.

The following results could be obtained: Pregnancy rates (*CIDR*: 46.2%, *CIDR Placebo*: 57.1%, *Zero*: 42.9%), luteal size and blood flow did not differ among groups.

Serum P4 concentrations of animals belonging to the *CIDR* group were significantly increased on the evening of day 4 and on day 6. Pregnant *CIDR* animals had increased P4 concentrations on days 9, 16, 20 and 42 ($p \leq 0.07$) in comparison to pregnant animals of both other groups.

In CL biopsies of animals allocated to the *CIDR Placebo* group the relative amount of CYP11A1 (on day 42) and HSD3B1 (on days 12 and 42) mRNA was increased.

The relative abundance of VEGF was significantly higher in CL tissue of *CIDR pregnant cows* on day 6 in comparison to pregnant animals of both other groups.

On day 42 the relative abundance of PGR was higher in CL biopsies of all *CIDR Placebo* animals in comparison to those of animals without treatment. The relative amount of LHCGR was increased in luteal tissue of all *CIDR* group animals the on day 42.

In conclusion, external P4 supplementation does not affect pregnancy rates, whereas serum P4 concentration can be increased by the application of exogenous P4. All analyzed luteal parameters remain unaffected by the CIDR® device.

The analysis of the relative mRNA abundance leads to the assumption that P4 and prostaglandin metabolism and angiogenesis are not directly affected by the exogenous P4 supplementation. Nevertheless, P4 supplementation influences the expression of LHCGR mRNA possibly resulting in the increased secretion of P4 on day 42.

Notes

TRANSCERVICAL EMBRYO COLLECTION IN LATE STAGE IN CATTLE.

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Transcervical embryo collection is used routinely in the bovine species throughout the world to collect D6 to D9 embryos (Early embryos) for genetic selection. For research purposes, however, the collection of embryos at later stages of pregnancy, i.e., D12 to D21 (late embryos), is needed. So far, for the recovery of late embryos, females are euthanized and embryo collection is performed after recovery of the genital tract. In order to reduce the number of animals used and still provide valuable material for embryo research, we have therefore developed a transcervical technique to collect late embryos. The objective of this study was to compare embryo recovery results at early and late stages within our laboratory.

Altogether, 232 cows were used for this study. 145 flushes were performed to collect D6-D9 embryos and 251 flushes were performed to collect D12 to D21 embryos. For the early embryos, a classical 3 way collection equipment (IMV, France) was used. To collect the late embryos, the same equipment was used but the extensible flexible catheter that goes inside the external rigid catheter was removed, so that larger embryos could be collected through the remaining larger hole (2 way collection). All females were submitted to ovum pick-up to remove the dominant follicle and were subsequently superovulated with FSH (Stimufol®, Reprobiol). Luteolysis was induced 48 hours prior to AI. Two AI were performed with frozen semen, 48 and 56 hours after PGF2□ injection. Before embryo collection, cows were treated with an epidural injection of 3-4 ml Lidocaine®. The presence of copora lutea (CL) was checked and they were counted by rectal palpation. For all collections, the cervix was prepared with the initial introduction of a dilator. Then the catheter was introduced in one horn and the cuff was inflated as low as possible. For the collection of late embryos, 30 ml (Euroflush, IMV) was injected slowly twice to suspend the embryos prior to flushing the horn with 500 ml, and the same operation was performed on the second horn.

There was no significant difference in the number of embryos collected per flush in the early and late stages (758 embryos collected, 5.22 ±6.02 per flush vs 1238 embryos collected, 4.93 ±5.07 per flush, respectively). The number of embryos collected per corpus luteum, however, was significantly lower in the early vs late group (0.39% ±0.32 vs 0.44% ±0.34, respectively). Late collection allowed the retrieval of full embryos (Inner Cell Mass + trophoblast), even at very late stages such as D18 to D21. Careful collection is needed, however, so that embryonic tissues are not damaged or torn: the horn must be massaged gently and the flush should be ideally recovered in one single flow.

This technique is a powerful tool to collect late stage embryos for research purposes. It can also be applied to recover single embryos after AI or ET. Since it is not traumatic, animals can be used again for the same procedure.

Notes

MITOCHONDRIAL ACTIVITY IN PRE-IMPLANTATION CULTURED PORCINE EMBRYOS AFTER SUBLETHAL HIGH HYDROSTATIC PRESSURE STRESS

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Despite the improvement of *in vitro* pig embryo production methods over the last years, the quality and developmental competence of *in vitro* produced blastocysts is lower compared with their *in vivo* counterparts. An innovative technique to increase the subsequent stress tolerance during embryo culture is a sublethal, high hydrostatic pressure (HHP) (Pribenszky et al., *Reprod Dom Anim* **46**(Suppl.2), 26-30, 2011). On the other hand, mitochondrial activity is an important determinant of the normal pre-implantation embryonic development because this activity correlates with cellular energy demand during embryo cleavage, compaction and blastulation. Therefore, manipulation of mitochondrial activity may improve performance of embryos produced *in vitro*. Our study describes in what way HHP treatment may alter mitochondrial activity during porcine embryo development from zygote to the blastocyst stage.

Porcine zygotes were recovered from superovulated gilts. After collection the embryos were cultured in NCSU-23 medium in an atmosphere containing 5% CO_2 in air at 39°C. Embryos at 2- to 4-cell, morula and blastocyst stages were selected on days 2, 4 and 6 of culture, respectively. In the experimental group, for all analyzed stages of development, embryos (N=69) were treated with 20 Mpa hydrostatic pressure for 60 min 39°C, with an interval of 60 min between HHP treatment with subsequent measurements of mitochondrial activity. In control group, embryos (N=59) were not treated HHP. To estimate mitochondrial activity, all embryos were labeled with 0.5 μM MitoTracker Orange CMTMROs (Molecular Probes) for 30 min. at 39°C and subsequently analyzed in LSM 510 META confocal microscope (Carl Zeiss GmbH).

Mitochondrial activity (A_v) measured in arbitrary unit (proportional to the amount of fluorescence emitted from mitochondria) was equal 5.91, 5.98, 42.39 and 99.19 for zygote, 2- to 4 cell, morula and blastocyst stage of control group, respectively, whereas after HHP treatment A_v was: 1.34 (zygote), 2.18 (2- to 4 cell), 13.61 (morula) and 31.44 (blastocyst). For both, the control and experimental groups, from zygote up to the 2- to 4 cell stage the mitochondrial activity was very low and remained unchanged, then significantly increased at morula stage and increased again in the blastocyst stage ($p<0.01$). Finally, we detected statistically significant difference ($p<0.01$) between developmentally matched, HHP treated and non-treated embryos. While the mean value of A_v was equal for HHP treated and HHP non-treated zygotes as well as 2- to 4 cell stage, the mitochondrial activity was three times lower in HHP treated morula and blastocyst in comparison with non-treated ones.

In conclusion, sublethal HHP stress resulted in decreasing of mitochondrial activity during porcine embryo development from morula up to the blastocyst stage. These results suggest that HHP could improve the developmental competence of pre-implantation pig embryos cultured *in vitro* because of lower production of reactive oxygen species (ROS). Further investigations regarding ROS and ATP level are required.

Notes

SERUM ANTI-MULLERIAN HORMONE (AMH) CORRELATES WITH OVARIAN RESERVE AND EMBRYO PRODUCTION IN SUPEROVULATED HOLSTEINS

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Serum AMH relations with oocyte numbers, and in vivo embryo production in Holstein heifers were evaluated at weekly intervals during the estrous cycles and monthly for six months in 15 unstimulated cows. In addition, serum AMH was evaluated over 3 consecutively induced cycles at ovum pick-up in 41 superovulated heifers and at embryo flushes in 125 others. A modified Ovsynch protocol with 4 days of decreasing FSH (Pluset H Minitube of America) was used. Blood samples were collected in serum tubes and spun within 2 hours. The samples were stored at -20C until evaluation using the Minitube of America AMH-Bovine specific immunoassay (AMH Fertility Assay). The statistical analyses were performed using Statview 5 with $P<0.05$.

Serum AMH among animals ranged from 43 to 960 pg/mL. The average AMH level of all cows was stable during the estrous cycle and for each of the 6 monthly consecutive measurements. There was a high correlation between all values per animal ($P<0.01$) suggesting AMH levels are consistent during the cycle and for at least several consecutive months. Animals which were repeatedly stimulated showed decreasing AMH levels (509±295; 299±210; 211±119) and a decrease in recovered embryos (7.4±4; 5.6±3.8; 4.2±3.2; P=0.02). The number of oocytes was not altered by multiple stimulations (10.4±9.8; 11.3±6.2; 8.5±7.6; P=0.75). As AMH and embryo numbers decreased after multiple stimulations, only the first AMH value and results of the first OPU or flush were used to establish following correlation. Serum AMH showed a positive correlation to the number of oocytes (r2=0.245) and embryos collected (r2=0.27).When separated into AMH categories, low (<100), normal (100-400), and high (>400 pg/mL), high AMH OPU animals yielded significantly higher numbers of oocytes than the animals in the normal or low AMH groups (13.8±9.2; 9.2±5.2; 5.6±3.9; P=0.001). Flushed animals with high AMH levels had significantly higher numbers of embryos than the ones with low AMH (10.9±7.9; 5.7±5; P=0.002). Comparison of the first AMH value to the average number of oocytes or embryos collected over the course of 3 collections/animal, showed a positive correlation to the average number of oocytes/collection from individual OPU donors (r2 =0.436) and a positive correlation to the average number of embryos/collection from individual donors (r2=0.176). When separated into AMH groups, high AMH flushed animals had significantly higher numbers of embryos than the normal or low AMH ones (9.3±3.1; 5.7±3.4; 4.5±2; P=0.0001). As OPU animals with low AMH were only used once, average oocyte/collection data was not available for this category. There is a significant difference between the high and normal AMH categories (12±3.6; 7±2; P=0.0001). Circulating AMH is stable over time in unstimulated but decreases in repetitively stimulated animals. AMH is highly associated with superovulation response, oocyte and embryo production and its use should improve animal selection to achieve improve efficiency of MOET.

Keywords: AMH, anti-mullerian hormone, bovine, ovarian reserve, fertility

Notes

BIRTH OF THE FIRST CALF OF MURCIANA-LEVANTINA BOVINE BREED DERIVED BY OPU, *IN VITRO* PRODUCTION AND EMBRYO VITRIFICATION

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In a conservation project, reproductive biotechnology was implemented for the recovery and conservation of an endangered bovine breed in Spain. The breed Murciana-Levantina, declared to be worthy of special protection status (FAO, 2006), is of great interest because of its hardness, longevity, docility and disease resistance. This contribution describes the birth of the first calf of this breed obtained by reproductive biotechnology, using ultrasound-guided punction and aspiration of ovarian follicles (OPU), *in vitro* embryo production, vitrification of embryos by a cryotop device and, finally, the transfer of cryopreserved embryos to recipient heifers of a commercial dairy herd. The biological mother of the calf presented here was a 30 month old Murciana-Levantina heifer. The recipient females were dairy heifers (Holstein).

OPU involved removal of the dominant follicle by GnRH (Dalmarelin®), followed 48 h later by 500 IU FSH-LH (Pluset®) and OPU 48 h later (Chaubal et al. 2006). *Cumulus*-oocyte complexes (COCs) with categories I-III were considered suitable for processing *in vitro* (Zaraza et al. 2010). COCs were cultured in maturation medium drops (TCM-199) (10 µl/oocyte). Semen of a Murciano-Levantino bull was obtained by electroejaculation and frozen with a classical freezing process in a computer-controlled freezer. Spermatozoa were capacitated by the swim-up procedure and the sperm concentration was adjusted to 10^6 spermatozoa/ml. The embryo culture medium was synthetic oviductal fluid (SOF) (Holm et al. 1999) supplemented with 5% FBS (5 µl/embryo). Cleavage rates were recorded 48 h after insemination and the number of blastocysts was determined on day 7 post insemination. The blastocysts were vitrified using a cryotop device (Kitazato®, Fujinomiya, Japan) following the protocol defined by Kuwayama et al. (2005) and the vitrification and warming media preparation for bovine embryos described by Morató et al. (2010). The synchronization protocol included the initial application of one dose of a GnRH (Dalmarelin®) together with the insertion of a CIDR® device (12 days). Two days before the withdrawal of the devices, a dose of $PGF_{2\alpha}$ (Dinolytic®) was applied. On the heat-day a second dose of GnRH was administered. ET was performed by placing the embryo in a deep position of the horn ipsilateral to the *corpus luteum*, after epidural anesthesia (Astiz et al. 2012).

A total of 6 OPUs were performed with an outcome of 66 total COCs and 35 viable COCs (53%). The mean number of viable COCs obtained by OPU in each session (5.8) was similar than that obtained in Holstein cows stimulated with FSH (De Roover et al. 2008). During the fourth OPU, a total of 30 follicles were aspirated from the biological mother, from which 17 total oocytes were recovered (56.7%) of which 10 were viable COCs (58.8%; 2 COCs type I, 1 type II and 7 type III). The cleavage rate was 40%, with a final outcome of 2 blastocysts of quality I. Embryos were vitrified and both vitrified-thawed embryos recovered their original shape within 2 h. One vitrified embryo quality I was transferred to a Holstein heifer with a *corpus luteum* of 3.3 cm^2 of luteal tissue. Pregnancy was diagnosed on day 30 post-transfer by transrectal ultrasound and reconfirmed on day 60. The pregnancy was normal, as were calving and the recovery of the newborn. The female calf named "Fuensanta" weighing 33 kg, which is within normal limits for this breed and therefore the calf was not suffering from large calf syndrome. At the moment, from four donor cows, a total of 9 embryos have been achieved. From these embryos, 7 have been transferred after cryoconservation and warming with a 100% of survival rate and 2 after fresh transfer, with the pregnancy that we referred in this communication. In addition, all four donor cows became pregnant after AI and had successful pregnancies. The work continues and in the near future we hope to present more Murciana-Levantina calves derived from this project.

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Notes

HIGHLY EFFICIENT *IN VITRO* PRODUCTION OF NUCLEAR-TRANSFERRED PIG EMBRYOS USING ADULT BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

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The current study was aimed at investigating the effect of adult bone marrow-retrieved mesenchymal stem cells (ABM-MSCs) that provided the source of nuclear donor cells on the extracorporeal development of porcine cloned embryos. In the somatic cell cloning procedure, oocytes that had acquired the meiotic maturity under *in vitro* culture conditions were utilised as nuclear recipient cells. The cumulus-oocyte complexes (COCs) were matured *in vitro* for 20 to 22 h in TC 199 medium supplemented with 10% foetal bovine serum (FBS), 10% porcine follicular fluid (pFF), 5 ng mL^{-1} recombinant human basic fibroblast growth factor (rh-bFGF), 10 ng mL^{-1} recombinant human epidermal growth factor (rhEGF), 0.6 mM *L*-cysteine, 1 mM dibutyryl cyclic adenosine monophosphate (db-cAMP; bucladesine), 0.1 IU mL^{-1} human menopausal gonadotropin (hMG) and 5 mIU mL^{-1} porcine follicle-stimulating hormone (pFSH). The COCs were subsequently cultured for an additional 22 to 24 h in the fresh maturation medium lacking db-cAMP, hMG and pFSH. To create the ooplast-nuclear donor cell couplets, the previously enucleated oocytes underwent microinjection of either contact-inhibited/trypsinised ABM-MSCs (Group I) or adult cutaneous fibroblast cells (AC-FCs; Group II) into their perivitelline spaces. The ooplasts were then electrofused with somatic cells and simultaneously electroactivated by application of two consecutive DC pulses of 1.2 kV cm^{-1} for 60 μs. The artificially activated nuclear-ooplasmic hybrids (clonal cybrids) were exposed to 5 μg mL^{-1} cytochalasin B (CB) for 2 h, followed by *in vitro* culture to morula and blastocyst stages in 0.4% bovine serum albumin (BSA) and 10% FBS-enriched NCSU-23 medium for 6 to 7 days. A total of 208 and 192 enucleated oocytes that were reconstituted via electrofusion with either ABM-MSC nuclei or AC-FC nuclei were simultaneously subjected to electrical activation in Groups I and II, respectively. In Groups I and II, 197/208 (94.7%) and 161/192 (83.9%) oocytes were effectively fused/activated and intended to be *in vitro* cultured, respectively ($P<0.05$; χ^2 test). Groups I and II yielded proportions of 189/197 (95.9%) and 96/161 (59.6%) for dividing cloned embryos, respectively ($P<0.001$; χ^2 test). The rates of embryos that reached the morula and blastocyst stages were 157/197 (79.7%) and 82/197 (41.6%) or 78/161 (48.4%) and 39/161 (24.2%) in Groups I or II, respectively ($P<0.001$; χ^2 test). In conclusion, the competencies of ABM-MSC nuclei to support both cleavage activity and *in vitro* development of nuclear-transferred pig embryos to morula and blastocyst stages were significantly higher than the competencies of AC-FC nuclei.

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Notes

EVALUATION OF THE *IN VITRO* DEVELOPMENTAL POTENTIAL OF PORCINE CLONED EMBRYOS CREATED USING NUCLEAR DONOR FIBROBLAST CELLS UNDERGOING SCRIPTAID-DEPENDENT EPIGENOMIC TRANSFORMATION

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The present study was undertaken in order to assess the developmental capacity of nuclear-transferred (NT) pig embryos descended from foetal fibroblast cells that had been epigenomically modulated via exposure to new-generation non-selective inhibitor of histone deacetylases, designated as scriptaid. Cumulus-oocyte complexes (COCs) were matured *in vitro* for 20 h in Tissue Culture Medium 199 (TCM 199) enriched with 1 mM L^{-1} dibutyryl cyclic adenosine monophosphate (db-cAMP), 10 IU mL^{-1} equine chorionic gonadotropin (eCG), 10 IU mL^{-1} human chorionic gonadotropin (hCG), 10% porcine follicular fluid (pFF), 10 ng mL^{-1} recombinant human epidermal growth factor (rhEGF), 5 ng mL^{-1} recombinant human basic fibroblast growth factor (rh-bFGF) and 0.6 mM L^{-1} *L*-cysteine. Afterwards, the COCs were cultured for 22 to 24 h in the db-cAMP- and eCG+hCG-depleted medium. Prior to use for somatic cell nuclear transfer, the permanent fibroblast cell lines (between passages 2 and 4) that had been established from the primary cultures originating from dermo-integumentary tissue explants of decapitated and eviscerated conceptus at Day 35 of gestation, were treated with 350 nM L^{-1} scriptaid (6-(1,3-dioxo-1*H*, 3*H*-benzo[de]isoquinolin-2-yl)-hexanoic acid hydroxyamide) during 24-h serum starvation. Reconstruction of enucleated oocytes was achieved by their fusion with epigenomically-modified fibroblast cells in an isotonic dielectric solution. The concentration of Ca^{2+} ions in the electroporation medium was increased up to 1.0 mM L^{-1}. Electrofusion of ooplast-somatic cell couplets was evoked by two successive DC pulses of 1.2 kV cm^{-1} for 60 μs. The same electric pulses that triggered the fusion of ooplast-nuclear donor cell complexes were simultaneously applied to initiate the activation of reconstituted oocytes (clonal cybrids). Immediately after electrofusion/electroactivation, clonal cybrids were incubated in North Carolina State University-23 (NCSU-23) medium supplemented with 5 μg mL^{-1} cytochalasin B for 1 to 2 h, followed by *in vitro* culture up to morula and blastocyst stages in NCSU-23/BSA/FBS medium for 144 to 168 h. It has been shown that the frequencies of cleaved embryos (137/156; 87.8%^{A}), morulae (108/156; 69.2%^{C}) and blastocysts (61/156; 39.1%^{C}) developing from NT embryos that were reconstructed with foetal fibroblast cells subjected to scriptaid exposure, were significantly higher than in the TSA-untreated group (89/125; 71.2%^{B}, 72/125; 57.6%^{D} and 34/125; 27.2%^{D}, respectively) [^{A,B} $P<0.001$, ^{C,D} $P<0.01$; χ^2 test]. Cumulatively, the enhancements in not only cleavage activity of porcine cloned embryos, but also their morula/blastocyst yields appear to result from increased functional capabilities for proper induction of architectural remodeling and epigenetic reprogramming of scriptaid-treated foetal fibroblast cell nuclei in a cytoplasm of reconstituted oocytes.

Notes

EFFECT OF CULTURE TEMPERATURE AND OOCYTE QUALITY ON IN VITRO MATURATION OF BOVINE OOCYTES

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IVM of bovine oocytes are performed at 38°C to 39°C, as this temperature is close to the rectal temperature in cattle. However recent reports demonstrated that the temperature in preovulatory follicles is 1.5 to 2°C cooler than their adjacent stroma in cattle. It was hypothesized that IVM at a temperature that mimics the thermal environment of the bovine preovulatory follicles in vivo rather than the conventional temperature practiced in vitro may provide a better culture condition for maturation of bovine oocytes.

The present study examined the effect of low culture temperature and oocyte quality on cumulus expansion and nuclear maturation of cumulus-oocyte complexes (COCs) obtained from bovine ovaries. COCs were classified as very good (n=179) and good (n=198) quality based on their homogeneity of the cytoplasm and the compactness of the cumulus investment. Very good and good quality COCs were separately cultured in tissue culture medium-199 (TCM-199) supplemented with 10% FCS for 22 hours filled with a humidified 5% CO_2 in air at either 36.5°C or 38.5 °C. Cumulus cell expansion of all COCs was evaluated at the end of the maturation period under a stereomicroscope (x10). Nuclear status of bovine oocytes in both groups was determined by nuclear staining and evaluated with interference phase contrast microscopy (x400). The data were analyzed by chi-square.

There were no significant differences between very good quality COCs matured at 36.5°C or 38.5°C with regard to percentage of cumulus expansion and oocytes reached to metaphase II (M II) stage. However, the percentage of oocytes reached to M II stage decreased (58.3 v.s 81.7%; $P<0.05$) when the incubation temperature was decreased in good quality COCs.

In conclusion decreasing the IVM temperature (36.5 °C) did not have dramatic effects on cumulus expansion and nuclear maturation. It was hypothesized that culture of bovine oocytes during IVM at a lower incubation temperature may provide a better thermal environment for the completion of nuclear maturation. Under certain circumstances oocyte maturation at a lower temperature could be useful to subsequent embryo development. However further studies are required to determine the developmental competence of oocytes matured at low temperature to the blastocyst stage in vitro.

Notes

ENHANCEMENT OF *IN VITRO* DEVELOPMENTAL OUTCOME OF CLONED GOAT EMBRYOS AFTER EPIGENETIC MODULATION OF BOTH ADULT DERMAL FIBROBLAST CELLS AND ACTIVATED NUCLEAR-TRANSFERRED OOCYTES

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The purpose of our study was to explore the effect of trichostatin A-mediated epigenomic transformation of nuclear donor fibroblast cells and artificially activated oocytes that had been reconstructed with them on the developmental abilities of caprine nuclear-transferred (NT) embryos. The enucleated *in vitro*-matured oocytes were subzonally-injected with adult ear skin-derived fibroblast cells exposed or not exposed to trichostatin A (TSA; Groups I and II, respectively). Enucleated oocyte-somatic cell complexes were subjected to simultaneous fusion and electrical activation that were initiated via a single DC pulse of 2.4 kV/cm for 15 μs. After a 1-h delay, nuclear-ooplasmic hybrids were additionally stimulated with the use of 5 μM calcium ionomycin, followed by treatment with 2 mM 6-dimethylaminopurine (6-DMAP) for 2 h. Afterwards, reconstituted oocytes were incubated for 24 h in Upgraded B2 INRA medium supplemented with TSA (Group I) or deprived of TSA (Group II). Dividing embryos were cultured in the TSA-free medium, enriched with FBS, for 144 to 168 h up to morula/blastocyst stages. Among 198 cultured NT embryos in Group I, 147 (74.2%) were cleaved. The percentages of cloned embryos that reached the morula and blastocyst stages were 87/198 (43.9%) and 53/198 (26.8%), respectively. In Group II, out of 103 cultured embryos, up to 65 (63.1%) underwent cleavage divisions, but 30 (29.1%) and 11 (10.7%) developed to morula and blastocyst stages, respectively. Altogether, TSA-based inducible epigenomic modification of both caprine dermal fibroblast cells and clonal cybrids contributed to the relatively high morula and blastocyst formation rates by improvement of donor cell nuclear reprogrammability.

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Notes

PROTEOMIC ANALYSIS OF CAT ZONA PELLUCIDA

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The mammalian oocyte is surrounded by a matrix called zona pellucida (ZP). This envelope participates in sperm-egg interactions during the first steps of fertilization and is involved in processes as acrosome reaction induction, sperm binding and it may be implicated in speciation. In cat (*Felis catus*), this matrix is considered to be composed of at least three glycoproteins (ZP2, ZP3 and ZP4). However, the last ZP composition studies have demonstrated the presence of a fourth protein in several mammals (rat, human, hamster or rabbit) being necessary a new consideration of its composition in other species. Therefore, the objective of this study was the analysis of cat ZP protein composition by means of proteomic analysis.

Partially purified ZP from 8 ovaries (4 females) and a total of 100 isolated ZP (from 3 females) were used for HPLC-MS/MS analysis. The results showed several peptides corresponding with four proteins. Specifically, 20 peptides from ZP1, 34 from ZP2, 22 from ZP3 and 24 from ZP4 were identified which correspond to a protein coverage of 33.17%, 71.50%, 50.23% and 49.64% for ZP1, ZP2, ZP3 and ZP4 respectively.

In conclusion, this study proves for the first time that cat ZP is formed by four glycoproteins. This work shows that the four ZPs composition is more common than previously thought and open the door for future research about the role of these four proteins in the structure of the ZP and its contribution to the gametes interaction.

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Notes

THE EFFECT OF HUMAN FOLLICULAR FLUID FROM WOMEN WITH DIFFERENTIAL ART OUTCOMES ON BOVINE *IN VITRO* OOCYTE MATURATION, FERTILIZATION AND BLASTOCYST FORMATION

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The micro-environment of the oocyte, the follicular fluid (FF), has been the subject of numerous studies, trying to determine a marker for oocyte quality. Up till now it remains unclear how the composition of the FF might affect the success of embryo development in ART. Interestingly, maternal metabolic disorders, such as obesity or type II diabetes, are associated with hormonal and biochemical changes in the serum, which are reflected in the FF. Such changes might possibly influence oocyte and embryo quality, since obesity has been associated with lower success rates during ART, possibly due to oocyte or embryonic developmental failure. Therefore, we hypothesized that the composition of the FF during final maturation might influence the quality of embryos generated during ART. To investigate this hypothesis, human FF from women with obesity (BMI $> 30 kg/m^2$, OBESE), $\leq 30\%$ top quality embryos at 3 days pi (NEG FF) and $\geq 50\%$ top quality embryos at 3 days pi (POS FF) was isolated and supplemented (25% FF) in a bovine *in vitro* maturation setting.

Bovine oocytes were aspirated from slaughterhouse ovaries and matured for 24h under CONTROL (no FF), NEG FF, POS FF or OBESE conditions. Matured oocytes (n = 87) and presumptive zygotes (n = 93) were stained with DAPI to determine maturation and fertilization grade. Presumptive zygotes (n = 1276, 4 replicates) were cultured to study cleavage rates and blastocyst formation.

Maturation and fertilization rates were not affected by treatment. Interestingly, the POS FF and CONTROL group presented with similar results on cleavage and blastocyst formation. However, cleavage rate was lower for NEG FF embryos compared to CONTROL embryos (74 vs. 80%, $P = 0.038$). Furthermore, the blastocyst percentages were lower for OBESE (25%, trend: $P = 0.053$) and NEG FF embryos (25%, trend: $P = 0.095$) compared to the CONTROL (31%). The blastocyst percentages from cleaved zygotes were reduced for OBESE embryos, compared to CONTROL embryos (33 vs. 38%, trend: $P = 0{,}098$). Hatching rates did not differ between treatments.

In conclusion, FF from obese women seemed to reduce bovine blastocyst formation when compared to the control treatment. Furthermore, FF from women with a low number of high quality embryos during ART, caused a reduction in cleavage as well as blastocyst formation, while the FF of women with a good number of top quality embryos did not affect *in vitro* embryonic development. Further research should focus on the mechanisms involved.

Notes

EFFECT OF α-LINOLENIC ACID ON GOAT OOCYTE DEVELOPMENTAL COMPETENCE AND EXPRESSION OF APOPTOTIC-RELATED GENES IN SUBSEQUENT BLASTOCYSTS

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Recently, it has been shown that α-linolenic acid (ALA), as a precursor of longer chain ω-3 fatty acids, enhances maturation rates as well as developmental rates in bovine embryos. Therefore, in this study we aimed to evaluate the effect of ALA on goat oocyte developmental competence as well as on expression of apoptotic-related genes (BAX, BCL2 and P53) in subsequent in vitro derived parthenogenetic blastocysts.

Therefore, goat ovaries were obtained from a local slaughterhouse and cumulus oocyte complexes (COCs) were recovered by slicing method. COCs were maturated either in media supplemented with 50 µM ALA (ALA-IVM) or in the same medium without ALA (CONTROL-IVM). Maturation was performed in a humidified atmosphere containing 5% CO2, 5% O2, and 90% N2 at 38.5°C for 24 h. After in vitro maturation, oocytes of ALA-IVM (n=287) and CONTROL-IVM (n=269) groups were subjected to parthenogenetic activation (2.5 µM ionomycin for 1 min followed by 2 mM 6-DMAP treatment for 3 h) followed by culture in CR1aa medium for 7 days under the conditions stated above. Subsequently, expression of BAX, BCL2 and P53 genes were analyzed by quantitative real-time PCR comparing blastocysts derived from ALA-IVM and CONTROL-IVM. Differences in developmental rates were analyzed by Chi-square test, considering $P<0.05$ to be significant.

As a result, cleavage rates were higher in oocytes of ALA-IVM than in CONTROL-IVM (65.2% vs. 52.8%). Likewise, blastocyst rates were higher in ALA-IVM compared to CONTROL-IVM (25.1% vs. 16.7%). Moreover, the relative transcript abundance of BAX and P53 genes were significantly reduced in blastocysts derived from ALA-IVM, whereas expression of BCL2 was increased compared to blastocysts derived from CONTROL-IVM.

Taken together, our results show that supplementation of maturation media with 50 µM ALA promotes developmental rates in goat parthenogenetic embryos indicating beneficial effects on embryo development and quality of subsequent blastocysts.

Notes

BIOLOGICAL PROPERTIES OF NEW GENERATION BIOMATERIALS PCL (POLY E-CAPROLACTONE, PLDLA (POLY-L/DL-LACTYDE) AND PLA/DBC (COPOLYMER POLY_L-LACTYDE AND DIBUTYRYL CHITIN) AS POTENTIAL MATERIALS FOR REPRODUCTION MEDICINE DEVICES

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The possibilities of employment of the new generation biomaterials in biotechnology of reproduction were evaluated. In our study previously selected biomarkers were evaluated, with confirmed biocompability in the culture *in vitro* in somatic cells and *in vivo* in contact with alive tissue. Tested biomaterials are routinely used in human medicine. The aim of our study was biological verification of new generation biomaterials PCL (poly ε-caprolactone, PLDLA (poly-L/DL-lactyde) and PLA/DBC (copolymer Poly L-lactyde and dibutyryl chitin) as potential materials for production of medical devices, as catheters for obtaining, transporting and transfer of embryos as well as laboratory equipment for cryopreservation. The possible practical application of these biomaterials needs further verification of their biological properties on embryo culture. The foil discs made of PCL, PLDLA and PLA/DBC, thickness 0.5 mm, diameter 35.0 mm, were prepared in two forms: the baseline one (PCL, PLDLA, PLA/DBC) and thermally modified by freezing with liquid nitrogen (PCL-LN, PLDLA-LN, PLA/DBC-LN). The verification of bioconcordance was performed by culture of 186 pig embryos at the development stage of 2 to 4 blastomeres (PCL n=17; PCL-LN n=20; PLA/DBC n=17; PLA/DBC-LN n=20; PLDLA n=33; PLDLA n=30; control group n= 49). We performed five days long culture of embryos after contact lasting 15 minutes with the evaluated material not frozen and frozen in liquid nitrogen. The control group consisted of embryos cultured in the same conditions, in medium NCSU-23 suitable for pig embryos culture in incubators in 39^0C, in the atmosphere of 5% CO_2 in air. The criteria of evaluation of biomaterial quality were the number of blastocysts obtained after *in vitro* culture, the total number of cells in blastocysts and the fragmentation of nuclear DNA analized by TUNEL.

In PCL and PLA/DBC biomaterials, both non frozen and frozen, the development of embryos was suppressed shortly after transfer to the culture with biomaterial and no embryo developed to the stage of 4 – 8 blastomeres. Embryos reached blastocysts stage only in frozen and not frozen PLDLA. After the culture in PLDLA we received 81.82% (27/33) blastocysts, in the control group: 65.31% (32/49) blastocysts. The mean number of cells in blastocysts was 31.4 (PLDLA), 31.2 (PLDLA-LN) and 28.7 (control group), no statistically significant difference was found between groups. The apoptotic index of PLDLA, PLDLA-LN and control group was 3.59, 3.56 and 2.33, respectively, no statistically significant difference was found between the groups. Because of cytotoxic effect of PCL (polycaprolacton) and PLA/DBC (copolymer poly_L-lactyde and dibutyryl chitin) on embryos, they cannot be used as the material for catheter production used in biotechnology of animal reproduction and other devices used for *in vitro* culture and cryopreservation. PLDLA (poly-L/DL-lactyde) can be used in reproduction biotechnology as new generation material e.g. for production of catheters for embryos collection and transfer.

Notes

A CONCEPT OF TRANSLATIONAL CONTROL IN THE PORCINE UTERINE TISSUE DURING EARLY PREGNANCY

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An early and probably rate limiting step of translation initiation is the activation of the mRNAs. This process stimulates their recruitment to ribosomes and relies on translation initiation factors (eIFs). eIF4E directly binds to the 5´-cap of the mRNA and in concert with other factors (eIF4G, eIF4A), secondary structures are resolved which enables ribosome entry. The canonical model describes that the interaction of the factors is regulated by phosphorylation and by the abundance and phosphorylation of specific repressors the so called eIF4E binding proteins, 4E-BP1, 2 and 3. We present evidence for an alternative and unique process of translational control in the porcine endometrium during early pregnancy at the noninvasive epitheliochorial implantation and placentation. Complex events of proteolytic modifications of various eIFs are characteristic for this mechanism. From a mechanistic point of view, these events comprise an N-terminally truncation of eIF4E (which only occur in the porcine endometrium) and 4E-BP2, degradation of 4E-BP1 and also a characteristic fragmentation of eIF4G. eIF4A and the poly(A)-binding protein PABP, however, are not affected. Systemically, the modification of the factors is strongly stimulated when the uterine horn harbors embryos or when ovariectomized pigs are substituted with estradiol and progesterone. Different Ca^{2+} dependent endometrial proteases, active in the luminal (eIF4E-cleavage) and/or glandular epithelium (4E-BP1 degradation), were responsible for the specific modifications of the factors. Functional effects were differential binding of the factors, which influence ribosome recruitment of different mRNA sub-classes. In detail, eIF4E truncation and 4E-BP1 degradation reduces the repressive action of 4E-BPs and in such a way stimulates translation. On the other hand, eIF4G fragmentation may have repressive effects. In general, it is suggested, that highly eIF4E sensitive mRNAs which are characterized by extensive structured 5´-UTRs might be differently regulated. Since the truncation of the indispensable factor eIF4E is unique for the porcine endometrium and highly stimulated by embryonic signals, it is suggested that this alternative mechanism of translational control contributes to establish the non-invasive implantation and placentation type as it is found in pigs.

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