UNIVERSITÀ DEGLI STUDI DI SASSARI

Scuola di Dottorato in
RIPRODUZIONE, PRODUZIONE, BENESSERE ANIMALE E SICUREZZA DEGLI ALIMENTI DI ORIGINE ANIMALE
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Indirizzo in
RIPRODUZIONE, PRODUZIONE E BENESSERE ANIMALE (XXII ciclo)
Coordinatore: prof. Sergio Ledda

QUALITY OF IN VITRO OVINE EMBRYO PRODUCTION: LAMBING RATE, BODY WEIGHT AND GENE EXPRESSION

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Anno Accademico 2008/2009

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ABSTRACT

Abstract

The objective of this study was to examine the effect of different culture media on in vitro embryo production in terms of pregnancy rate, lambing rate and lamb body weight. Moreover the objective was to compare, in vivo vs in vitro embryos, the mRNA abundance of genes involved in embryo quality.

Oocytes were obtained from ovaries of slaughtered adult ewes, matured in vitro in TCM199 supplemented with 0.4 mg/ml BSA in A(BSA_4), B(BSA_8), C(BSA_8–HA), D(BSA_8-CH) groups or with 10% FBS in E (FBS_{10}) group for 24h and then fertilized with fresh ram semen. Zygotes were cultured for 6-7 days in 20 µl droplets of SOF supplemented with 4 mg/ml fatty acid free BSA for A group, 8 mg/ml fatty acid free for B, C, D groups, and 10% FBS for E group at 39°C under 5% CO_2, 7% O_2 and 88% N_2. On the third, fifth and seventh day of culture (day 0= fertilization day) 4mg/ml fatty acid-free BSA, 8mg/ml fatty acid-free BSA, 8mg/ml fatty acid-free BSA+6mg/ml HA, 10% Charcoal stripped FBS and 10% FBS were added to the culture A, B, C, D and E respectively. Blastocysts (A, B, C, D groups) on day 6-7 were transferred into recipient ewes either fresh or after vitrification. Pregnancy was confirmed by ultrasonography at 40 days and pregnancies were allowed to go to term. Lambing and body weight were recorded soon after the birth. For gene expression in vitro (A, B, C, D, E groups) and in vivo derived (F group) fresh embryos were snap frozen in LN_2 in groups of 10 and analyzed by quantitative Real Time PCR. The relative mRNA abundance of blastocysts in vitro produced and in vivo derived for genes implicated in: imprinting (*IGF2R, GRB10*, *UBE2A*), embryo development (*LAMA1, IL-6*, *FGF4*), apoptosis (*BAX*), methylation (*DNMT3A*) and resistance to stress (*HSPB1*) was analyzed.

Pregnancy and lambing rate were similar within groups only for fresh embryos while among vitrified groups, as expected, A(BSA_4) vs D(BSA_8-CH) ($P<0.05$) were significantly different. The birth weight was higher for B(BSA_8) and C(BSA_8-HA) within groups in both fresh and vitrified embryos.

However, the level of expression of imprinting genes *(IGF2R, UBE2A)* was significantly upregulated ($P<0.05$) in the 5 in vitro cultured groups than in vivo derived group. Furthermore, *UBE2A* was significantly upregulated ($P<0.05$) in C(BSA_8-HA) compared with to the rest of in vitro groups. *LAMA1* was significantly upregulated ($P<0.05$) in group A(BSA_4) compared to other groups.

Our data confirm that the use of different culture media affects the blastocysts quality in terms of cryotolerance, body weight and gene expression.

ACKNOWLEDGEMENTS

I would like to express my cordial respect and thanks to my supervisor ***Professor Salvatore Naitana***. I want to thank the Animal Biology Department, Faculty of Veterinary Medicine as well as the University of Sassari for accepting me as PhD candidate.

Sincere thanks to my tutor ***Dr. Maria Dattena*** for successfully completing my PhD research, for her scientific advice, enthusiasm, criticism and encouragements during the course of my study and thesis writing.

I want to show my gratefulness to whole Agris-Sardegna DIRPA agency for giving me the opportunity to undertake my PhD research in its laboratories. Specially thanks to: ***Dr. Antonello Carta, Dr. Sebastiano Ligios, Dr. Gianni Piredda***. In addition, ***Dr. Andrea Fraghì*** for his helpfulness and the librarian ***Miss Marina Leoni*** for her precious help in my study.

I am grateful to ***Dr. Pablo Bermejo-Alvarez, Dr. Dimitros Rizos*** and ***Alfonso Gutierrez-Adan*** of INIA-Madrid (Spain) and ***Dr. Angela Sanna*** and ***Dr. Andrea Mastinu*** of Neuroscienze PharmaNess Scarl Pula (CA) for providing me with the access to their laboratories for my gene expression experiments.

Thanks to ***Laura***, I couldn't have finished this thesis without your advise and help, thank you very much for your friendship.

Very special thanks to my "chilean sister" ***Margarita,*** my friends ***Fabrizio*** and ***Lia***, for their help and support, especially thanks for their friendship.

Great thanks to whole "Reproduction division": ***Marilia, Susanna, Giorgio, Davide, Andrea, Luigi, Loredana, Antonio, Giampiero, Tore*** and ***Tore*** for all their help and kindness.

Thanks to "DIRPA genetic girls": ***Annalisa***, ***Giuliana*** and ***Sabrina*** for performing sequencing and their generosity.

Thanks to DIRPA Microbiology division: specially for "-80°C" use!

Thanks to administration of Animal Biology Department specially to ***Davide Scarpa*** for his perfect assistance in the paper, dossier and deadline relative to PhD.

Special thanks to ***Mario Salis*** for critical reading, but most importantly thanks for his absolute friendship.

Thanks to ***Luca*** for his love, patience and for supporting me during this study.

Thanks to my best friend ***Giannella*** for her generosity and great friendship.

……and thanks a lot to all my friends.

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1.

INTRODUCTION

1. Introduction

In vitro embryos production (IVP) plays a very important role in biotechnologies of reproduction with innumerable applications ranging from transfer of embryos for genetic improvement, preservation of endangered species, developmental study, and more recently embryonic stem cell production. However, IVP embryo can lead to alteration of the quality of embryos with reduction of embryo viability and aberrant gene expression.

IVP is a three-step process involving in vitro oocyte maturation, in vitro fertilization and in vitro culture. A variety of embryo culture systems were developed for the production of viable blastocysts (Bavister 1995; Thompson 1997), but their quality is far from the in vivo counterpart. Usually the basic culture media are supplemented with serum (FBS/FCS) or bovine serum albumin (BSA) as a source of protein (Leibfried-Rutledge et al., 1986; Gardner et al., 1994 and 1999; Thompson 2000). Serum inhibits the early cleavage divisions and has an accelerating effect later in development (Langendonckt et al., 1997; Thompson et al., 1998), in addition an increased birth weight was attributed to the presence of serum in the medium (Holm et al., 1996; Sinclair et al., 1998; Young et al., 2001), while the BSA balances the osmolality, binds growth factor and improves embryo development (Walker et al., 1992; Thompson et al., 1995; Lane et al., 2003; Dattena et al., 2007). These supplements and some other unknown factors can lead to changes in mRNA abundance influencing gene expression in a global manner (Niemann and Wrenzycky, 2000; Young et al., 2001; Rizos et al., 2002a; Lazzari et al., 2002; Lonergan et al., 2003a).

Efficient IVP system should lead into viable offspring either after transfer of fresh or cryopreserved embryos.

The aim of present research was to evaluate the quality of embryo in vitro produced with different culture media, in term of pregnancy, lambing rate and lamb body weight.

Moreover, the relative mRNA abundance of genes involved on quality embryos was compared between in vivo and in vitro production. In addition, the appendix of this thesis report the gene expression related to staminality of embryonic stem-like cells obtained from embryos in vitro produced.

2.

LITERATURE REVIEW

2. Literature review

2.1. In vitro embryo production

In vitro embryo production (IVP) in ruminants provides an excellent source of low-cost embryos for research on developmental biology and physiology or several applications that involves reproduction biotechnologies and other applications such as stem cell production.

2.1.1. In vitro maturation, in vitro fertilization and in vitro culture

The IVP involves collection of oocytes and three main steps: maturation of primary oocytes from large antral follicles, fertilization of the matured secondary oocytes with frozen-thawed or fresh semen and culture of presumptive embryos for up to 6/7 days until blastocyst stage that can be transferred to recipients, cryopreserved for future use or submitted to biological study.

- *Collection of oocytes*

Oocytes can be obtained from two different sources: live or slaughtered animals. The first method is widely used in cattle (Reichenbach et al., 1994; Farin and Farin 1995), while is limited in small ruminants. The oocytes and specially individual collection of oocytes can be recovered by several techniques: laparoscopic method (Snyder and Nellor, 1975; McKelvey et al., 1986; Tervit, 1995), laparoscopy- guided "ovum pick up" LOPU technique (Tervit, 1996; Baldassare et al., 1996; Aguillar et al., 2002) or transvaginal ultrasound guided aspiration (TUGA) technique also known as ovum pick up (OPU) (Bousquet et al., 1995). But in the laboratory routine to this day in order to cut down on time and costs the oocytes are obtained from abbatoir-derived ovaries. Ovaries are

transported to the labarotory within 2-3 hours in phosphate buffered saline (PBS) or saline solution at 25-37°C and follicles 2-8 mm in diameter are aspirated or sliced to obtain oocytes. Aspiration can recover only superficial follicles while slicing can recover a higher amount of oocytes both superficial and inner (Vajta et al., 1996; Mantovani et al., 1999), but it is necessary more time than aspiration with the risk of compromising vitality of oocytes. The immatured oocytes can be treated with PBS or in tissue culture medium 199 (TCM 199) tamponed with hepes. Method, medium, temperature, follicle dimension, age animal (Yang et al., 1998) are very important factors because they influence following steps of IVP.

Oocytes quality represent another important factor affecting success of IVP system. Usually, the the oocytes quality is morphologically evaluated. There is evidence in the literature suggesting oocytes matured in vivo are more developmentally than those matured in vitro (Bordignon et al., 1997; Leibfried-Rutledge et al., 1987) one of the causes is represented by the high degree of homogeneity amongst oocytes matured in vivo compared with heterogeneity exhibited by in vitro matured oocytes (de Loos et al., 1992). Thus the oocytes are classified as ***Grade A*****:** Compact cumulus oocyte complexes (COCs) with an unexpanded cumulus mass having ≥ 4 layers of cumulus cells, and with homogenous evenly granular cytoplasm. ***Grade B***: COCs with 2-3 layers of cumulus cells and a homogenous evenly granular cytoplasm. ***Grade C***: Oocytes partially or wholly denuded or with expanded or scattered cumulus cells or with an irregular and dark cytoplasm. Furthermore, Eckert and Niemann (1995) demonstrated that there are not difference in the potential developmental between grade A and B oocytes.

- *In vitro maturation (IVM)*

The maturation of oocytes is a crucial moment of IVP. With *in vivo* conditions the oocyte run into a series of events that are fundamental to achieve its meiotic competence from the diplotene to the metaphase II stage (nuclear maturation). Acquisition of oocyte maturation correlates with folliculogenesis (Mermillod et al., 1999a). Initially, follicle and oocyte growth are highly correlated, in a second phase the follicle continues to grow, while the oocyte remains stable until it reaches full adult size depending on granulosa cell-oocyte interactions. Moreover the oocyte maturation into the follicle is conditioned by the presence of hormones: FSH (follicle stimulating hormone), LH (lutinizing hormone) and estradiol. For this reason also in vitro oocytes need these components and special culture media that can mimick as much as possible the in vivo conditions.

Components of the culture media can affect the meiotic regulation of mammalian oocytes (Kito and Bavister, 1997). Normally, meiotic maturation occurs spontaneously after removal of the oocytes from the follicle, but the addition of gonadotrophins in vitro increases the number of oocytes that progress to metaphase II (Moor and Trounson, 1977; Galli and Moore, 1991).

Maturation is usually performed in TCM 199 (Staigmiller and Moor, 1984) supplemented with FSH, LH, estradiol 17 β, serum or albumin (Younis and Brackett, 1989; Thompson, 2000). FSH and LH accelerate meiotic progression, Accardo et al. (2004) demonstrated that the highest maturation rate of sheep oocytes was reached with use recombinat FSH/LH. Estradiol 17 β contributes with FSH and LH to improve oocyte maturation (Funahashi and Day, 1993; Bing et al., 2001; Accardo et al., 2004).

Many types of serum were used: fetal calf serum (FCS), fetal bovine serum (FBS), bovine serum albumin (BSA). Serum contains a wide variety of components: fatty acid, proteins, growth factors, trace elements and growth promoters. Most IVM protocols add

the media with 5-20% serum (Takagi et al., 1991; Krisher et al., 1999). Serum influences embryo development, morphology, metabolism (Carolan et al., 1995; Thompson, 1997). In addition, FBS avoids zona pellucida hardening that may prevent fertilization. Albumin is a protein source, it balances the osmolality and promotes development of embryos to the blastocyst stage (Takagi et al., 1991; Takahashi and First, 1992; Shamsuddin et al., 1994; Edwards et al., 1997).

More recently, the use of media with little or without serum were used. Addition of BSA in synthetic oviductal fluid (SOF) is currently the most popular culture medium that contributes to formation and expansion of blastocoele (Tervit et al., 1972; Gardner et al., 1994; Accardo et al., 2004; Dattena et al., 2006). In addition, some researchers using media without serum (Bavister et al., 1992; Eckert and Niemann, 1995; Krisher et al., 1999) improved the viability of embryos, but others demonstrated poor rates of embryonic development (Takagi et al., 1991).

Addition of growth factors (GF) have been shown to stimulate oocyte maturation and to reduce the requirement for gonodotrophins during maturation (Mermillod et al., 1993; Harper and Brackett, 1993; Kobayashi et al., 1994; Gandolfi et al., 1996; Majerus et al., 1999), in particular addition of epidermal growth factor (EGF) to the maturation medium increases development and formation of blastocyst in several species (Abeydeera et al., 1998; Lonergan et al., 1996), insulin-like growth factor I (IGF-I) plays an important role in intra-ovarian regulation of follicular growth (Monniaux et al., 1997) and transforming growth factors-α (TGF-α) augment meiotic maturation. In contrast Ledda et al. (1996) observed that the presence of growth factors did not modify the percentage of oocytes able to complete meiosis.

The glutathione protects cells in oxidative stress that represents an impediment in development of in vitro mammalian embryos (De Matos et al., 1995; Cogniè et al., 2002).

The addition of cysteamine in IVM medium, as a glutathione precursor, in sheep and in goat was successfully adopted (Wells et al., 1997; Ptak et al., 1997).

Moreover, Holm and Callesen (1998) suggest that IVM was influenced by specific condition of culture environment as osmolality (Yamamuchi et al., 1999), temperature (Lenz et al., 1983), pH, CO_2 (Geshi et al., 1999), oxigen tension and culture volume that are very important parameters in IVM step.

In addition studies on apoptosis, molecular control of cells pathway and transduction signals could lead up to an IVM system improvement.

- *In vitro fertilization (IVF)*

During oocyte maturation, nuclear and cytoplasmic changes occur that render the COCs able to be fertilized. Fertilization represents a very critical step of the entire procedure, which needs to be optimized.

IVF step consists of preparing the spermatozoa and the oocytes for syngamy and co-incubation for a certain period of time, but this is not sufficient, because the spermatozoa must first undergo capacitation in the female reproductive tract for some time before being able to penetrate the Zona Pellucida (Chang and Hunter, 1977). Capacitation involves changes to the structure and physical properties of the acrosomal sperm head membrane rendering it more fusigenic (acrosome reaction). This change is facilitated by the removal of cholesterol and non-covalently bound epididymal/seminal glycoproteins (Galantino-Homer et al. 1997). The result is a more fluid membrane with an increased membrane permeability to Calcium (Ca^{2+}) (Handrow et al. 1989) that produces increased intracellular cAMP levels and a hyperative spem motility (Parrish et al. 1994; Visconti et al. 1995). The beginning of hyperactivated motility is accompanied by an efflux of intracellular H^+ causing an increase in intracellular pH (Parrish et al., 1989). Most IVF procedures in sheep are carried out between a pH of 7.4 and 8.0 (Wani, 2002).

In 1963, Yanagimachi and Chang first reported that mammalian spermatozoa can be capacitated in vitro. Washing of the spermatozoa before incubation is reported to accelerate capacitation in vitro (Wani, 2002) also heparin, this is a glucoseaminoglycans added to sperm treatment medium. The use of heparin reflects the in vivo mechanism that heparin-binding proteins present in seminal plasma. Evidence suggests that heparin-binding proteins play a role in fertilization by attaching to the sperm surface, enabling heparin-like CAGs in the female tract to induce capacitation, with eventually, an improved fertilization rate in cattle and in sheep.

Nowadays the commonly used media for in vitro fertilization are: TCM199 (Slavik et al, 1992), Synthetic oviductal fluid (SOF) (Tervit et al, 1972; Sun et al, 1994), modified Tyrode and Krebs-Ringer's solution supplemented with energy source (glucose, lactate and piruvate) and bovine serum albumin (Watson et al, 1994). Protein is an essential component of the media used for in vitro capacitation of spermatozoa. Sperm capacitation in sheep and in goat is obtained in media supplemented with heat-inactivated oestrus serum at different concentrations: 20% with fresh semen (Crozet et al., 1987) and 2% with frozen-thawed semen (O'Brien et al., 1997). A gamete co-incubation time of 17/24-h is normally required in IVF/sheep system, but this time can change because differences in sperm capacitation kinetics exist between IVF systems.

- *In vitro culture (IVC):*

The final step of IVP system is IVC. The major purpose of embryo culture is to sustain the zygote in a suitable environment until blastocyst stage. Two culture systems are routinely used for IVP: co-culture (or medium conditioned on cells); (2) semidefined conditions in media designed to suit embryo requirements.

The co-culture technique was described by Gandolfi and Moor (1987). The cells of choice are granulosa, ovuduct epithelial or buffalo rat liver cells producing growth-

promoting factors and/or removing inhibitory components from the medium, such as glucose and oxygen (Watson et al., 1994). Co-culture of sheep/embryos is generally performed with TCM199 medium usually supplemented with serum (FCS).

Serum is the most commonly used protein source, generally used at a concentration of 5-10%. Serum provides a source of albumin that balances the osmolality of the medium, also it provides numerous amino acids, growth factors and other macromolecules necessary for the hatching of the mammalian blastocyst. Unfortunately the serum induces premature blastulation, alterations in mitochondria and other abnormalities (Dorland et al, 1994) and also contamination problems. Cultured in 20% serum sheep and cow embryos frequently increased gestation length and greater birth weights (Sinclair et al., 1996; Young et al., 2001). Moreover another alternative is represented by enrichment of the culture medium with Charcoal-treated FBS. It is used to elucidate the effects of hormones in a variety of in vitro systems (Accardo et al., 2004).

Anyway, the most commonly used semi-defined system for the culture of embryos is SOF medium enriched with Eagle's 20 amino acids and BSA (SofaaBSA; Gardner et al., 1994). This method appears to have the most infuence on embryo development, morphology and metabolism. BSA has several functions including: balancing the osmolality, chelating heavy metals providing some pH buffering (Mehta and Kiessling, 1990) and binding growth factors. Indeed, albumin provides beneficial factors such as energy substrate (Bavister et al., 1983; Maurer et al., 1993).

Alternatively, recombinant albumin preparation (Lane et al., 2003), hyaluronan (HA) and synthetic macromolecules such PVP (Keskintepe et al., 1996) were used instead of BSA to prepare a completely defined culture media. Accardo et al. (2004) demonstrated that recombinant albumin was able to support development to the blastocyst stage at the same rate as BSA.

HA is a macromolecule present in the female reproductive tract (Laurent et al., 1995) it that affects different physical or biochemical reactions, most of all cell movement and differentiation (Scott and Heatley, 2002), also it regulates protein secretion and distribution. Tirone et al. (1997) observed that HA is correlated with normal oocyte development. Addition of HA with BSA to the culture medium stimulates embryo development in mouse, pig, cow culture (Furnus et al., 1998; Gardner et al., 1999). Moreover, several studies examined the ability of HA to improve cryo-tolerance of IVP embryos, but it did not make significant differences in development to the blastocyst stage (Lane et al., 2003; Dattena et al., 2007).

Despite progress in the culture of embryos, there are still some differences between in vitro and in vivo derived embryos.

2.1.2. Embryo evaluation

The appearence of an embryo on day 7 is the first parameter that counts for the selection of embryos for immediate transfer or cryopreservation for later use. There are many methods to evaluate a good quality blastocyst and they ground on: metabolism, apoptosis (Van Soom et al., 2002), enzyme activity (O'Fallon and Wrigth, 1986), glucose uptake, kinetics of development, post- cryopreservation survival (Furnus et al., 1997), staining (Purcell et al., 1985), and gene expression (Lonergan et al., 2003a,b), but total morphology and blastocyst cell number (Ellington et al., 1990) are the most often used criteria to assess embryo viability.

Generally, a good quality blastocyst contains a well-expanded blastocoelic cavity, an homogeneus trophoblast with multiple cell-cell contacts and distinct nuclei and an inner cell mass (ICM) that is clearly visible and intact.

International Embryo Transfer Society elaborated a guide by morphological methods to classify embryo quality or 'viability' in four categories or grades: 1) ***excellent*** or good (symmetrical and spherical embryo mass with individual blastomeres that are uniform in size, colour and density. Zona Pellucida should be smooth and have no concave or flat surface. At least 85% of the cellular material should be an intact viable embryonic mass); 2) ***fair*** (moderate irregularities in overall shape of the embryonic mass or in size, colour and density of individual cells. At least 50% of the cellular material should be an intact viable embryonic mass); 3) ***poor*** (major irregularities in overall shape of the embryonic mass or in size, colour and density of individual cells. At least 25% of the cellular material should be an intact viable embryonic mass), and 4) ***death or degenerated*** (degenerating embryos, no viable).

Currently, only microscopic morphology is used for evaluation embryos. Despite morphological evaluation is a simple, non invasive and rapid procedure, is a subjective method of analysis and requires experienced evaluators to accurately classify or "score" embryos (Overström, 1996). Moreover metabolic and genetic disorders are not detectable.

2.1.3. Embryo transfer

Embryo transfer (ET) is a valuable research technique by which an embryo is collected from a female donor or produced in vitro and then transferred into selected female recipients.

ET procedures have become very successful. In commercial embryo transfer programs with cattle, pregnancy rate of 70% with unfrozen embryos and 65% with frozen embryos have been accomplished routinely.

The first successful ET in farm animals was reported in sheep and goats by Warwick et al. (1949). Extraordinary progress and diversification in this embryo

technology were performed across decades, however its success still depends from several practical and technical factors. Sheep represent the only species of domestic animal, next to cattle, in which large numbers of in vitro-derived embryos have been transferred. Walker et al. (1992) also reported day 50 pregnancy rates of 48% for ewes receiving two in vitro embryos. The transfers in the preceding studies were performed either via mid-ventral laparotomy or with the aid of a laparoscope (Cappai et al., 1988; Naitana et al., 1992). ET in sheep is usually carried out by surgical method.

There are two methods of ET: ***indirect transfer*** this technique is considered a standard technique and has been widely used for both fresh and vitrified embryos. Briefly, after cryoprotectant removal the frozen-thawed embryos are put into a petri and morphological evaluation is performed under microscope. Soon after a laparatomy or laparascopy is performed and when the reproductive tract is exteriorised a fine TomCat catheter connected to a 1 ml syringe is used to transfer the embryos into the tip of the ipsilateral uterine horn bearing at least one corpus luteum. According to Dattena et al. (2000) increased pregnancy rates were observed following transfer of fresh embryos (93.7%) compared to the frozen ones (70.3%) ($p<0.05$) while lambing rates (81.2 % vs 75 %) do no show statistical differences between groups, on the other hand, Baril et al. (2001) observed similar results after transfer between fresh and vitrified embryo in terms of pregnancy rate (72% both cases) and the number of born lamb (60% vs 50%) respectively; ***direct transfer*** this embryo transfer technique is limited in small ruminants compared with cattle, probably due to the excessive costs of procedures when compared with the value of the animal, but the vitrification technique as cryopreservation method in combination with the direct embryo transfer technique can offer a real possibility to reduce costs of procedures and increase the use of this techniques in ewes (Baril et al., 2001). The advantages of this method are that warming is easily and rapidly achieved and embryo

transfer is directly performed after thawing, without the need for optical equipment (microscope-free) because embryo quality evaluation is based on the fact that only good quality embryos become vitrified. This method of transfer is considered ideal for in field conditions use (Isachenko et al., 2003).

In the last years, both techniques (indirect and direct) showed to have similar results in terms of pregnancy rates (60-75%) and lambing rates (55-75%) (Baril et al., 2001; Isachenko et al., 2003; Dattena et al., 2004).

2.2. Effect of different culture media on embryos production

2.2.1. Gene expression

Recently, the expression pattern of many genes that could be potentially used as molecular markers for embryo quality, viability and pre/post-implantation progress, has been estimated and compared between in vitro and in vivo systems.

The vast majority (85%) of the differentially expressed transcripts were downregulated in in vitro-cultured blastocysts, which lead us to suggest that the primary reason why in vitro embryos are of inferior developmental competence compared with in vivo-cultured embryos is because of a deficiency of the machinery associated with transcription and translation, the lower gene expression was contolled by trancsription (Corcoran et al. 2006). Such observations highlight the importance of the postfertilization culture environment for the quality of the resulting blastocyst.

The recognition of different genes and characterization of their expression pattern gives useful information about the genes involved during the development and about genes whose expression is altered during abnormal development. Furthermore, the relationship between phenotype and gene expression profile would help to determine the effect of particular genes and to elucidate the expression pattern of genes required to ensure a desirable development.

Thus, particular conditions of culture in vitro can alter gene expression in the embryo (Doherty et al., 2000; Rizos et al., 2002b). Analysis of expression patterns of developmentally important genes essential in early development provides a useful tool to assess the normality of the embryos produced and a tool to optimize assisted reproduction technologies.

Genes thought to play important roles during pre- and post-implantation development were mainly chosen for analysis (Rizos et al., 2008). The products of these genes are involved in various biological processes including imprinting, epigenetic regulation of transcription, methylation, apoptosis, stress adaptation, metabolism, growth factor/cytokine signalling, transcription and translation, compaction and blastocyst formation (Wrenzycki et al. 2005).

Imprinting is a particularly important genetic mechanism in embryo of large domestic livestock, aberrant imprinting disturbs are the cause of various disease syndromes. The study of imprinting also provides new insights into epigenetic gene modification during development. The gene related to imprinting widely analysed in preimplantation embryos are: *IGF2* that is only expressed from the allele inherited from the father, *IGF2R* and *H19* maternal copies are expressed in offspring. The IGFs are a group of structurally related polypeptides that regulate the growth of many types of mammalian cells (Yaseen et al., 2001), epigenetic changes in pre-implantation embryos may affect their gene expression during embryonic, placental, later fetal and neonatal development.

GRB10 is able to interact with a variety of receptor tyrosine kinases, including, notably, the insulin receptor and it also forms a link between fetal growth and glucose-regulated metabolism in postnatal life and it is a candidate for involvement in the process of fetal programming (Smith et al., 2007). Disruption of *GRB10* gene expression was previously shown to cause increased animal size at birth and increased insulin sensitivity (Wang et al., 2007).

Genes involved in embryo development and highly sensitive to environmental conditions during in vitro embryo production are: *UBE2A* (Ubiquitin-conjugating enzyme E2A), *LAMA1* (laminin alpha 1), *IL6* (Interleukin 6) involved specifically in

preimplantation, implatation or early postimplantation development, *FGF4* (fibroblast growth factor 4 precursor) involved in pluripotency.

Expression of heat shock protein *HSPB1* is particularly sensitive to slight changes of pH, which has strong effects on the structure of those proteins and can substantially influence their functioning in the cell (Chernik et al., 2004). Heat shock proteins are known to play an important role not only in various stress conditions such as exposure to heat shock or freezing, but also in a variety of biological events including gene activation cell cycle arrest, differentiation and apoptosis (Palasz et al., 2008). Heat shock proteins also appear to be involved in activities such as cell motility and muscle movement, stabilizing the cell's structural framework (the cytoskeleton), assisting with folding and stabilizing newly produced proteins, and repairing damaged proteins

Gjorret et al. (2001) reported that apoptosis is more frequent in blastocysts produced in vitro that those produced in vivo. Distortions of apoptosis in the blastocysts may lead to either early embryonic death or to the formation of anomalies in the fetus that produce early abortions (Brill, 1995). The genes involved in resistance to apoptosis are for example *SURVIVIN* or *BAX*. Normally, the presence of serum during the culture period resulted in a significant increase in the level of expression of *BAX* (Rizos et al., 2003), expression of this apoptotic gene was higher in low/degenerated embryos than in good quality embryos (Yang et al., 1997). *BAX* is also related to oxidative stress.

In normal embryos, maintenance of a correct DNA methylation pattern requires both the expression and the correct regulation of *DNMT* genes involved in X chromosome inactivation, cell differentiation and imprinting, and usually associated with gene silencing. Deletion of *DNMT1* results in embryonic lethality. *DNMT3A* and *DNMT3B* are highly expressed in the developing mouse and mammalian embryos. Loss of methylation causes apoptosis in embryos.

In domestic species, there is a large body of evidence demonstrating that culture media can perturb gene expression in the developing embryo (Lonergan et al. 2003a; Wrenzycki et al. 2005). This is the case, not only when comparing in vitro and in vivo culture systems, but also when comparing among different in vitro culture systems (Eckert and Niemann 1998; Rizos et al. 2002b; Rinaudo and Schultz 2004; Wrenzycki et al. 2005). For example, when a comparison between serum free and serum-supplemented SOF (Rizos et al. 2003) was studied, the presence of serum during the culture period resulted in a significant increase in the level of expression of *Mn-SOD*, *SOX, BAX,* LIF and LIF-Rb and a decrease in the relative abundance of transcripts for *CX43* and *INT-t.*

Sanna et al. (2007) showed the effects of two different ovine culture systems containing BSA and BSA-HA on the relative abundance of a set of developmentally important gene transcripts: *SURVIVIN, HSPB1*, *GLUT-1* (metabolism), *CX43* (compaction), *DNMT1, DNMT3A* and *DNMT3B*, *OCT-4* and *FGF-4*, *RHAMM* (modulate cellular binding Hyaluronan Mediated Motility Receptor), *E-CADHERIN* (cell to cell adhesion), *IGF-II, IL-6* and *SOX17* (transcription factor for endoderm differentiation).

Thus, expression of mRNAs relevant for early embryonic development may show striking differences in relative abundance dependent on the composition of the medium (Van Soom and de Kruif, 1992). Moreover, identification of these genes would provide necessary information to adjust the existing protocols.

In addition, when preimplantation embryos are exposed to adverse culture conditions, they undergo an enormous resiliency to adapt themselves to the environment or to adjust their developmental programme to the suboptimal culture conditions (Wrenzycki et al., 2004). Embryos can have a special embryonic plasticity in particular culture systems and this depends on their genetic background (Payne et al., 1992). However, when the

capacity for compensation is overloaded, development is compromised and may result in pathological abnormalities (Young et al., 1998).

Quantitative measurements of mRNA levels can be made by several methodological approaches. The classical method of detecting gene transcripts, such as Northern blotting, requires pools of hundreds of embryos for a single analysis, while with the advent of quantitative real time PCR (qRTPCR) (Watson, 1992), using primers for specific candidate genes, it has become possible to detect mRNAs of low abundance in a few (5/10) or even in single embryos. Such gene by gene analysis also provides a narrow view of the potentially complex underlying regulatory network involved in embryo pre-implantation development, in addition this sensitive technology provides to characterize the expression profile and the role of the genes, and can facilitate the progress of the embryo culture systems and the recognition of the genes that may be implicated in the origin of the clinical abnormalities associated to IVP.

Depending on the species availability, DNA microarray analyses provide a genome-wide perspective by profiling the expression of thousands of genes simultaneously. In a recent study, mRNA expressions across a wide range of biological processes in vivo- and in vitro-cultured bovine blastocysts using cDNA microarrays were compared (Corcoran et al. 2006).

2.2.2. Cryotolerance

The purpose of cryopreservation is to hold embryos in a state of hypobiosy (reduction of the biological activity) so that when they are thawed, the normal biochemical and developmental processes will be resumed.

Through the years, different techniques of cryopreservation were developed, such us controlled slow freezing (Whittingam, 1971), fast or rapid freezing (Takeda et al., 1984;

Trounson et al., 1987) and ultra rapid freezing techniques/Vitrification (Rall and Fahy, 1985; Isachenko et al 2003; Rypka et al., 2006).

Vitrification is defined as "the solidification of a solution brought about not by crystallization but by extremely boosting viscosity during cooling" (Fahy et al, 1984). The glass state has the ionic and molecular distribution of the liquid state, thus avoiding both chemical and mechanical damage. Glass formation is usually observed when the cryoprotectant concentration is higher than 40% w/v (MacFarlane 1987) exposing the embryos to osmotic and toxic effects, but these effects can be limited by selecting the most appropriate cryoprotectants: ethylene glycol followed by glycerol and DMSO (Kasai 1996) or a mixture of two or more cryoprotectants at room temperature or after cooling to 4°C. Non-permeating agents such as sucrose added to a vitrification solution reduce the toxicity probably because the amount of intracellular cryoprotectant is reduced. A second condition for vitrification is high cooling and warming rates. The higher the cooling rate, the easier the glass formation. Thus, a practical approach to vitrification is to reach the fastest cooling rate technically possible. Usually, vitrification is carried out by plunging 0.25 ml plastic straws directly into liquid nitrogen. This alternative form of cryopreservation is more appropriate for biopsied embryos, cloned or in vitro produced (Massip et al, 2005; Vajta et al., 2000).

The ability of embryos to survive cryopreservation can be considered as an indicator for embryo quality and viability both in vitro and in vivo (Kaidi et al. 1998; Rizos et al. 2003; Moore et al., 2007; Leoni et al., 2008). In vitro-produced embryos are more sensitive to cryoinjury than in vivo-derived embryos (Leibo 1986; Hasler et al. 1995), probably the high sensibility to cryopreservation could be due to ultrastructural and functional differences, such us poorly cell to cell contact development, evaluation of total blastocyst cell number (Iwasaki et al. 1994, Kaidi et al., 1998), differences in the zona

pellucida composition and in re-expansion (Naitana et al., 1997; Berlinguer et al., 2004, Donnay et al., 2007), high lipid content (Pollard and Leibo, 1993), and viability that can alter the post-thawing metabolism (Massip, 2001; Rizos et al., 2002c).

In addition, in vitro conditions under which the embryos have been generated affect their susceptibility to freezing, Thus, it seems that culture conditions and media have a major influence on survival and development of in vitro-produced embryos following cryopreservation. Mucci et al. (2006) found no difference in blastocyst production for cultures supplemented with amino acids and oestrous cow serum and ⁄ or BSA, but post-thaw survival rates were higher for medium containing amino acids and BSA (51.9% vs.25%). The presence of serum in the medium can affect the speed of embryo development and the quality of the resulting blastocysts.

Rizos et al. (2003) demonstrated that omission of serum during the postfertilization culture period can significantly improve the cryotolerance of the blastocysts to a level intermediate between serum-generated blastocysts and the in vivo derived ones. In addition, embryos cultured in SOF with serum or BSA show a different ability to cryo-surviving, BSA embryos had a higher cryotolerance than the ones cultured with serum (Dinnyés et al., 1996; Abe et al., 2002; Dattena et al., 2007), also Lee et al. (1997) reported high pregnany rates for embryos that were cultured in SOF containing BSA.

Ovine embryos were also successfully vitrified (Gajda et al. 1989; Széll et al. 1990). The lower efficiency observed in vitrification of in vitro produced sheep embryos in the study of Traldi et al. (1999) compared to other studies (Ptak et al. 1999) could be partly due to the differences in IVP protocols. Thus, also Dattena et al. (2007) demonstrated that 6 mg/ml of glycosaminoglycan (HA) supplemented with BSA improve the cryotolerance such us affirmed by other authors (Gardner et al., 1999; Furnus et al., 1998; Lane et al., 2003; Kano et al., 1998). In addition, different doses of BSA in IVP system could improve

the cryotolerance of ovine embryos (Mara et al., 2009). In fact different culture systems could lead to differences in lambing rate after vitrification and warming showing a different embryo crytolerance.

2.2.3. Difference between in vitro and in vivo embyos

Transfer of blastocysts derived from in vivo and in vitro production into recipients would be the ultimate proof to test any step in the IVP of embryos. However, at the blastocysts stage several traits can be examined to appraise the potential of blastocysts to develop into normal, viable, and fertile offspring. Conditions during embryo culture affect the quality of the deriving blastocysts (Wrenzycki et al., 2001).

In general, differences between in vitro produced and in vivo-derived embryos were reported according to different parameters such as ***morphology,*** the IVP system has reflected morphogenetic alterations as darker cytoplasm, in fact embryos cultured in serum appear "dark" when visualised under a dissecting microscope and are less reflective of incidence light compared with serum-free cultured or in vivo derived embryos (Gardner et al., 1994; Thompson et al., 1995), this is due to the accumulation of lipid droplets within the cytoplasm (Rizos et al., 2002a) as triglycerides. Other characteristic can be less dens, swollen blastomeres, more fragile ZP, gap junction and cytoplasmatic appearances (Van Soom et al., 1996), furthermore, IVP embryos tend to contain more vacuoles and have a fewer and shorter junction complex (Shamsuddin and Rodriguez-Martinez, 1994), compaction is less pronounced in vitro embryos. In IVP embryos was observed higher incidence of chromosome abnormalities. ***Timing of development***, the timing of development in vitro, specifically the timing of completion of the third cell cycle, is a critically important parameter for predicting successful embryogenesis, normally faster developing embryos are considered to be developmentally more competent (Holm et al.,

2002; Wrenzycki et al., 2003; Gutierrez-Adan et al., 2004). ***Cell number,*** the developmental rate of embryos cultured in different media is influenced by the medium used, resulting in a significant difference of embryonic cell number, in fact blastocysts produced in vitro have fewer cells than their in vivo counterparts (Walker et al., 1992). (Van Soom et al., 1997). ***Metabolism,*** a greater degree of glycolysis occurs within embryos cultured in serum than those derived in vivo, furthermore the level of glucose oxidation is lower for in vitro embryos (Khurana and Niemann, 2000; Thompson, 2000). This observations may be related to an increased incidence of mitochondrial degeneration. Pyruvate oxidation was markedly higher for cultured embryos than in vivo derived embryos. ***Apoptosis*** morphologically high quality blastocysts of several farm animal species a significant difference exists in the percentages of apoptotic cells between in vivo and in vitro produced embryos (Van Soon et al., 2002). ***Resistance to freezing*** (Rizos et al., 2003 and 2008) and ***expression of specific mRNAs*** (Corcoran et al., 2005; Badr et al., 2007) were described in previous paragraphs.

2.3. In vivo embryo production

The in vivo embryo production (IVD) includes the following stages: ovarian stimulation, fertilization and collection of embryos.

2.3.1. Superovulation treatment

The principle of superovulation is to provide to the female a high level of exogenous gonadotrophins (typically FSH) so a greater number of follicles are recruited and selected for ovulation.

Progestagen treatments with vaginal sponge have to precede ovarian stimulation of gonadotrophins. The progestagen commonly used may be fluorogestone acetate (FGA), or medroxy-progesterone acetate (MAP) for 12 or 14 days by the insertion of an intravaginal sponge. (Thibier and Guérin, 2000). The principle of this method is to simulate the action of a natural corpus luteum, therefore, during treatment the pituitary output of gonadotrophins is suppressed. Once the sponge is removed, the pituitary gland releases increasing amounts of gonadotrophins which stimulated follicular growth and subsequent ovulation. However, despite the benefits of females synchronisation, progestagen treatments have been identified as causal agents of alterations both at systemic level, in the patterns of LH release (Scaramuzzi et al., 1988) and at ovarian level, in the patterns of follicular growth and dominance (Nöel et al., 1994; Leyva et al., 1998).

Alternatively, to usual 12-14 days progestagen treatment a shorter duration of progestagen treatment (5-9 days) was demonstrated to avoid detrimental effects of low progesterone/progestagen concentration at the end of treatment and to improve reproductive performance (Viñoles et al 2001; Dixon et al., 2006). According to this results (Mayorga et al., 2007) using short progestagen treatment in superovulatory protocols on

Sarda sheep, observed a higher tendency in terms of ovulation rate, total embryos recovered, number of viable embryos and fertility rates in ewes treated with short progestagen versus long progestagen treatment.

The ovarian response to a superovulation treatment seems to be strongly related by protocol of gonadotropin administration. The superovulatory treatment is usually given at the end of the synchronization treatment twice daily for a period of 4 days, several superovulation treatments have been tested to reduce the frequency of FSH injections (Dattena et al., 1994). Moreover a basal LH concentration is required for the optimal follicle growth (Mc Neilly et al., 1986) and an adequate FSH/LH ratio provides the best superovulatory yield (Amstrong et al., 1989).

It has been reported that, the ovarian response to superovulation protocols in terms of the mean ovulation rate and mean numbers of recovered and viable embryos generally tend to be higher in sheeps treated with decreasing FSH dosages (step-down) than in those treated with constant dosages (Gonzalez-Bulnes et al., 2004; D'Alessandro et al., 2005).

2.3.2. Fertilization

The fertilization can be achieved either by natural mating or by artificial insemination (AI). (Thibier and Guérin, 2000).

Natural mating has been widely applied in superovulatory programs because represents an easy and practical method (Gonzalez-Bulnes et al., 2002; Bartlewski et al., 2008). Breeding should be supervised and repeated every 8 hours until the donor is out of heat. Males used for breeding should be established on the property for 3-4 weeks before to be used in a multiple ovulation program and should have no history of recent illness. Stress and elevated body temperatures due to fever commonly cause temporary infertility of males. Alternatively to natural mating ***artificial insemination*** can be achieved with fresh

or frozen semen usually by vaginal or cervical techniques, but the convoluted structure of the ewe cervix makes precise deposition of semen difficult, thus reducing the chances of fertilization (Boland et al., 1983; Buckrell et al., 1994). Some studies have reported to enhance fertilization rates in superovulatory programs with artificial insemination plus natural mating (Bari et al, 2000; D'Alessandro et al., 2005).

2.3.3. Flushing technique

Embryos are collected between the 6th and 7th day after the onset of oestrus.

The more common techniques used for embryo recovery, are the ***mid-ventral laparotomy*** that ensures over 80% of embryo recovery (Wallace, 1992), however, the acceptability of this procedure has been questioned due to the reduced fertility as a result of postoperative adhesions when several collections are required from a value donor (Boundy et al., 1985; Thibier and Guérin, 2000), and the ***laparoscopy*** technique that offers several advantages in terms of animal welfare, when it is done correctly the adverse sequellae on the genital tract seems to be less than after laparotomy technique (McKelvey et al., 1986). However, it has been described that this technique is associated with a significantly lower embryo recovery rate than full laparotomy (65 to 70%) (Mc Kelvey et al., 1986). More recently semi-laparoscopic approach was described by (Bari et al., 2000) and demonstrated to improve until 83% of recovery rate embryos.

2.4. Offspring

2.4.1. Pregnancy and lambing

Bovine and ovine IVC embryos are usually transferred on day 6 or 7 (when day 0 is the day of fertilization), and the most advanced embryos are usually selected because they tend to be more viable (Hasler et al., 1995). The conditions of embryo culture have a marked effect on the quality of the resulting blastocysts and on number of new-born.

In mammals, pregnancy is the period of reproduction during which a female carries one or more live offspring from implantation in the uterus through gestation. It begins when a fertilized zygote implants in the uterus, and ends once it leaves the uterus. The length of gestation is genetically determined, although it can be modified by maternal, fetal and environmental factors.

Usually, the early diagnosis of pregnancy is required as soon as possible after mating or insemination to identify pregnant animals, Radford et al. (1960) were first described a method based on harness teaser that mark, during breeding season, the animals that will return on heat. Many literatures on pregnancy diagnosis in sheep described clinical and laboratory methods which had been employed up to now, for example ultrasound technology can be considered a very important skill to provide diagnosis pregnancy. There are two important kinds of ultrasonagraphy: ***transabdominal*** and ***transrectal.***

Transabdominal ultrasonography offers an accurate, non invasive and a practical method of pregnancy diagnosis and determining fetal numbers in sheep. This technique is reliable to determinate pregnancy after day 28-30 of gestation (Blasco and Folch, 1989; Celorrio et al., 1994) while for determining fetal number, the optimal time is between 45-90 day of gestation, after that the fetuses become too large to be consistently differentiated

from each other. (White and Russel, 1984; Haibel, 1990). The more commonly probes used in transabdominal ultrasonography are linear and sectorial tranducers with frequencies of 3,5 and 5 MHZ.

While, transrectal ultrasonography is a technique for early diagnosis of pregnancy. It is described that allows identification of the conceptus from 19-20 days after mating (Chevalier, 1988), but for an accurate determination of pregnancy and fetal number is better if is used between 25-40 days of gestation. This technique is described to be efficient to observe pregnancy until day 90-91 because the foetus is not easily accessible later (Gonzalez-Bulnes et al., 1998). The more commonly probes used in transrectal ultrasonography are linear transducers with frequencies of 5 and 7,5 MHZ.

The average gestation length in sheep varies from 144 to 151 days according to several factors. There are breed differences in gestation length. Ewes carrying multiple births tend to have shorter gestations. Moreover, IVP embryos is associated with increase gestation length (Walker et al., 1992; Thompson et al., 1995) However, the cause of this abnormalities has not been identified, Thompson et al. (1995) suggests that it is not the period of in vitro culture, but the culture system used that gives rise to these phenomena, anyway the average gestation in sheep is 146 days.

The production of offspring involving available technologies like ovum pick-up, in vitro embryo production and cryopreservation. In the last years, many authors showed successfully lambing in sheep after embryo transfer of in vivo and in vitro embryos. Several studies were found no significant difference between fresh and vitrified in vivo embryos in term of lambing rate. Mermillod et al. (1999b) obtained 60% versus 50%, Dattena et al. (1999) 81% vs 62%, Mayorga et al. (2009) 60% versus 59%. While, there were significant differences when fresh and vitrified in vitro embryos were compared. In fact, the percentages of lambing rate vary considerably under different culture media used.

For example, with serum Ptak et al. (1999) obtained a lambing rate of 41% and 23% in fresh and vitrified embryos respectively. When Charcoal-treated FBS in in vitro culture was added Accardo et al. (2004) found 66.6% (fresh) and 20.6% (vitrified). While, Dattena et al. (2007) using bovine serum albumin obtained 53.6% and 46.9% in fresh and vitrified embryos respectively; and a lambing rate of 57.4% and 43.7% for fresh and vitrified embryos when hyaluronan was added to the in vitro culture.

2.4.2. Body weight

Pregnancy rates following transfer of in vitro produced embryos are lower than in vivo (Agca et al., 1998).The influence of various in vitro procedures on embryo survival and the production of normal offspring was investigated in different species. The birth of unusually large lambs, was reported to be probably due to an inappropriate environment where the embryo is stored for a relatively long time or to an extensive manipulation in laboratory. Several factors give rise to the heavy calf or lamb and they include: asynchronous transfer, progesterone treatment, (Kleeman et al., 1994), nuclear cloning of embryos (Willadsen et al.,1991), in vitro culture (Walker et al., 1996; Holm et al., 1996), and vitrification procedures (Naitana et al., 1995; Leoni et al., 2003) and gene expression (Yazawa et al., 1997).

Van Wagtendonk-de Leeuw et al. (1998) demonstrated that the bovine IVP effect increased the birth weight by 10% (4-5 kg) and it increased by 3 days the gestation period, too. They also demonstrated that exposing in vivo matured and fertilized sheep zygotes to different culture systems, the expression of fetal oversize was depending on the nature of serum source. Farin and Farin (1995) showed that fetuses derived from IVC embryos were heavier than those from in vivo embryos. Moreover, Walker et al. (1992) reported that

lamb birth weights were heavier and gestation lengths longer when ewes carried in vitro-cultured embryos than when ewes carried in vivo-cultured embryos. Thompson et al. (1995) showed a study of IVC, with and without serum, of embryos derived from both in vivo and vitro sources demonstrating that lambs derived from IVC containing 20% human serum were an average of 20% heavier at birth than lambs from the same culture medium containing 8% BSA and no serum. Anyway, others authors reported that the presence or absence of serum didn't result in birth weight compared to control in vivo (Thompson et al., 1995; Sinclair et al., 1997). Thompson et al. (1995) also reported that, although gestation length was significantly increased by an average of 2 days in the serum group, gestation length did not account for the heavier birth weights. Holm et al. (1996) reported that lambs produced from SOF cultures containing 20% serum were heavier and had longer gestations than lambs derived from control embryos that were in vivo matured and cultured. The authors concluded that IVM-IVF, independently of culture conditions, was capable of altering birth weight and the duration of gestation. Instead, Sinclair et al. (1998) demonstrated that the exposing ovine embryos to in vitro conditions leads to increase 18-36% in mean birth weight at day 125 of gestation, depending on the culture system used. Also Wenigerkind et al. (1999) reported that birth weight is influenced by IVC system, IVC systems based on SOF may decrease the mean birth weight and a two-step culture of embryos in SOF without serum for the first 3 days is not advantageous in respect to birth weight of calves. But, when SOF with BSA and amino acids were used, the mean birth weights and incidence of abnormalities were similar to those the deriving from in vivo embryos (Walker et al., 1992; Thompson et al., 1995; Sinclair et al., 1997). In addition Merton and van Wagtendonk-de Leeuw (1998) demonstrated that the freezing seems to increase birth weight of IVP offspring calves.

Additionally, some components of IVC may be responsible of alterations of birth weight in relation to aberrant gene expression of IVP embryos.

2.4.3. The large offspring syndrome

In IVP, technology has been significantly improved in cattle and sheep, but the quality of the embryos produced is impaired in comparison with their in vivo counterparts (Niemann, et al., 2002). A considerable proportion of the offspring born from transfer of IVP embryos is affected by the large offspring syndrome (LOS). This syndrome is characterized by a variety of pathologies, including abnormal phenotypes, placental and organ defects, increased gestation length, elevated abortion and increased perinatal death, but the most important feature is a significant increase in birth weight (Walker et al., 1996; Young et al., 1998; Sinclair et al., 1998; Niemann and Wrenzycki, 2000).

The reported abnormalities in fetuses and calves following transfer of IVP embryos include increased rates of early embryonic death and abortion, production of large-size fetuses and calves, musculoskeletal deformities, disproportionate fetal growth, abnormal organ growth, failures in development of the allantois, and abnormalities of placental vasculature and development including hydrallantois. This syndrome has also been observed in sheep (Walker et al. 1996), and more recently, in mice (Eggan et al. 2001; Fernandez-Gonzalez et al. 2004).

Innumerable researches suggest that there are environmental factors which can influence the LOS: asynchronous embryo transfer into an advanced uterine environment, nuclear transfer, maternal exposure to excessively high urea diets, but one of the most important factor is the in vitro culture, in fact environmental changes due to an extended exposure of in vitro culture media can be probably linked to inappropriate epigenetic

modifications of imprinted genes (Walker et al., 1996; Kruip and den Daas, 1997; Lorraine et al., 1998; Lonergan et al., 2003b; Corcoran et al., 2005).

Lazzari et al. (2002) analysed gene expression in bovine embryos and the incidence of LOS in calves derived from transfer of embryos produced with different in vitro and in vivo systems. In vitro culture of bovine embryos in the presence of high concentrations of serum or BSA significantly increased the number of cells in day 7 blastocysts, the size of the blastocyst on day 12 and the relative abundance of transcripts for several genes including glucose transporter (*GLUT1*, *GLUT2, GLUT3, GLUT4),* heat shock protein (*HSP70.1*), Cu /Zn-superoxidave dismutase (*SOD*), basic fibroblast growth factor *(bFGF)* insulin-like growth factor (*IGF1R* and *IGF2R)* when compared with embryos cultured in vivo. These deviations were linked to gestation length and birth weight of the derived calves. Changing culture supplements from serum to BSA were previously shown to reduce the frequency of LOS.

IGF2R role in LOS is very important because its epigenetic change creates oversized offspring due to the production of a protein which blocks the normal growth of cells (Yaseen et al., 2001; Young et al., 2001). When the receptor is deactivated the embryos grow too large. Other symptoms can include enlarged hearts, immature lungs and damaged kidneys, but Lazzari et al. (2002) reported that the relative abundance of *IGF2R* transcripts was not affected by different production systems (Serum and BSA).

3.

MATERIALS AND METHODS

3. Materials and methods

The experiments were done at the Agris – DIRPA (Dipartimento della Ricerca nelle Produzioni Animali) – Servizio Zootecnico – Settore Scientifico Riproduzione S.S. 291 Km 18.6, 07100 Sassari (Italy) together with Dpto. de Reproducción Animal y Conservación de Recursos Zoogenéticos, INIA, Ctra de la Coruña Km 5.9, 28040 Madrid (Spain).

Except where otherwise indicated, all chemicals were obtained from Sigma-Aldrich.

First experiment: "Pregnancy, lambing and birth weight of ovine embryos produced in vitro in 4 different culture media"

3.1. Collection of ovaries and oocytes

Ovaries of adult Sarda sheep (Fig. 1) were obtained from local slaughterhouses and transported in a cool bag to the laboratory within 2-3 hours in saline solution at a temperature of ~35-37 °C. Oocytes were collected by cutting the ovaries (Fig. 2). Only the follicular oocytes recovered by at least 2 layers of granulosa cells and an even cytoplasm were selected for in vitro maturation.

Fig. 1. Ovaries of sarda sheep

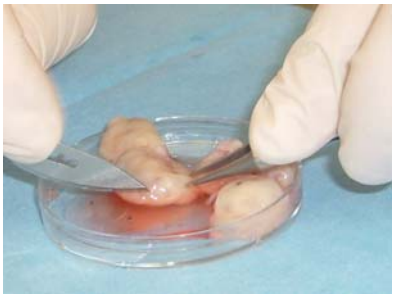


Fig. 2. Ovaries cutting

3.2. In vitro embryo production (IVP)

3.2.1. Culture treatment

In this experiment we have considered 4 groups of embryos produced in vitro in different culture media and transferred into recipient ewes. The groups are indicated with the letters A(BSA_4), B(BSA_8), C(BSA_8-HA), D(BSA_8-CH) and with parenthetical initials identifying the kind of culture in accordance with the supplement used (see Table 1). The rationale of these 4 groups was the following: in previous paper Dattena et al. (2007) showed that BSA (8 mg/ml) gives heavy lambs, for this reason we decided to halve the dose from 8 to 4 mg/ml. In previous studies other authors reported that use of hyaluronan improves in general embryo quality (Furnus et al., 1998; Kano et al., 1998; Lane et al., 2003; Dattena et al., 2007), for this reason we used BSA with hyaluronan during culture period. D(BSA_8-CH) is the protocol traditionally used in our laboratory (Accardo et al., 2004).

Table 1. Groups of culture media with different supplements of BSA and serum.

Culture Groups	**IVM**	**IVC Day 1**	**IVC Day 3-5-7**
A (BSA_4)	4mg/ml BSA	4mg/ml BSA_{faf}	4mg/ml BSA_{faf}
B (BSA_8)	4mg/ml BSA	8mg/ml BSA_{faf}	8mg/ml BSA_{faf}
C (BSA_8-HA)	4mg/ml BSA	8mg/ml BSA_{faf}	8mg/ml BSA_{faf} + 6mg/ml HA
D (BSA_8-CH)	4mg/ml BSA	8mg/ml BSA_{faf}	10% Charcoal Stripped FBS

3.2.2. In vitro maturation (IVM)

Oocytes were washed in Hepes-buffered TCM199 (H-TCM199) supplemented with 4 mg/ml BSA (bovine serum albumin) and 2 mM glutamine. The medium used for maturation was bicarbonate-buffered TCM199, containing 2 mM glutamine, supplemented with 4 mg/ml BSA for A, B, C and D groups, 100 μM cysteamine, 0.3 mM sodium pyruvate, 1 μg/ml estradiol-17β plus 0.1 i.u./ml r-FSH (follicle stimulating hormone) and 0.1 i.u./ml LH (luteinizing hormone). The PH was adjusted to 7.2/7.4 and the osmolarity to 275 mOsm. The oocytes were incubated in 400 μl of medium in 4-well dishes (Nunc, Nunclun Denmark) with each well containing 20-30 oocytes covered with mineral oil. IVM conditions were 5% CO_2 in humidified air at 39 °C for 24 h.

3.2.3. In vitro fertilization (IVF)

After maturation the oocytes were partially denuded of granulosa cells with 300 i.u./ml of hyaluronidase in H-TCM199 using a glass capillary pipette in order to help the spermatozoa penetration and then they were washed three times in H-TCM199 with BSA to remove the hyaluronidase residuals. Fresh semen of Sarda breed ram of proven fertility was collected and kept at room temperature for up to 2 h and then it washed in synthetic oviduct fluid (SOF) supplemented with 4 mg/ml of fatty acid free BSA (BSA_{faf}), centrifuged at 200xg for 5 min at 20°C, discarded supernatant was added directly to the fertilization medium (Fig. 3). The fertilization medium was consisting of SOF (Tervit et al., 1972) enriched with 20% heat inactivated estrous sheep serum. The oocytes was washed three times in medium of fertilization and a maximun of 10-15 oocytes per drop were fertilized in 50 μl with $1x10^6$ sperm/ml at 39°C and 5% CO_2 in a humidified air (Fig. 4).

Fig. 3. In vitro fertilization

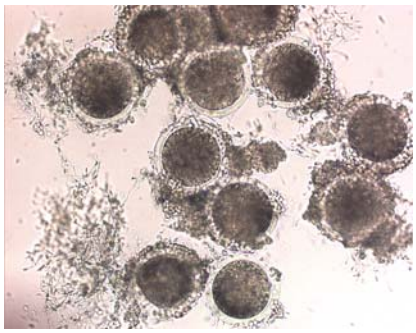


Fig. 4. Fertilized oocytes

3.2.4. In vitro culture (IVC)

The day after the fertilization, presumptive zygotes were washed in SOF to remove spermatozoa debris. They were allocated to 20 µl-culture drops (5-6 embryos/drops) consisting of SOF supplemented with 1% (v/v) Basal Medium Eagle (BME)-essential amino acids, 1% (v/v) minimum essential medium (MEM) - non-essential amino acids, 1 mM glutamine and specific supplements (see Table 1 "IVC day 1"). Embryos were incubated in an humidified air of 7% O_2, 5% CO_2, 88% N_2 at 39°C. On the third, fifth and seventh day of culture (day 0 defined as the day of fertilization) different supplements were added to the culture (see Table 1). The culture was continued for 6-7 days until blastocyst stage (Fig. 5).

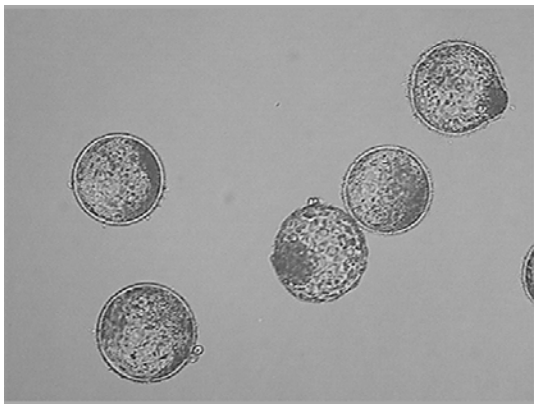


Fig. 5. Blastocysts (6/7 days) produced with BSA_4

3.3. Vitrification embryos

A part of embryos of A, B, C, D groups produced by IVP were vitrified before to be transferred in recipients according to Dattena et al. (2004). Briefly, all vitrification solutions were prepared using Dulbecco's PBS supplemented with 0.3mM sodium pyruvate, 3.3 mM glucose and 20% FBS. Expanded blastocysts were exposed at room temperature to equilibration solution (V1) 10% ethylene glycol (EG) and 10% dimethylsulfoxide (DMSO) for 4-5 min, then to the vitrification solution (V2) 20% EG; 20% DMSO and 0.5 M sucrose for ≤ 45 sec, then were loaded into open pulled straws (OPS) and immediately plunged into liquid N_2 (2 blastocysts per straw).

3.4. Embryo transfer

Only embryos of high quality (Q1) were utilised for embryo transfer. The embryo evaluation was observed using a stereomicroscope (magnification 80x). Totally 194 embryos in vitro produced (Q1) were used: n° 90 fresh and n° 104 vitrified.

Fresh and vitrified embryos of A, B, C, D groups in pairs were transferred into sheep (synchronized or with natural oestrus) usually at 6-7 days of culture and carried out by inguinal mini-laparatomy by indirect (fresh embryos) and direct (vitrified embryos) transfer methods with the help of a Tom-cat. The OPS containing the vitrified embryos were warmed before embryo transfer by holding for 6 sec in air and then dipped into a Falcon tube with H-TCM199 + 20% FBS + 0.5 M sucrose in a water bath at 37° C for 15 sec.

3.5. Pregnancy diagnosis

Pregnancy of transferred ewes was confirmed by abdominal ultrasonography using 50S Tringa Esaote (U.S.A.) MHz ultrasound scanner with 5MHz transducer at day 40 post embryo transfer.

3.6. Lambing

At 146 day of pregnancy ewes were allocated indoor for monitoring the onset of lambing. The lambs borned were weight and identified within 6 h after birth.

Second experiment: "Gene expression analysis of ovine embryos produced in vitro in 5 different culture media versus in vivo derived embryos"

3.7. In vitro embryos production

Embryos produced in vitro in the first experiment (A, B, C, D) were used in this second experiment, and was added an other group: E(FBS_{10}), 10% FBS (fetal bovine serum) in IVM and IVC steps, this is of the most conventional protocol used in most part of the laboratory.

3.8. In vivo embryo production (IVD)

In this study in vivo embryos were used as control in gene expression analysis and this group was indicated as F(in Vivo).

3.8.1. Synchronisation of oestrus

Six Sarda ewes in adult age (2-5 years) and healthy conditions were selected through oestrus detection by the introduction of a vasectomised ram in the flock twice daily (8:00 a.m.–16:00 p.m.). The ewes were considered to be on oestrus when they show oestrus behaviour and were mated by the vasectomised ram. The visualisation of oestrus behaviour take at least one hour and was performed according to Ortman R. (2000) with some modifications.

3.8.2. Superovulation treatment

Superovulatory treatment consisted in 350 I.U. of porcine FSH (Folltropin®, Bioniche Animal Health, Ireland) administered in eight (i.m.) decreasing doses at every 12 h (2 ml x 2, 1.5 ml x 2, 1.0 ml x 2 and 0.5 ml x 2) starting on day 4 after oestrus (day 0). A single dose of 125 µg (i.m.) cloprostenol (Estrotek®, Azienda Terapeutica Italiana, Italy) was injected on day 6 after oestrus detection to induce luteolysis.48 h before sponge removal. All donors were naturally mated 24 h after sponge removal.

3.8.3. Oestrus detection and mating

Oestrus detection was performed twice daily (8:00 a.m.–16:00 p.m.) with help of the vasectomised ram for visualizations of oestrus behaviour from cloprostenol injection. When oestrus was detected donors were mated with fertile rams which were replaced a another one 12 h intervals until the animals refused mating.

3.8.4. Recovery embryos

Surgical embryo recovery was performed at day 8 after cloprostenol injection. All donors were deprived of food and water for 24 h prior to surgery. The ewes were sedated with acepromazine maleate (Prequillan®, Fatro, Italy) with a dose of 3 mg/ewe (i.v.) and general anaesthesia was induced with penthotal sodium (Penthotal sodium®, intervet, Italy) with a dose of 10 mg/kg body weight (i.v.). In order to asses the number of Corpora Lutea (CL) the reproductive tract was extracted with an incision of 5-6 cm by inguinal mini-laparatomy. According to Tervit and Havik (1976) and minor modifications embryos were obtained by surgical flushing technique. After flushing, the recovered embryos were kept less than 30 min at room temperature in HTCM199 + BSA 0.4% and evaluated morphologically using a stereomicroscope (magnification 80 X),

then 30 embryos (Q1) in group of 10 were collected and stored at -80°C prior to gene expression analysis.

3.9. Gene expression analysis

Totally 180 embryos (Q1) were used for gene expression analysis: n° 150 fresh and n° 30 in vivo.

In vitro fresh blastocysts of A, B, C, D, E groups and in vivo embryos of F group were snap frozen in liquid N_2 for 3-5 min, three pools of 10 embryos per culture group and then were stored at -80°C prior to use for gene expression analysis. In this experiment 8 genes important for imprinting (*IGF2R, GRB10*, *UBE2A*), embryo development (*LAMA1, IL-6*, *FGF4*), apoptosis (*BAX*) and methylation (*DNMT3A*) were analysed.

In addition, the gene implicated in response to stress (*HSPB1*) was tested. Unfortunatelly it was possible to test this gene only in B (BSA_8) and C (BSA_8-HA) groups because there were not enough cDNA in the other groups.

3.9.1. mRNA isolation

Poly(A)$^+$ RNA was isolated using a Dynabeads® mRNA Direct™ Micro Kit (Dynal Oslo, Norway) according to the manufacturer's instructions with minor modifications (Fig. 6). Briefly, fresh blastocysts were lysed by adding 50 µl of lysis/binding buffer (100 mM Tris-HCl, pH 7.5; 500 mM LiCl; 10 mM EDTA, pH 8.0; 1% LiDS; 5 mM DTT). Then the lysate was transferred to a tube containing 10 µl prewashed Dynabeads Oligo $(dT)_{25}$. After 5 min incubation at room temperature, necessary for binding poly(A)$^+$ RNAs to oligo (dT) Dynabeads, the Dynabeads-mRNA

complex was washed twice using 50 µl washing buffer A (10 mM Tris-HCl, pH 7.5; 0.15 M LiCl; 1 mM EDTA; 0.1 % LiDS) and twice with 50 µl washing buffer B (10 mM Tris-HCl, pH 7.5; 0.15 M LiCl; 1 mM EDTA). Then the complex was resuspended in 10 µl of ice-cold 10mM Tris-HCl. The whole process was carried out employing a Dynal MPC®-M magnetic separator to discard the supernatant at every step.

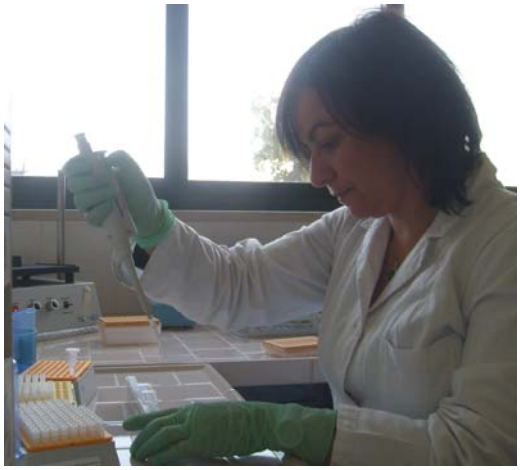


Fig. 6. mRNA isolation from blastocysts

3.9.2. cDNA synthesis by Reverse Transcriptase PCR (RT-PCR)

The Dynabeads-mRNA complex was directly reverse transcribed into cDNA in a total reaction volume of 20 µl according to the M-MLV Reverse Transcriptase manufacture's instructions (Invitrogen) with minor modifications. The oligo $(dT)_{25}$ bound to the bead surface used to capture the mRNA was also employed as a primer. The RT reaction was carried out at 50°C for 5 min, 65°C for 5 min followed by flash cooling on ice then briefly centrifuged. The mixture was then added to 4 µl of First-Strand Buffer, 2 µl of 0.1 M DTT and 1 µl of RNaseOUT™ (40U/µl; Invitrogen). The mixture was then incubated at 37°C for 2 min, added to 1 µl (200 U) of M-MLV RT, incubated at

37°C for 50 min and inactivated at 70°C for 15 min. The synthesized cDNA by RT-PCR was then used for gene expression analysis.

3.9.3. PCR primers

Primers were used to amplify histone H2AFZ *(H2AFZ),* insulin-like growth factor 2 receptor (*IGF2R*), growth factor receptor-bound protein 10 (*GRB10*), ubiquitin-conjugating enzyme E2A (*UBE2A*), laminin alpha 1 (*LAMA1*), interleukin 6 (*IL6*), fibroblast growth factor 4 (*FGF4*), Bcl-2-associated X protein (*BAX*), DNA-methyltransferase 3 alpha (*DNMT3A*) and heat shock protein binding protein 1 (*HSPB1*). Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 2.

3.9.4. Quantitative Real Time PCR (qRTPCR)

The quantification of all mRNA transcripts was carried out by quantitative real-time (qRTPCR). For qRTPCR, 3 groups of cDNA per experimental group, each obtained from 10 embryos, were used with two repetitions for all genes of interest. Experiments were conducted to contrast relative levels of each transcript and histone *H2AFZ* in every sample. PCR was performed by adding a 2 µl aliquot of each cDNA sample to the PCR mix containing the specific primers (Table 2.). Primer sequences, annealing temperature, the approximate sizes of the amplified fragment of all transcripts and the GeneBank® accession number are shown in Table 2. For quantification, PCR was performed using a Rotorgene 2000 Real Time Cycler™ (Corbett Research, Sydney, Australia) (Fig. 7) and SYBR Green (Molecular Probes, Eugene, OR) as a double-stranded DNA-specific fluorescent dye. PCR conditions were optimized to achieve efficiencies close to 1 and then the comparative cycle threshold (CT) method was used to quantify expression levels

as described by Schmittgen and Livak (2008). Quantification was normalized to the endogenous control, *H2AFZ*. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative CT method, the ΔCT value was determined by subtracting the *H2AFZ* CT value for each sample from each gene CT value of the sample. Calculation of ΔΔCT involved using the highest sample ΔCT value (i.e., the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$

Fig. 7. Rotorgene 2000 Real Time Cycler

Table 2. Details of primers used for qRTPCR

Functions	Gene	Primer Sequence (5'- 3') Forward and Reverse	Fragment size (bp)	Annealing temperature (°C) x cicle number	Gene Bank® Accession No.
Endogenous Control	***H2AFZ***	(F) AGGACGACTAGCCATGGACGTGTG (R) CCACCACCAGCAATTGTAGCCTTG	212	56 x 35	NM174809.2
Imprinting	***IGF2R***	(F) GCTGCAGTGTGCCAAGTGAAAAAG (R) AGCCCCTCTGCCATTGTTACCT	201	56 x 35	NM174352.2
	GRB10	(F) GAAGACGGGACAAGCAAAGT (R) CTGGCACCACGTAACCATCTG	291	56 x 35	XM602256.4
	UBE2A	(F) GGGCTCCGTCTGAGAACAACATC (R) CATACTCCCGCTTGTTCTCCTGG	336	56 x 35	XM864331
Embryo development	***LAMA1***	(F) CCCTGCCAGCAATGCACACATC (R) TCGGATGCCGTTCTGTTGAAGG	341	56 x 35	AF010231
	IL6	(F) CGCCTTCACTCCATTCGCTGTC (R) CGCCTGATTGAACCCAGATTGG	307	56 x 35	NM173923
	FGF4	(F) AACGTGAGCATCGGCTTCCACC (R) TTGCTCAGGGCGATGAACATGC	284	56 x 35	NM001040605.1
Apoptosis	***BAX***	(F) CTACTTTGCCAGCAAACTGG (R) TCCCAAAGTAGGAGAGGA	158	56 x 35	NM173894.1
Methylation	***DNMT3A***	(F) CTGGTGCTGAAGGACTTGGGC (R) CAGAAGAAGGGGCGGTCATC	317	56 x 35	AY271299
Stress	***HSPB1***	(F) TCCCTGGACGTCAACCACTTCG (R) AGGTTTGGCGGGTGAGGATGTC	391	56x35	NM174068.1

3.10. Analysis of data

3.10.1. Statistical analysis of pregnancy, lambing and birth weight.

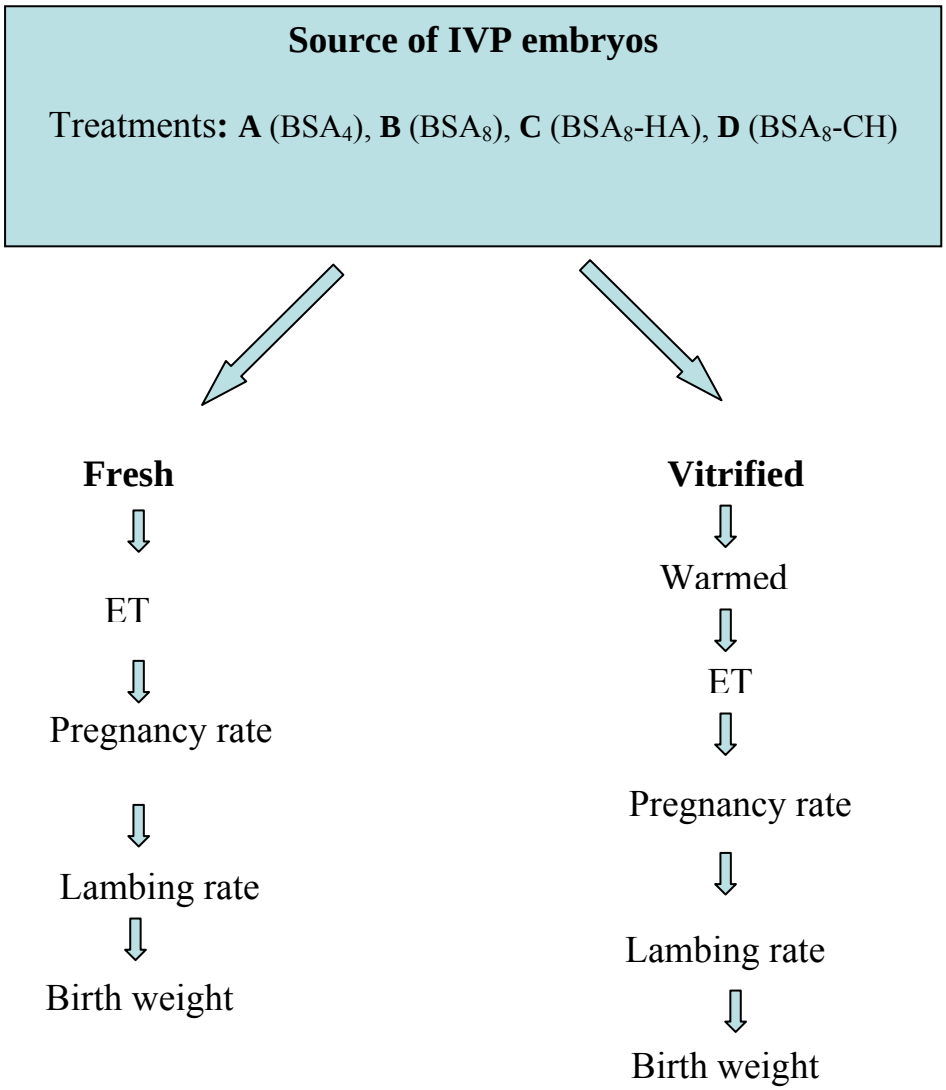
Pregnancy rate, lambing rate and birth weight between fresh and vitrified embryos and among culture groups were performed using Chi-Square analysis. Differences with $P<0.001$, $P<0.01$ and $P<0.05$ were considered statistically significant.

3.10.2. Statistical analysis of gene expression data

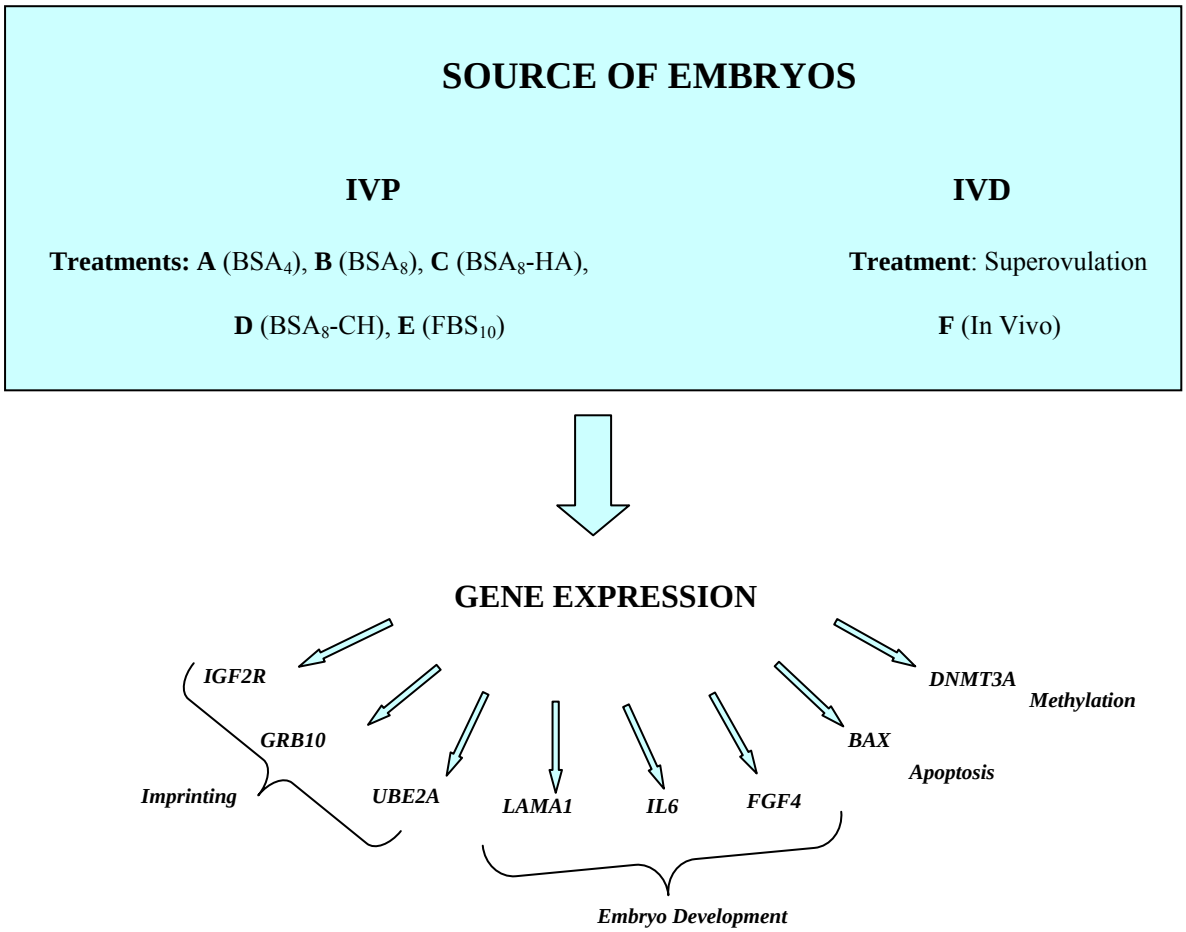
Data on mRNA expression were analyzed using the SigmaStat (Jandel Scientific, San Rafael, CA) software package. One-way repeated-measures ANOVA followed by Student- Newman-Keuls methods was used for the analysis of differences in mRNA expression assayed by qRTPCR. Differences with $P<0.05$ were considered statistically significant.

3.11. Experimental design

3.11.1. First experiment



3.11.2. Second experiment



Source of embryos
IVP

Treatments: B (BSA_8) and **C** (BSA_8-HA)

GENE EXPRESSION of *HSPB1* (*Stress*)

(Because shortage of cDNA was tested only in two groups)

4.

RESULTS

4. Results

4.1. Pregnancy rate

After embryo transfer, among the groups A, B, C, D and between fresh and vitrified embroys there were not significant differences in the pregnancy rate at 40 d (Table 3) different culture media.

Table 3. Pregnancy rate (at 40 days) of embryos produced in vitro after culture in SOF with different supplements and transferred as fresh and vitrified after warming into recipient ewes.

	Culture medium	N° of blastocysts transferred	Recipients	Pregnancy (%)	Significance
FRESH	**A** (BSA_4)	26	13	8/13 (61.5)	NS
	B (BSA_8)	20	10	7/10 (70)	
	C (BSA_8 + HA)	20	10	6/10 (60)	
	D (BSA_8 + CH)	24	12	10/12 (83.3)	
VITRIFIED	**A** (BSA_4)	24	12	7/12 (58.3)	NS
	B (BSA_8)	24	12	9/12 (75)	
	C (BSA_8 + HA)	24	12	8/12 (66.6)	
	D (BSA_8 + CH)	32	16	9/16 (56.2)	

4.2. Lambing rate

When lambing rate of fresh embryo was compared within groups no statistical difference was found (see Table 4).

When lambing rate of vitrified embryos was compared within groups statistical difference ($P<0.05$) was found between A vs D group (see Table 4). However differences close to significance was found in the vitrified embryos also for B and C groups.

Because of similar results among fresh and vitrified embryo groups it was decided to compare also fresh and vitrified groups with statistical difference only when group D fresh was compared with D vitrified ($P<0.001$) (Table 4).

Table 4. Lambing rate of embryos produced in vitro after culture in SOF with different supplements and transferred as fresh and vitrified after warming into recipient ewes.

	Culture medium	N° of blastocysts transferred	Born lambs/transferred embryos (%)	Significance
FRESH	**A** (BSA_4)	26	14/26 (53.8)	NS
	B (BSA_8)	20	10/20 (50)	
	C (BSA_8 + HA)	20	12/20 (60)	
	D (BSA_8 + CH)	24	16/24 (66.6)	
VITRIFIED	**A** (BSA_4)	24	12/24 (50) [a]	
	B (BSA_8)	24	11/24 (45.8)	
	C (BSA_8 + HA)	24	10/24 (41.6)	
	D (BSA_8 + CH)	32	7/32 (21.8) [b]	

Significance: a, b = $P<0.05$

4.3. Birth weight

The body weight of Sardinian lambs was considered heavy when it was ≥ 4.5 Kg.

Thus heavy body weight was found in B (2/10, 20%) and C (2/12, 16,6%) when compared with the other groups. Similar results were obtained with vitrified groups: B (3/11, 18%) (Fig. 8) and C (BSA+HA) (1/10, 20%). While, A (Fig. 9) and D groups did not gave heavy lambs.

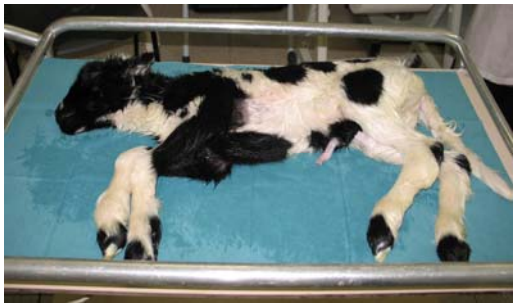


Fig. 8. Heavy lamb (4.6 Kg), after E.T. from B(BSA_8) vitrified embryo

Fig. 9. Lamb born after E.T. from A(BSA_4) fresh embryo

4.4. Relative mRNA abundance of selected gene transcripts

The relative mRNA abundance of the gene transcripts studied is showen in Fig.10 and in Fig.11.

Imprinting genes

The expression of three imprinted genes related with fetal overgrowth, *IGF2R*, *GRB10* and *UBE2A* were analyzed. *IGF2R* was significantly upregulated in all in vitro groups compared to in vivo produced embryos, while *GRB10* expression did not differ between groups. Moreover, *UBE2A* was significantly ($P<0.05$) upregulated in the in vitro groups compared with in vivo derived group. *UBE2A* in the C group was downregulated compared with the other four in vitro groups

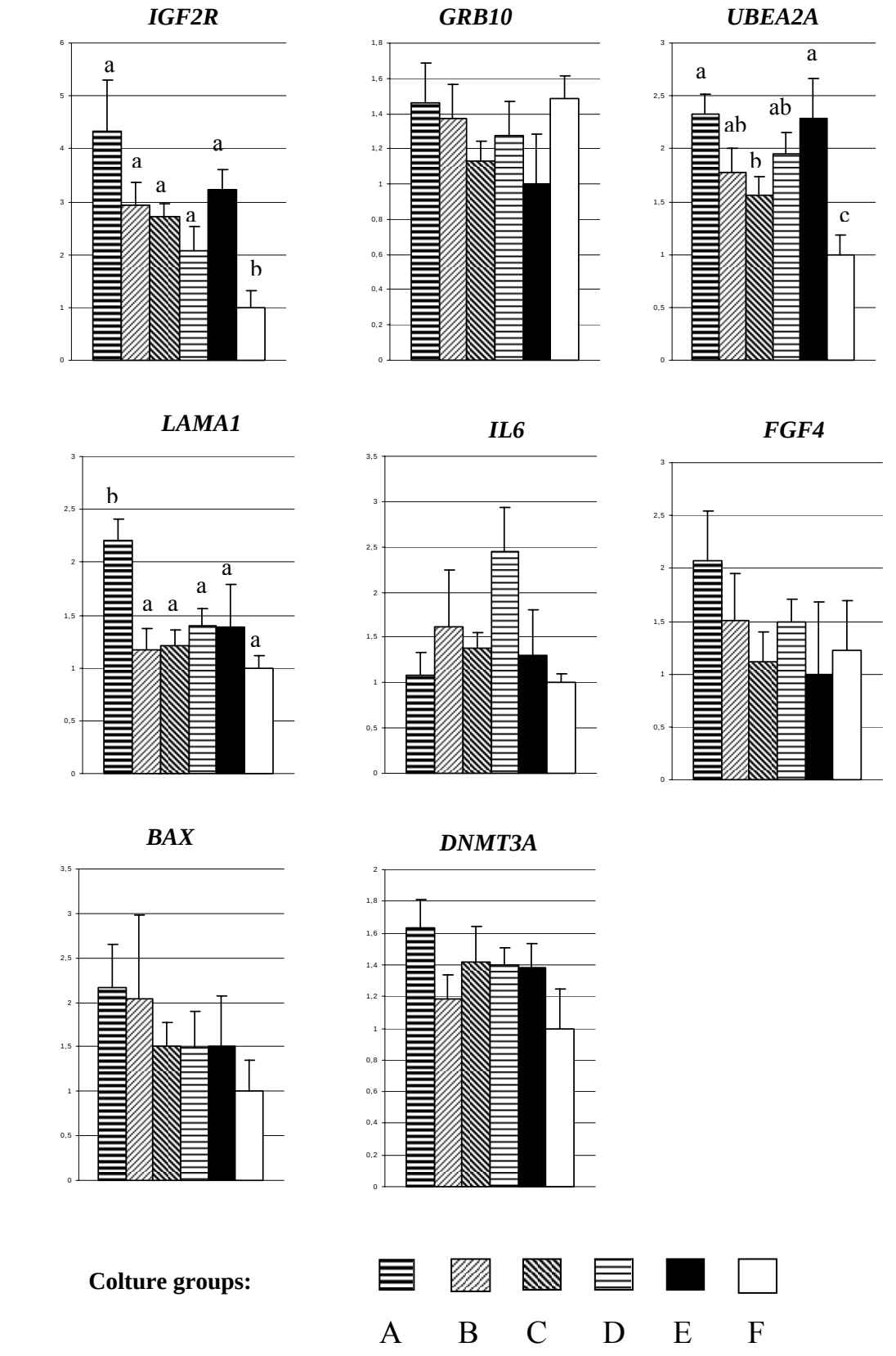
Genes of embryo development

The transcript abundance of three genes known to be affected by embryo culture conditions was analyzed. *LAMA1* was significantly upregulated ($P<0.05$) only in group A when compared with in vivo embryos. In addition, when *LAMA1* was compared within the vitro groups the A group was also upregulated ($P<0.05$). There were not differences for *IL6* and *FGF4* in all groups.

Apoptosis and methylation genes

Relative mRNA abundance of two genes related with apoptosis (*BAX*) and de novo methylation (*DNMT3A*) were analyzed. No differences were observed in *BAX* and *DNMT3A* in all groups.

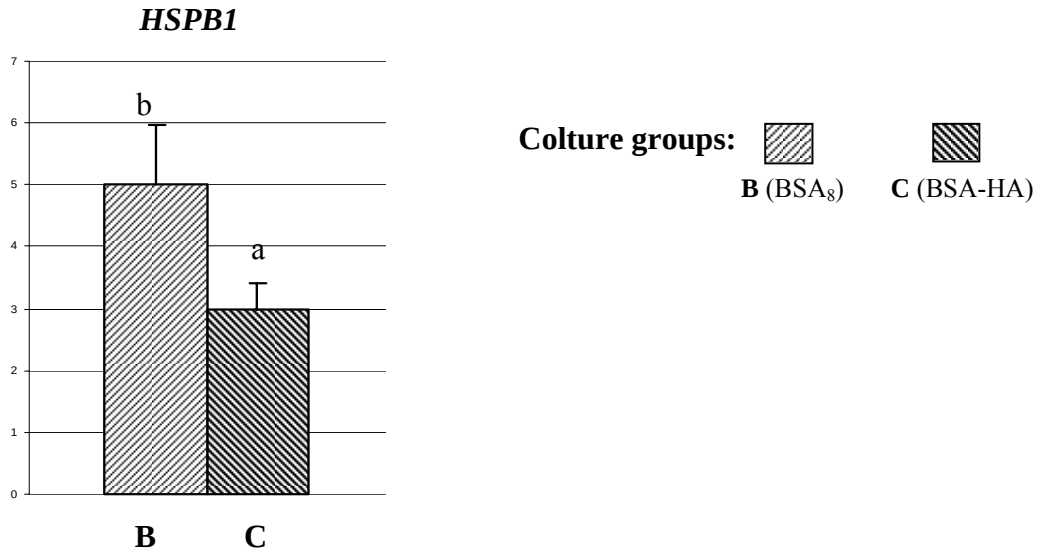
Fig. 10. Relative mRNA abundance of various developmentally important genes transcripts in fresh sheep embryos cultured in vitro in SOF with different supplements.



Response to stress

Finally, the level of expression was decreased in presence of HA. The relative abundance of *HSPB1* transcripts in two different ovine culture systems containing bovine serum albumin and bovine serum albumin with hyaluronan was significantly ($P<0.05$) upregulated in B group compared with C group.

Fig. 11. Relative mRNA abundance of *HSPB1* gene transcript in fresh sheep embryos cultured in vitro in SOF with two different supplements.



5.

DISCUSSION AND CONCLUSIONS

5. Discussion and Conclusions

5.1. First Experiment

"Pregnancy, lambing and birth weight of ovine embryos produced in vitro in 4 different culture media"

The results presented in this study demonstrated that the use of different supplements can affect embryo viability in terms of cryotolerance and lamb body weight.

The pregnancy rates were not significantly different within treatments, both in fresh and vitrified groups, when embryos were transferred into ewes. The serum treated group (D) of freshly transferred embryos had higher pregnancy rate (83.3%) among all considered groups, but when the same treatment was used in the vitrified group the lower pregnancy rate was recorded (56.2%). These data, although they were not statistically compared, suggested that the serum treatment had reduced cryotolerance.

Moreover, the lambing rate following transfer of fresh embryos although it did not reach significant differences, was higher in the serum group. However, when we consider the vitrified serum group, the lambing rate was significantly different when compared with BSA_4. These data are similar to those reported by Mara et al. (2009) in sheep and by Lazzari et al. (2002) in cows.

In addition, although not statically different, possibly due to small number of observations, all the other BSA groups (B and C) showed tendency to be more cryotolerant than serum group.

In our experimental conditions, the addition of hyaluronan did not improve the lambing rate. These results are in contradiction with other study where it is reported an enhanced lambing rate when this macromolecule is used (Lane et al., 2003).

As reported by previous authors in vitro culture (Walker et al., 1992; Holm et al., 1994) and vitrification of embryos (Young et al., 1998; Sinclair et al., 1999) may result in fetuses that are heavier at birth than those obtained in vivo. Although there were not significant differences among the groups analyzed in term of birth weight, in both BSA_8 and BSA_8-HA groups (fresh and vitrified) heavy lambs were born.

Similarly, Lazzari et al. (2002) and Dattena et al. (2007) reported the presence of heavy born animal from blastocysts produced in vitro with BSA. On the contrary, other studies reported reduction of large offspring when BSA is added in place of serum during culture (Thompson et al., 1995; van Wagtendonk-de Leeuw et al., 2000). These conflicting findings underscore that incidence of heavy lambs varies greatly and that these conditions are difficult to reproduce (Young et al., 1998). In addition, serum doses or BSA, number of batches and the culture system used vary greatly from lab to lab making data difficult to compare.

Thus, in order to reduce the heavy lamb syndrome, keeping good cryotolerance, we decided to halve the dose of BSA from 8 mg/ml to 4 mg/ml. It was reported by Lazzari el al. (2002) (that) high doses of BSA are considered between 16 mg/ml and 8 mg/ml. Indeed, in this study when 4 mg/ml BSA treatment was used no heavy lambs were born.

For all these reasons to asses a possible effect of these culture media, expression analysis of some genes involved in embryonic welfare were evaluated and discussed in the second experiment.

5.2. Second experiment

"Gene expression analysis of ovine embryos produced in vitro in 5 different culture media versus in vivo derived embryos"

It is known that gene expression can provide a useful tool to test quality of blastocysts. In parallel to transfer of fresh embryos in vitro produced the expression of nine genes known to be involved in imprinting, embryo development, apoptosis, methylation and oxidative stress was examined and compared with the in vivo embryo production counterpart.

Presumably, current in vitro culture systems can lead to either persistent silencing or enhanced expression of a particular gene throughout critical phases of fetal development. Normally, 85% of genes are downregulated in IVP blastocysts. Corcoran et al. (2006) found 384 genes differentially regulated in IVF embryos, and there was in their study a general downregulation of genes involved in transcription and translation.

On the contrary, the genes selected for this study seems to be upregulated in vitro compared to their in vivo counterpart, although this is not statistically significant for all genes. The genetic aberration, which normally leads to altered phenotypes relating to weight (large calf/lamb), is determined in vitro by a downregulation of genes involved in embryo development. For this reason an involvement of transcription mechanisms is possible since it is demonstrated that they determine gene deficiency.

Imprinting genes analysed were *IGF2R, GRB10* and *UBE2A.* In culture systems, *IGF2R* is expressed throughout preimplantation development up to the blastocyst stage in a varying pattern. Normally, the concentration of mRNA for this gene seems to follow a pattern similar to most genes expressed during embryo culture while in the case in point

it increases, indicating that culture may depend on increased mRNA from this gene (Yaseen et al., 2001). In addition, it is demonstrated that reduced fetal methylation and expression of *IGF2R* in vitro suggest that pre-implantation embryos may be vulnerable to epigenetic alterations in imprinted genes (murine: Lau et al., 1994; bovine: Lonergan et al., 2003b; ovine: Young et al., 2001). *IGF2R* is a maternally imprinted gene which encodes a trans-membrane receptor that transport mannose-6-phosphate tagged proteins and IGF2 to lysosomes, reducing the amount of IGF2 and thereby decreasing embryonic growth. Disruption of *IGF2R* imprinting (failure to paternal allele repression) caused a two-fold increase in its expression and it is associated with reduction in fetal and adult weight in mice (20%) (Wutz et al., 2001), and a reduction of its expression and methylation level in sheep fetuses has been associated with fetal overgrowth (Young et al., 2001). In contrast, in this experiment the *IGF2R* level was upregulated in in vitro embryos groups compared with in vivo counterparts. To our knowledge, no study has analyzed *IGF2R* expression in in vivo produced ovine embryos. Thurston et al. (2008) found that *IGF2R* was biallelically expressed in ovine blastocysts by comparison of its expression levels in embryos produced by parthenogenesis and IVF. However, in vitro culture conditions may be associated with disruption of imprinting (Sinclair et al., 2000) and therefore the present results may be a consequence of monoallelic expression of *IGF2R* in biallelic in in vitro produced and in vivo derived blastocysts, which may lead to aberrant imprinting regulation in later stages (Young et al., 2001).

GRB10 plays an important role in regulating insulin and is involved in IGF signalling. It is a maternally expressed gene in some tissues and biallelically expressed in others (Charalambous et al., 2003) The disruption of the imprinted *GRB10* in mice (maternal allele transcriptional suppression) is associated with overgrowth by an IGF2-independent mechanisms (Charalambous et al., 2003) and it has been suggested to be

implicated in Silver-Russel syndrome which is related to a growth disorder (Hitchins et al., 2001). As *IGF2R* was found to be biallelically expressed in ovine blastocysts (Thurston et al., 2008), no differences were observed among embryo groups suggesting that transcriptional regulation of *GRB10* gene could be not involved in weight increase of lambs.

UBE2A is an X-linked gene which encodes a ubiquitin-conjugating enzyme (E2) in the proteasome pathway of protein degradation. Its expression is known to be affected by embryo culture conditions in bovine (Palasz et al., 2008) and *UBE2A*-knockout mice body weight was significantly decreased (Roest et al., 2004). Although no evidence of imprinting was found in mice, *UBE2A*-knockout females fail to produce offspring in spite of normal ovulation because embryos arrested at the 2-cell stage, whereas male fertility was not affected (Roest et al., 2004), which may suggest a maternal imprinting in a tissue specific manner. In this perspective, this experiment showed significantly higher transcript abundance in the in vitro groups compared with the in vivo derived group. This fact may be a consequence of biallelic expression of *UBE2A* in in vitro produced blastocysts and monoallelic expression in the in vivo derived ones. It was recently found that *UBE2A* is paternally imprinted in bovine blastocysts. There is a programmed epigenetic process of X-inactivation during preimplantation development (Fernandez-Gonzalez et al., 2007) and IVC is thought to affect the transcription levels of X-linked genes (Nino-Soto et al., 2007). This disruption of X-inactivation produced by IVC could be the reason of the increased expression of *UBE2A* observed in the embryos produced in vitro.

LAMA1 is a gene involved in organization of cells into tissues during embryonic development, it was upregulated in group D compared with the rest of the groups. This gene was found to be affected by culture conditions (Palasz et al., 2008) and it encodes a

glycoprotein present in both embryonic and extraembryonic basement membranes. Transcription changes may reflect changes in the integrity and molecular composition of cellular membranes (Miner et al., 2004), which in our study may be linked to a lower surfactant tension in group D (BSA_4) compared with the rest of the groups.

In addition, two genes related with embryo development were analysed: *IL6* and *FGF4.* The first appeared to support embryonic compaction, blastocyst formation, hatching and mother signalling (Meisser et al., 1999); the second is very important for early preimplantation development and pluripotency of inner cell mass. In these genes known also to be affected by embryo culture conditions (Palasz et al., 2008) no differences were found.

The main de novo methyltransferase (*DNMT3A*) and one apoptosis-related gene (*BAX*) did not differ among groups, although the lowest expression of *BAX* was obtained for the in vivo derived group. In spite of the fact that in previous studies (Park et al., 2003) in bovine blastocysts *BAX* transcript was significantly increased in blastocyst stages compared with the in vivo counterpart.

Finally, the author wanted to test expression of *HSBP1*, a gene related to response to stress that helps to protect cells under adverse conditions such as infection, inflammation, exposure to toxins, elevated temperature, injury, and disease. *HSBP1* gene expression were compared in B and C only B resulting upregulated in respect to C. Normally, high levels of expression cause senescence in population of cells or apoptosis, thus these results demonstrated that a high level of expression of B was linked to less tolerance to freezing stress. Unfortunately, this result was possible to compare with the in vivo derived embryos because shortage of cDNA.

5.3. Conclusions

Severals conclusions can be drawn from the present study:

i. The different culture media used in this study affect the quality of embryos in term of cryotolerance and body weight.

ii. In our experimental condition none of the gene studied was able to give any indication on the quality of embryo when this quality was referred to pregnancy rate, lambing rate and body weight.

iii. When gene expression of fresh embryos in vitro produced was compared with their in vivo counterpart, differently from in cow, the genes studied were always upregulated. Moreover, comparison is made harder because there are just a few studies in which embryo was produced, transferred and analysed by gene expression.

REFERENCES

Abe H, Yamashita S, Satoh T, Hoshi H. Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. Mol Reprod Dev 2002; 61:57–66.

Abeydeera LR, Wang WH, Cantley TC, Rieke A, Prather RS, Day BN. Presence of epidermal growth factor during in vitro maturation of pig oocytes and embryo culture can modulate blastocyst development after in vitro fertilization. Mol Reprod Dev 1998; 51:395-401.

Accardo C, Dattena M, Pilichi S, Mara L, Chessa B, Cappai P. Effect of recombinant human FSH and LH on in vitro maturation of sheep oocytes; embryo development and viability. Anim Reprod Sci 2004; 81:77-86.

Agca Y, Monson RL, Northey DL, Mazni OA, Schaefer DM, Rutledge JJ. Transfer of fresh and cryopreserved IVP bovine embryos: normal calving, birth weight and gestation lengths. Theriogenology. 1998; 50:147-62.

Aguillar B, Roche A, Olivera J, Folch J, Alabart JL. Oocytes retrieval after repeated ovum pick-up in unstimulated sheep and goat. Proc 18th AETE meeting 2002: p 130-1.

Amstrong DT, Siuda A, Opavsky MA, Chandrasekar Y. Bimodal effects of luteinizing hormone and role of androgens in modifying the superovulatory responses of rats to infusion of purified porcine follicle-stimulating hormone. Biol. Reprod 1989; 40:54-62.

Antczak M, Van Blerkom J. Oocyte influences on early development: the regulatory proteins leptin and STAT3 are polarized in mouse and human oocytes and differentially distributed within the cells of the preimplantation stage embryo. Mol Hum Reprod. 1997; 3:1067-86.

Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev. 2003; 17:126-40.

Badr H, Bongioni G, Abdoon ASS, Kandil O, Puglisi R. Gene expression in the *in vitro*-produced preimplantation bovine embryos. Zygote 2007; 15:355-67.

Baldassarre H, Furnus CC, De Matos DG, Pessi H. In vitro production of sheep embryos using laparoscopic folliculocentesis: alternative gonadotrophin treatments for stimulation of oocyte donors. Theriogenology 1996; 45:707-17.

Bari F, Khalid M, Haresign W, Murray A, Merrel B. Effect of mating system, flushing procedure, progesterone dose and donor ewe age on the yield and quality of embryos within a MOET program in sheep. Theriogenology 2000; 53:727-42.

Baril G, Traldi AL, Cognié Y, Leboeuf B, Beckers JF, Mermillod P. Successful direct transfer of vitrified sheep embryos. Theriogenology. 2001; 56:299-305.

Bartlewski PM, Alexander BD, King WA. Ovarian and endocrine determinants of superovulatory responses in anestrouts ewes. Small Ruminant Res 2008; 75:210-6.

Bavister BD. Co-colture for embryo development: is it really necessary . Human Reprod 1992; 7:1339-41.

Bavister BD. Culture of preimplantation embryos: facts and artifacts.Hum Reprod Update. 1995; 1:91-148.

Berlinguer F, Leoni G, Bogliolo L, Pintus PP, Rosati I, Ledda S, Naitana S. FSH different regimes affect the developmental capacity and cryotolerance of embryos derived from oocytes collected by ovum pick-up in donor sheep. Theriogenology 2004; 61:1477–86.

Bing YZ, Naga T, Rodriguez-Martinez H. Effects of cysteamine, fsh and estradiol-17beta on in vitro maturation of porcine oocytes. Theriogenology 2001; 55:867-76.

Blasco I, Folch J.. Diagnostico precoz de gestacion y determinacion del nmero de fetos en el Ganado ovino. ITEA 1989; 82: 22-31.

Boland MP, Crosby TF, Gordon I. Factors influencing the superovulatory response in sheep. Theriogenology 1983; 19: 114 (Abstract).

Bordignon V, Morin N, Durocher J, Bousquet D, Smith LC. GnRH improves the recovery rate and the in vitro developmental competence of oocytes obtained by transvaginal follicular aspiration from superstimulated heifers. Theriogenology 1997; 48:291-8.

Boundy T, Clarkson MJ, Winter AC. Embryo transfer in sheep under practice conditions. Vet. Rec 1985. 12:379-81.

Brill A, Torchinsky A, Carp H, Toder V. The role of apoptosis in normal and abnormal embryonic development. J Assist Reprod Genet. 1999; 16:512-9.

Buckrell BC, Buschbeck C, Gartley CJ, Kroetsh T, McCutcheon W, Martin J, Penner WK, Walton JS. Further develoment of a transcervical technique for artificial insemination in sheep using previously frozen semen. Theriogenology 1994; 42: 601-11.

Bousquet D, Milovanov C, Bell JC, Durocher J, Smith LC. Nuclear and cytoplasmatic maturation of oocytes aspirated from large follicles in superovulated heifers. Theriogenology 1995; 43:172 (Abstract).

Cappai P, Branca A, Casu S, Naitana S, Ledda, Loi P. Embryo transfer da Ovis A. musimon, a Ovis aries. Atti. Soc. Ital. Sci. Vet. 1998; 42:179-181.

Carolan C, Lonergan P, Van Langendonckt A, Mermillod P. Factors affecting bovine embryo development in synthetic oviduct fluid following oocyte maturation and fertilization in vitro. Theriogenology. 1995; 43:1115-28.

Celorrio I, Carbajo M, Dominguez JC, Anel L, Boixo JC, Chamorro JC, Gutierrez G. Estudio de la eficacia de la ultrasonografia tipo B como metodo de diagnotico de gestacion en Ganado ovino.In proceedings: 7as Jornadas Internacionales de Reproduccion Animal. Murcia 1994; p.300.

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 2003; 113:643-55.

Chang MC, Hunter RHF. Capacitation of mammalian sperm: Biological and experimental aspects. In: Handbook of physiology, section 7, vol. 5,edited by R.O. Greep 1977; pp. 339-51.

Charalambous M, Smith FM, Bennett WR, Crew TE, Mackenzie F, Ward A. Disruption of the imprinted Grb10 gene leads to disproportionate overgrowth by an Igf2 independent mechanism. Proc Natl Acad Sci U S A. 2003; 100:8292-7.

Chernik IS, Panasenko OO, Li Y, Marston SB, Gusev NB. pH-induced changes of the structure of small heat shock proteins with molecular mass 24/27kDa (HspB1). Biochem Biophys Res Commun. 2004; 324:1199-203.

Chevalier F. Échographie de l'appareil génital des femelles domestiques. Recuil de Médecine Vétérinaire 1988; 164: 81-100.

Choi YH, Harding HD, Obermiller AD, Hinrichs K. Oct4 expression pattern in the equine embryo. Reprod Fertil Dev 2007; 19:189-90.

Cognié Y, Poulin N, Mermillod P. Cysteamine improves in vitro goat oocytes maturation in defined medium. Proc 18th AETE meeting 2002; p 154 (Abstract).

Corcoran D, Fair T, Lonergan P. Prediction embryo quality: mRNA expression and the preimplantation embryo. Reprod Biomed Online 2005; 11:340-8.

Corcoran D, Fair T, Park S, Rizos D, Patel OV, Smith GW, Coussens PM, Ireland JJ, Boland MP, Evans AC, Lonergan P. Suppressed expression of genes involved in transcription and translation in in vitro compared with in vivo cultured bovine embryos. Reproduction 2006; 131:651–60.

Crozet N, Huneau D, Desmedt V, Théron MC, Szöllösi D, Torrès S, Sévellec C. In vitro fertilization with normal development in the sheep. Gamete Res. 1987; 16:159-70.

D'Alessandro AGD, Martemucci G, Taibi L. How the FSH/LH ratio and dose numbers in the p-FSH administration treatment regimen, and insemination schedule affect superovulatory response in ewes. Theriogenology 2005; 63:1764-74.

D'Alessandro AGD, Martemucci G, Taibi L. How the FSH/LH ratio and dose numbers in the p-FSH administration treatment regimen, and insemination schedule affect superovulatory response in ewes. Theriogenology 2005; 63:1764-74.

Dattena M, Ptak G, Loi P, Cappai P. Survival and viability of vitrified in vitro and in vivo produced ovine blastocysts. Theriogenology 2000; 53:1511-19.

Dattena M, Accardo C, Pilichi S, Isachenko V, Mara L, Chessa B, Cappai P. Comparison of different vitrification protocols on viability after transfer of ovine blastocysts in vitro produced and in vivo derived. Theriogenology. 2004; 62:481-93.

Dattena M, Chessa B, Lacerenza D, Accardo C, Pilichi S, Mara L, Chessa F, Vincenti L, Cappai P. Isolation, culture, and characterization of embryonic cell lines from vitrified sheep blastocysts. Mol Reprod Dev 2006; 73:31-9.

Dattena M, Mara L, Bin T AA, Cappai P. Lambing rate using vitrified blastocysts is improved by culture with BSA and hyaluronan. Mol Reprod Dev. 2007; 74:42-7.

de Loos F, van Maurik P, van Beneden T, Kruip TA. Structural aspects of bovine oocyte maturation in vitro. Mol Reprod Dev. 1992; 31:208-14.

de Matos DG, Furnus CC, Moses DF, Baldassarre H. Effect of cysteamine on glutathione level and developmental capacity of bovine oocyte matured in vitro. Mol Reprod Dev. 1995; 42:432-6.

Dinnyés AC, Carolan C, Lonergan P, Massip A, Mermillod P. Survival of frozen or vitrified bovine blastocysts produced in vitro in synthetic oviduct fluid. Theriogenology 1996; 46:1425-39.

Dixon AB, Kinhts M, Pate JL, Lewis PE, Inskeep EK. Reproductive performance of ewes after 5-day treatment with intravaginal inserts containing progesterone in combination with injection of prostaglandin F2α.. Reprod. Domest. Anim. 2006; 41:142-8.

Doherty AS, Mann MRW, Tremblay KD, Bartolomei MS, Schultz RM. Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. Biol Reprod 2000; 62:1526-35.

Donnay I, Auquier P, Kaidi S, Carolan C, Lonergan P, Mermillod P, Massip A. Vitrificaion of in vitro produced bovine blastocysts: methodological studies and developmental capacity. Anim Reprod Sci 1998; 52:93-104.

Eckert J, Niemann H. In vitro maturation, fertilization and culture to blastocysts of bovine oocytes in protein-free media. Theriogenology 1995; 43:1211-25.

Eckert J, Niemann H. mRNA expression of leukaemia inhibitory factor (LIF) and its receptor subunits glycoprotein 130 and LIF-receptor-beta in bovine embryos derived in vitro or in vivo. Mol Hum Reprod 1998; 4:957–65.

Edwards LJ, Batt PA, Gandolfi F, Gardner DK. Modifications made to culture medium by bovine oviduct epithelial cells: changes to carbohydrates stimulate bovine embryo development. Mol Reprod Dev. 1997; 46:146-54.

Eggan K, Akutsu H, Loring J, Jackson-Grusby L, Klemm M, Rideout WM, Yanagimachi R, Jaenisch R. Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. Proc Natl Acad Sci U S A 2001; 98:6209–14.

Ezashi T, Ghosh D, Roberts RM. Repression of Ets-2-induced transactivation of the tau interferon promoter by Oct4. Mol Cell Biol 2001; 21:7883-91.

Fahy GM, MacFarlane DR, Angell CA, Meryman HT. Vitrification as an approach to cryopreservation. Cryobiology 1984; 21:407-26.

Farin PW, Farin CE. Transfer of bovine embryos produced in vivo or in vitro: survival and fetal development. Biol Reprod 1995; 52:676-82.

Fernandez-Gonzalez R, Moreira P, Bilbao A, Jimenez A, Perez-Crespo M, Ramirez MA, Rodriguez De Fonseca F, Pintado B, Gutierrez-Adan A. Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. Proc Natl Acad Sci U S A, 2004; 101:5880–5.

Fernandez-Gonzales R, Angel-Ramirez M, Bilbao A, Rodriguez-De Fonseca F, Gutierrez-Adan A. Suboptimal in vitro culture conditions: an epigenetic origin of long-term health effects. Molecular reproduction and development 2007, 74:1149-56

Funahashi H, Day BN. Effects of the duration of exposure to hormone supplements on cytoplasmic maturation of pig oocytes in vitro. J Reprod Fertil. 1993; 98:179-85.

Furnus CC, de Matos DG, Martínez AG, Matkovic M. Effect of glucose on embryo quality and post-thaw viability of in-vitro-produced bovine embryos. Theriogenology. 1997; 47:481-90.

Furnus CC, de Matos DG, Martínez AG. Effect of hyaluronic acid on development of in vitro produced bovine embryos. Theriogenology. 1998; 49:1489-99.

Gajda B, Smorag Z, Wierzbowski S, Jura J, Wieczorek B. Transfer of vitrified sheep morula. Zuchthyg 1989; 24:97-100.

Galantino-Homer HL, Visconti PE, Kopf GS. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3'5'-monophosphate-dependent pathway. Biol Reprod. 1997; 56:707-19.

Galli C, Moore RM. Gonadotrophin requirements for the in vitro maturation of sheep oocytes and their subsequent embryonic development. Theriogenology 1991; 35:1083-93.

Gandolfi F, Moor RM. Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. J Reprod Fertil1987; 81:23-8.

Gandolfi F, Pocare P, Luciano AM, Geiger D. Effects of EGF and IGF-I during in vitro maturation of cattle oocytes on subsequent embryo development and metabolism. Theriogenology 1996; 45:277 (Abstract).

Gardner DK, Lane M, Spitzer A, Batt PA. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. Biol Reprod. 1994; 50:390-400.

Gardner DK, Rodriegez-Martinez H, Lane M. Fetal development after transfer is increased by replacing protein with the glycosaminoglycan hyaluronan for mouse embryo culture and transfer. Hum Reprod. 1999; 14:2575-80.

Geshi, M., M. Yonai, M. Sakaguchi, and T. Nagai.. Improvement of in vitro coculture systems for bovine embryos using a low concentration of carbon dioxide and medium supplemented with beta-mercaptoethanol. Theriogenology 1999; 51:551-8.

Gjorret JO, Avery B, Larsson LI, Schellander K, Hyttel P. Apoptosis in bovine blastocysts produced in vivo and in vitro. Theriogenology 2001; 55:321 (Abstract).

Gonzalez-Bulnes A, Santiago Moreno J, Lopez Sebastian A. Estimation of fetal development in Manchega dairy ewes by tranrectal ultrasonographic measurements. Small Ruminant Res 1998; 27:243-50.

Gonzalez-Bulnes A, Garcia-Garcia RM, Santiago-Moreno J, Lopez-Sebastian A, Cocero MJ. Effect of follicular status on superovulatory response in ewes is influenced by presence of corpus luteum at first FSH dose. Theriogenology 2002; 58: 1607-14.

Gonzalez-Bulnes A, Baird DT, Campbell BK, Cocero MJ, Garcia-Garcia RM, Inskeep KE, Lopez-Sebastian A, McNeilly AS, Santiago-Moreno J, Souza CJH, Veiga-Lopez A. Multiple factors affecting the efficiency of multiple ovulation and embryo transfer in sheep and goats. Reprod. Fertil. Dev 2004; 16:421-35.

Gutierrez-Adan A, Rizos D, Fair T, Moreira PN, Pintado B, De La Fuente J, Boland MP, Lonergan P. Effect of speed of development on mRNA expression pattern in early bovine embryos cultured in vivo or in vitro. Mol Reprod Dev 2004; 68:441-8.

Haibel GK. Use of ultrasonography in the reproductive management of sheep and goat herds. Veterinary Clinics of North America, Food Anim. Pract. 1990; 6:597-613.

Handrow RR, First NL, Parrish JJ. Calcium requirement and increased association with bovine sperm during capacitation by heparin. J Exp Zool. 1989; 252:174-82.

Handyside A, Hooper ML, Kaufman MH, Wilmut I. Towards the isolation of embryonal stem cell lines from the sheep. Roux's Arch Dev Biol 1987; 196:185-90.

Hansis C, Grifo JA, Krey LC. Oct4 expression in inner cell mass and trophectoderm of human blastocysts. Mol Hum Reprod 2000; 6:999-1004.

Harper KM, Brackett BG. Bovine blastocyst development after in vitro maturation in a defined medium with epidermal growth factor and low concentrations of gonadotropins. Biol Reprod. 1993; 48:409-16.

Hasler, JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes JE, Trimmer SA. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology 1995; 43:141-152.

He S, Pant D, Bischoff S, Gavin W, Melican D, Keefer C. Expression of pluripotency-determining factors Oct4 and Nanog in pre-implantation goat embryos. Reprod Fertil Dev 2005; 17:203 (Abstract).

Hitchins MP, Stanier P, Preece MA, Moore GE. Silver-Russell syndrome: a dissection of the genetic aetiology and candidate chromosomal regions.J Med Genet. 2001; 38:810-9.

Holm P, Walker SK, Seamark RF. Embryo viability, duration of gestation and birth weight in sheep after transfer of in vitro matured and in vitro fertilized zygotes cultured in vitro or in vivo. J Reprod Fertil. 1996; 107:175-81.

Holm, P. and H. Callesen.. In vivo versus in vitro produced bovine ova: similarities and differences relevant for practical application. Reprod Nutr. Dev 1998: 38:579-94.

Holm P, Booth PJ, Callesen H. Kinetics of early *in vitro* development of bovine in vivo- and in vitro-derived zygote produced and/or cultured in chemically defined or serum –containing media. Reproduction 2002; 123:553-65.

Isachenko V, Folch J, Isachenko E, Nawroth F, Krivokharchenko A, Vajta G, Dattena M, Alabart JL. Double vitrification of rat embryos at different developmental stages using an identical protocol. Theriogenology. 2003; 60:445-52.

Iwasaki S, Yoshikane Y, Li X, Watanabe S, Nakahara T. Effects of freezing of bovine preimplantation embryos derived from oocytes fertilized in vitro on survival of their inner cell mass cells Mol Reprod Dev 1994; 37:272-5.

Kaidi S, Donnay I, Van Langendonckt A, Dessy F, Massip A. Comparison of two co-culture systems to assess the survival of in vitro produced bovine blastocysts after vitrification. Anim Reprod Sci 1998; 52:39-50.

Kano K, Miyano T, Kato S. Efeffects of glycosaminoglycans on the development of in vitro-matured and fertilized porcine oocytes to blastocysts stage in vitro. Biol Reprod 1998; 58:1226-32.

Khurana NK; Niemann H. Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo. Biol Reprod 2000; 62:847-56.

Kito S, Bavister BD. Male pronuclear formation and early embryonic development of hamster oocytesmatured in vitro with gonadotrophins, amino acids and cysteamine. J Reprod Fertil. 1997; 110:35-46.

Kleeman DO, Walker SK, Seamark RF. Enhanced foetal growth in sheep administered progesterone during the first three days of pregnancy. J. Reprod. Fertil 1994; 102:411-17.

Kobayashi K, Yamashita S, Hoshi H. Influence of epidermal growth factor and transforming growth factor-alpha on in vitro maturation of cumulus cell-enclosed bovine oocytes in a defined medium. J Reprod Fertil. 1994; 100:439-46.

Krisher RL, Lane M, Bavister BD. Developmental competence and metabolism of bovine embryos cultured in semi-defined and defined culture media. Biol Reprod. 1999; 60:1345-52.

Kurosaka S, Eckardt S, McLaughlin KJ. Pluripotent lineage definition in bovine embryos by Oct4 transcript localisation. Biol Reprod 2004; 71:1578-82.

Lane M, Maybach JM, Hooper K, Hasler JF, Gardner DK. Cryo-survival and development of bovine blastocysts are enhanced by culture with recombinant albumin and hyaluronan. Mol Reprod Dev. 2003; 64:70-8.

Laurent C, Hellström S, Engström-Laurent A, Wells AF, Bergh A. Localization and quantity of hyaluronan in urogenital organs of male and female rats. Cell Tissue Res. 1995; 279:241-8.

Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruip T, Niemann H, Galli C. Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. Biol Reprod 2002; 67:767-75.

Ledda S, Bogliolo L, Leoni G, Calvia P, Naitana S. Influence of vasoactive intestinal peptide (VIP), atrial natriuretic peptide (ANP) and insulin-like growth factor-I (IGF-I) on in vitro maturation of prepubertal and adult sheep oocytes. Zygote 1996; 4:343-8.

Lee, E. S., Y. Okamoto, H. Yamashina, and Y. Fukui. Pregnancy rates after transfer of fresh or frozen bovine blastocysts developed from serum-free or protein-free media. Theriogenology 1997; 47:350 (Abstract).

Leibfried-Rutledge ML, Critser ES, First NL. Effects of fetal calf serum and bovine serum albumin on in vitro maturation and fertilization of bovine and hamster cumulus-oocyte complexes. Biol Reprod. 1986; 35:850-7.

Leibfried-Rutledge ML, Critser ES, Eyestone WH, Northey DL, First NL.Development potential of bovine oocytes matured in vitro or in vivo. Biol Reprod. 1987; 36:376-83.

Leibo SP. Cryobiology: preservation of mammalian embryos. Basic Life Sci 1986; 37:251-72.

Lenz, R. W., G. D. Ball, M. L. Leibfried, R. L. Ax, and N. L. First. Invitro Maturation and Fertilization of Bovine Oocytes Are Temperature-Dependent Processes. Biol Reprod 1983; 29:173-9.

Leoni G, Berlinguer F, Rosati I, Bogliolo L, Ledda S, Naitana S. Resumption of metabolic activity of vitrified/warmed ovine embryos. Mol. Reprod. Dev 2003; 64:207-213.

Leoni GG., Berlinguer F., Succu S, Bebbere D, Mossa F, Madeddu M., Ledda S, Bogliolo L., Naitana S. A new selection criterion to assess good-quality ovine blastocysts after vitrification and to predict their transfer into recipients. Mol. Reprod. Dev 2008; 75:373–82.

Leyva V, Buckrell BC., Walton JS. Regulation of follicular activity and ovulation in ewes by exogenous progestagen. Theriogenology 1998; 50:395-416.

Li J, Pan G, Cui K, Liu Y, Xu S, Pei D. A dominant-negative form of mouse SOX2 induces trophectoderm differentiation and progressive polyploidy in mouse embryonic stem cells. J Biol Chem. 2007; 282:19481-92.

Lonergan P, Carolan C, Van Langendonckt A, Donnay I, Khatir H, Mermillod P. Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development in vitro. Biol Reprod. 1996; 54:1420-9.

Lonergan P, Rizos D, Gutiérrez-Adán A, Fair T, Boland MP. Effect of culture environment on embryo quality and gene expression - experience from animal studies. Reprod Biomed Online. 2003a; 7:657-63.

Lonergan P, Rizos D, Gutierrez-Adan A, Moreira PM, Pintado B, De La Fuente J, Boland MP. Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage in vitro or in vivo. Biol Reprod 2003b; 69:1424-31.

Lau MM, Stewart CE, Liu Z, Bhatt H, Rotwein P, Stewart CL. Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. Genes Dev. 1994; 8:2953-63.

MacFarlane DR. Physical aspects of vitrification in aqueous solutions. Cryobiology 1987; 24:181-95.

Majerus, V., R. De Roover, D. Etienne, S. Kaidi, A. Massip, F. Dessy, and I. Donnay. Embryo production by ovum pick up in unstimulated calves before and after puberty. Theriogenology 1999; 52:1169-1179.

Mantovani, R., Rota, A., Penzo, N., and Bittante, G. Comparison among oocytes recovery methods for bovine embryo production in vitro.. Istituto di Urologia, IRCSS Ospedale Maggiore, Milano. 1999; Proc ASPA 13th Congress. pp. 235-7.

Mara L, Mayorga IM, Sanna D, Dattena M. In vitro culture system affects lambing and lamb birth weight of vitrified ovine embryos. Proc 25th AETE meeting 2009; p 222 (Abstract).

Massip A, Mermillod P, Dinnyes A. Morphology and biochemistry of in vitro produced bovine embryos: implications for their cryopreservations. Hum Reprod 1995; 10:103-8.

Massip A. Cryopreservation of embryos of farm animals. Reprod Domest Anim 2001; 36:49-55.

Mayorga I, Masia F, Mara L, Chessa F, Casu S, Dattena M. Superovulation treatment with FSH-p durino natural oestrus:comparison with progestagen synchronized superovulation protocols in sarda ewes. Proc 23th A.E.T.E. 2007; p. 204 (Abstract).

Mayorga IM, Mara L, Sanna D, Gallus M, Dattena M. Good quality sheep embryos produced by superovulation treatment without the use of sponge. GEMINI Workshop Working (Group 1 Experimental Models in Animal Reproduction: Theoretical and practical approaches) 7th to 8th May Lansk, Poland; 2009. (Abstract on line and poster).

McNeilly AS, Jonassen JA, Fraser HM. Supression of follicular development after chronic LH/RH inmunoneutralization in the ewe. J. Reprod Fertil 1986; 76:481-90.

McKelvey WAC, Robinson JJ, Aitken RP. Repeated recoveries of embryos from ewes by laparoscopy. Theriogenology 1986; 25:855-65.

Mehta TS, Kiessling AA. Development potential of mouse embryos conceived in vitro and cultured in ethylenediaminetetraacetic acid with or without amino acids or serum. Biol Reprod. 1990; 43:600-6.

Meisser A, Cameo P, Islami D, Campana A, Bischofl P. Effects of interleukin-6 (IL6) on cytotrophoblastic cells. Mol Hum Reprod 1999; 5:1055-8.

Mermillod, P., A. Vansteenbrugge, C. Wils, J. L. Mourmeaux, A. Massip, F. Dessy. Characterization of the Embryotrophic Activity of Exogenous Protein-Free Oviduct-Conditioned Medium Used in Culture of Cattle Embryos. Biol Reprod 1993; 49:582-7

Mermillod P, Oussaid B, Cognié Y. Aspects of follicular and oocyte maturation that affect the developmental potential of embryos. J Reprod Fertil Suppl. 1999a; 54:449-60.

Mermillod P, Traldi AL, Baril G, Becker JF, Massip A, Cogniè Y. A vitrification method for direct transfer of sheep embryos. Proc 13th AETE meeting 1999b; p: 212. (Abstract).

Merton JS, van Wagtendonk -de Leeuw. Factors affecting birth weight of IVP calves, Theriogenolgy 1998; 49:239 (Abstract).

Miner JH, Li C, Mudd JL, Go G, Sutherland AE. Compositional and structural requirements for laminin and basement membranes during mouse embryo implantation and gastrulation. Development. 2004; 131:2247-56.

Mitalipov MS, Kuo HC, Hennebold JD, Wolf DP. Oct4 expression in pluripotent cells of the Rhesus Monkey. Biol Reprod 2003; 69:1785-92.

Mitalipova M, Beyhan Z, First NL. Pluripotency of bovine embryonic cell line derived from precompacting embryos. Cloning 2001; 3:59-67.

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M,Maeda M, Yamanaka S. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell. 2003; 113:631-42.

Monniaux D, Huet C, Besnard N, Clément F, Bosc M, Pisselet C, Monget P, Mariana JC. Follicular growth and ovarian dynamics in mammals. J Reprod Fertil Suppl. 1997; 51:3-23.

Moor RM, Trounson AO. Hormonal and follicular factors affecting maturation of sheep oocytes in vitro and their subsequent developmental capacity. J Reprod Fertil. 1977; 49:101-9.

Moore K, Rodriguez-Sallaberry CJ, Kramer JM, Johnson S, Wroclawska E, Goicoa S, Niasari-Naslaji A. *In vitro* production of bovine embryos in medium supplemented with a serum replacer: effects on blastocyst development, cryotolerance and survival to term. Theriogenology 2007; 68:1316-25.

Mucci N, Aller J, Kaiser GG, Hozbor F, Cabodevila J, Alberio RH. Effect of estrous cow serum during bovine embryo culture on blastocyst development and cryotolerance after slow freezing or vitrification. Theriogenology 2006; 65:1551–62.

Naitana S, Loi P, Ledda S, Cappai P. Embryo transfer and new technology in sheep reproduction. Embryonic Development and Manipulation in Animal Priduction.1992; 15:183-194.

Naitana S, Dattena M, Gallus M, Loi P, Branca A., Ledda S, Cappai P.. Recipient synchronization affects viability of vitrified ovine blstocysts. Theriogenology 1995; 43:1371-78.

Naitana S, Ledda S, Loi P, Leoni G, Bogliolo L, Dattena M, Cappai P. Polyvinyl alcohol as a defined substitute for serum in vitrifications solutions to cryopreserve ovine embryos at different stages of development. Anim Reprod Sci 1997; 48:247-56.

Niemann H, Wrenzycki C. Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: implications for subsequent development. Theriogenology. 2000; 53:21-34.

Nino-Soto MI, Basrur PK, King WA. Impact of in vitro production techniques on the expression of X-linked genes in bovine (bos taurus) oocytes and pre-attachment embryos. Mol Reprod Dev. 2007; 74:144-53.

Niwa H, Burdon T, Chambers I, Smith A. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev. 1998; 12:2048-60.

Niwa H. Open conformation chromatin and pluripotency. Genes Dev 2007a; 21:2671–6.

Niwa H. How is pluripotency determined and maintained? Development 2007b; 134:635–46.

Nöel B, Bister JL, Pierquin B, Paquay R. Effects of FGA and PMSG on follicular growth and LH secretion in Suffolk ewes. Theriogenology 1994; 41:719-27.

O'Brien JK, Catt SL, Ireland KA, Maxwell WM, Evans G. In vitro and in vivo developmental capacity of oocytes from prepubertal and adult sheep. Theriogenology. 1997; 47:1433-43.

O'Fallon JV, Wright RW Jr. Quantitative determination of the pentose phosphate pathway in preimplantation mouse embryos. Biol Reprod. 1986; 34:58-64.

Ortman R. Monitoring of oestrus cycle of ewes by ram-seeking behaviour. Small Rum Res 2000; 133-7.

Overström EW. In vitro assessment of embryo viability. Theriogenolgy 1996; 45:3-16.

Palasz AT, Breña PB, De la Fuente J, Gutiérrez-Adán A. The effect of different zwitterionic buffers and PBS used for out-of-incubator procedures during standard in vitro embryo production on development, morphology and gene expression of bovine embryos. Theriogenology. 2008; 70:1461-70.

Palmieri SL, Peter W, Hess H, Scholer HR. Oct4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. Dev Biol 1994; 166:259-67.

Park SH, Park SB, Kim NH. Expression of early development-related genes in bovine nuclear transferred and fertilized embryos. Zygote. 2003; 11:355-60.

Parrish JJ, Susko-Parrish JL, Handrow RR, Ax RL, First NL. Effect of sulfated glycoconjugates on capacitation and the acrosome reaction of bovine and hamster spermatozoa. Gamete Res. 1989; 24:403-13.

Parrish JJ, Susko-Parrish JL, Uguz C, First NL. Differences in the role of cyclic adenosine 3',5'-monophosphate during capacitation of bovine sperm by heparin or oviduct fluid. Biol Reprod. 1994; 51:1099-108.

Payen E, Pailhoux E, Gianquinto L, Hayes H, Le Pennec N, Bezard J, Cotinot C. The ovine SOX2 gene: sequence, chromosomal localization and gonadal expression. Gene 1997; 189:143-7.

Payne SR, Munday R, Thompson JG. Addition of superoxide dismutase and catalase does not necessarily overcome developmental retardation of one-cell mouse embryos during *in-vitro* culture. Reprod Fertil Dev 1992; 4:167-74.

Pesce M, Schöler HR. Oct4: control of totipotency and germline determination. Mol Reprod Dev 2000; 55:452-7.

Pollard JW, Leibo S, Comparative cryobiology of in vitro and in vivo derived bovine embryos. Theriogenology 1993; 39:287 (Abstract).

Ptak G, Loi P, Dattena M, Cappai P, Tischner M. IVM/IVF techniques in prepubertal lamb oocytes. Proc 13th AETE meeting 1997: p 192 (Abstract).

Ptak G, Dattena M, Loi P, Tischner M, Cappai P. Ovum pick-up in sheep: efficiency of in vitro embryo production, vitrification and birth of offspring. Theriogenology 1999; 52:1105-14.

Purcell VG, Wall RI, Rexroad CE, Hammer RE, Brinster RL. A rapid whole mount staining procedure for nuclei of mammalian embryos. Theriogenology 1985; 24:687-700.

Radford HM, Watson RH, Wood GF. A crayon and associated harness for the detection of mating under field conditions. Aust. vet. J 1960; 36:57-66.

Rall WF, Fahy GM.Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. Nature. 1985; 313:573-5.

Reichnbach HD, Wiebke NH, Wenigerkind H, Modl J, Brem G. Bovine follicular oocytes collected by laparoscopic guided transvaginal aspiration. Theriogenology 1994: 41:283 (Abstract).

Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 2000; 18:399-404.

Rinaudo P, Schultz RM. Effects of embryo culture on global pattern of gene expression in preimplantation mouse embryos. Reproduction 2004; 128:301–11.

Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. Mol Reprod Dev 2002a; 61:234–248.

Rizos D, Lonergan P, Boland MP, Arroyo-Garcia R, Pintado B, De la Fuente J, Gutierrez-Adan A. Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture system. Implications for blastocyst quality. Biol Reprod 2002b; 66:589-95

Rizos D, Fair T, Papadopoulos S, Boland MP, Lonergan P. Developmental, qualitative, and structural differences between ovine and bovine embryos produced in vivo and in vitro. Mol Reprod Dev 2002c; 62:320-7.

Rizos D, Gutierrez-Adan A, Perez-Garnelo S, De La Fuente J, Boland MP, Lonergan P. Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. Biol Reprod 2003; 68, 236–43.

Rizos D, Clemente M, Bermejo-Alvarez P, de La Fuente J, lonergan P, Gutierrez-Adan A. Consequences of *In vitro* culture conditions on embryo development and quality. Reprod Dom Anim 2008; 43:44-50.

Roest HP, Baarends WM, de Wit J, van Klaveren JW, Wassenaar E, Hoogerbrugge JW, van Cappellen WA, Hoeijmakers JH, Grootegoed JA. The ubiquitin-conjugating DNA repair enzyme HR6A is a maternal factor essential for early embryonic development in mice. Mol Cell Biol. 2004; 24:5485-95.

Rypka M, Cervenková K, Uherková L, Poczatková H, Florschutz AV, Veselý J. A novel simplified ultra-rapid freezing technique for cryopreservation of tissue slices. Cryobiology. 2006; 52:193-9.

Sanna D, Rizos D, Mara L, Gutierrez-Adan A, Bermejo-Alvarez P, Dattena M. Effect of BSA and hyaluronan during in vitro culture of ovine embryos and their quality in terms of cryotolerance, gene expression, lambing rate and birth weight. 23th AETE meeting 2007; p 228 (Abstarct).

Scaramuzzi RJ, Downing JA, Campbell BK, Cognie Y. Control of fertility and fecundity of sheep by means of hormonal manipulation. Aust. J. Biol. Sci. 1988; 41:37-45.

Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C_T method. Nat Protoc 2008; 3:1101-6.

Scott JE, Heatley F. Biological properties of hyaluronan in aqueous solution are controlled andsequestered by reversible tertiary structures, defined by NMR spectroscopy. Biomacromolecules. 2002; 3:547-53.

Shamsuddin M, Rodriguez-Martinez H. Fine structure of bovine blastocysts developed either in serum-free medium or in conventional co-culture with oviduct epithelial cells. Zentralbl Veterinarmed A. 1994; 41:307-16.

Sinclair KD, Maxfield EK, Robinson JJ, Maltin CA, McEvoy TG, Dunne LD, Young LE, Broadbent PJ. Culture of sheep zygotes can alter fetal growth and development. Theriogenology 1997; 47:380 (Abstract).

Sinclair KD, McEvoy TG, Carolan C, Maxfield EK, Maltin CA, Young LE, Wilmut I, Robinson JJ, Broadbent PJ. Conceptus growth and development following in vitro culture of ovine embryos in media supplemented with bovine sera. Theriogenology 1998; 49:218 (Abstract).

Sinclair KD, Young LE, Wilmut I, McEvoy TG. In-utero overgrowth in ruminants following embryo culture: Lessons from mice and a warning to men. Hum Reprod 2000; 15:68–86

Slavik T, Fulka J, Goll I.Pregnancy rate after the transfer of sheep embryos originated from randomly chosen oocytes matured and fertilized in vitro. Theriogenology. 1992; 38:749-56.

Smith FM, Holt LJ, Garfield AS, Charalambous M, Koumanov F, Perry M, Bazzani R, Sheardown SA, Hegarty BD, Lyons RJ, Cooney GJ, Daly RJ, Ward A. Mice with a disruption of the imprinted Grb10 gene exhibit altered body composition, glucose homeostasis, and insulin signaling during postnatal life. Mol Cell Biol. 2007; 27:5871-86.

Snyder DA, Nellor JE. Development of the laparoscopic technique for in vivo collection of follicular oocytes in sheep. Fed. Proc. 1975; 338.

Staigmiller RB, Moor RM. Effect of follicle cells on the maturation and developmental competence of ovine oocytes matured outside the follicle. Gamete Res 1984; 9:221-9.

Sun FJ, Holm P, Irvine B, Seamark RF. Effect of sheep and human follicular fluid on the maturation of sheep oocytes in vitro. Theriogenology. 1994; 41:981-8.

Széll A, Zhang J, Hudson R. Rapid cryopreservation of sheep embryos by direct transfer into liquid nitrogen vapour at -180°C. Reprod Fertil Dev 1990; 2:613-18.

Takagi Y, Mori K, Tomizawa M, Takahashi T, Sugawarw S, Masaki J. Development of bovine oocytes matured, fertilized and cultured in a serum-free, chemically defined medium. Theriogenology 1991; 35:1197-207.

Takahashi Y, First NL. In vitro development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins Theriogenology 1992; 37:963-78.

Takeda T, Elsdeo RP, Seidel GE jr. Cryopreservation of mouse embryos by direct plunged into liquid nitrogen. Theriogenology 1984; 121:268 (Abstract).

Takeda J, Seino S, Bell GI. Human Oct3 gene family: cDNA sequences, alternative splicing, gene organization, chromosomal location, and expression at low levels in adult tissues. Nucleic Acids Res 1992; 20:4613-20.

Tervit HR, Whittingham DC, Rowson LEA. Successful culture in vitro of sheep and cattle ova. J Repr Fert 1972; 30:493-7.

Tervit HR, Smith JF, McGowan LT, Pugh PA. Birth of lambs from embryos produced in vitro following laparoscopic recoveryof follicular oocytes. Proc. Aust. Soc. Reprod Biol 1995; 27:68.

Tervit HR. Laparoscopic/laparotomy oocytes recovery and juvenile breeding. Anim Reprod Sci 1996; 42:227-38.

Thibier M, Guérin B. Embryo transfer in small ruminants: The method of choice for health control in germplasm exchanges. Livest. Prod. Sci. 2000; 62:253-270.

Thompson JG, Gardner DK, Pugh PA, McMillan WH, Tervit HR. Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. Biol Reprod. 1995; 53:1385-91.

Thompson JG. Comparison between in vivo-derived and in vitro-produced pre-elongation embryos from domestic ruminants. Reprod Fertil Dev. 1997; 9:341-54.

Thompson JG, Allen NW, McGowan LT, Bell AC, Lambert MG, Tervit HR. Effect of delayed supplementation of fetal calf serum to culture medium on bovine embryo development in vitro and following transfer. Theriogenology. 1998; 49:1239-49.

Thompson JG. In vitro culture and embryo metabolism of cattle and sheep embryos - a decade of achievement. Anim Reprod Sci. 2000; 60:263-75.

Thurston A, Taylor J, Gardner J, Sinclair KD, Young LE. Monoallelic expression of nine imprinted genes in the sheep embryo occurs after the blastocyst stage. Reproduction. 2008; 135:29-40.

Tirone E, D'Alessandris C, Hascall VC, Siracusa G, Salustri A. Hyaluronan synthesis by mouse cumulus cells is regulated by interactions between follicle-stimulating hormone (or epidermal growth factor) and a soluble oocyte factor (or transforming growth factor beta1). J Biol Chem. 1997; 272:4787-94.

Traldi AS, Leboeuf B, Cognié Y, Poulin N, Mermillod P. Comparative results of in vitro and in vivo survival of vitrified in vitro produced goat and sheep embryos. Theriogenoly 1999; 51:175 (Abstract).

Trounson A, Peura A, Kirby C. Ultrarapid freezing: a new low-cost and effective method of embryo cryopreservation. Fertil Steril. 1987; 48:843-50.

Van Soom A, de Kruif A. A comparative study of in vivo and in vitro derived bovine embryos. Proc 12th Int Congr Anim Reprod, 1992; 3:1363-65.

Van Soom A, Boerjan ML, Ysebaert MT, de Kruif A. Cell allocation to the inner cell mass and the trophectoderm in bovine embryos cultured in two different media. Mol Reprod Dev 1996; 45:171-82.

Van Soom A, Boerjan ML, Bols PE, Vanroose G, Lein A, Coryn M, de Kruif A. Timing of compaction and inner cell allocation in bovine embryos produced in vivo after superovulation. Biol Reprod 1997; 57:1041-9.

Van Soom A, Yuan YQ, Peelman LJ, de Matos DG, Dewulf J, Laevens H, de Kruif A. Prevalence of apoptosis and inner cell allocation in bovine embryos cultured under different oxygen tensions with or without cysteine addition. Theriogenology. 2002; 57:1453-65.

Vajta G, Holm P, Greve T, Callesen H. Overall efficiency of in vitro embryo production and vitrification in cattle. Theriogenology. 1996; 45:683-9.

Vajta G. Vitrification of the oocytes and embryos of domestic animals. Anim Reprod Sci 2000; 60/61: 357-64.

van Eijk MJ, van Rooijen MA, Modina S, Scesi L, Folkers G, van Tol HT, Bevers MM, Fisher SR, Lewin HA, Rakacolli D, Galli C, de Vaureix C, Trounson AO, Mummery CL, Gandolfi F. Molecular cloning, genetic mapping, and developmental expression of bovine POU5F1. Biol Reprod 1999; 60:1093-103.

Van Langendonckt A, Donnay I, Schuurbiers N, Auquier P, Carolan C, Massip A, Dessy F. Effects of supplementation with fetal calf serum on development of bovine embryos in synthetic oviduct fluid medium. J Reprod Fertil. 1997; 109:87-93.

van Wagtendonk-de Leeuw AM, Aerts BJ, den Daas JH. Abnormal offspring following in vitro production of bovine preimplantation embryos: a field study. Theriogenology. 1998; 49:883-94.

Viñoles C, Forsberg M, Banchero G, Rubianes E. Effect of long-term and short term progestagen treatment on follicular development and preganancy rate in cyclic ewes. Theriogenology 2001; 55: 993-1001.

Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P,Kopf GS. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development. 1995; 121:1139-50.

Wallace JM.. Artificial insemination and embryo transfer. In: Speedy A. W. (ed), Progress in Sheep and Goat Research. Wallingford, Oxon: CAB International, 1992; p. 1-24.

Walker, SK, Heard TM; Seamark RF. In vitro culture of sheep embryos without co-culture successes and perspectives. Theriogenology 1992; 37:111-126.

Walker SK, Hartwich KM, Seamark RF. The production of unusually large offspring following embryo manipulation: concepts and challenges. Theriogenology 1996; 45:111-120

Wang L, Balas B, Christ-Roberts CY, Kim RY, Ramos FJ, Kikani CK, Li C, Deng C, Reyna S, Musi N, Dong LQ, DeFronzo RA, Liu F. Peripheral disruption of the Grb10 gene enhances insulin signaling and sensitivity in vivo. Mol Cell Biol. 2007; 27:6497-505.

Wani NA. In vitro maturation and in vitro fertilization of sheep oocytes. Small Rum Res 2002; 44:89-95.

Warwick BL, Berry RO. Inter-generic and intra-specific embryo transfers in sheep and goats. J Hered. 1949; 40:297-303.

Watson AJ, Watson PH, Warnes D, Walker SK, Armstrong DT, Seamark RF. Preimplantation development of in vitro-matured and in vitro-fertilized ovine zygotes: comparison between coculture on oviduct epithelial cell monolayers and culture under low oxygen atmosphere. Biol Reprod. 1994; 50:715-24.

Wells DN, Misica PM, Day TA, Tervit HR. Production of cloned lambs from an established embryonic cell line: a comparison between in vivo- and in vitro-matured cytoplasts. Biol Reprod. 1997; 57:385-93.

Wenigerkind H, Glibotic J, Kiessling S, Stojkovic M, Aumüller R, Schernthaner W, Wolf E, Brem G. Effects of different systems for IVP of bovine embryos on birth weight and sex ratio of calves—a field study. Abstract 591 cited in van Wagtendonk et al, Theriogenology, 49, 883-894 (1998).

White I.R, Russel AJF. Determination of fetal number in sheep by relative ultrasonic scanning. Vet. Pract. 1984; 1:200-3.

Whittingam DG. Survival of mouse embryos after freezing and thawing. Nature (Lond) 1971; 233:125-6.

Willadsen SM, Janzen RE, McAlister RJ, Shea BF, Hamilton G, McDermand D. The viability of late morulae and blastocysts produced by nuclear transplantation in cattle. Theriogenology 1991; 35: 161-172

Wrenzycki C, Hermann D, Keskintepe L, Martins jr A, Sirisathien S, Brackett B, Niemann H. Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos. Hum Reprod 2001; 16:893-901.

Wrenzycki C, Hermann D, Niemann H. Timing of blastocyst expansion affects spatial messenger RNA expression patterns of genes in bovine blastocysts produced in vitro. Biol Reprod 2003; 68:2073-80.

Wrenzycki C, Hermann D, Lucas-Hahn A, Lemme E, Korsawe K, Niemann H. Gene expression patterns in *in vitro*- produced and somatic nuclear transfer derived pre-implantation bovine embryos: relationship to the large offspring syndrome. Anim Reprod Sci 2004; 83 593-603.

Wrenzycki C, Herrmann D, Lucas-Hahn A, Korsawe K, Lemme E, Niemann H. Messenger RNA expression patterns in bovine embryos derived from in vitro procedures and their implications for development. Reprod Fertil Dev 2005; 17:23–35.

Wutz A, Theussl HC, Dausman J, Jaenisch R, Barlow DP, Wagner EF.Non-imprinted Igf2r expression decreases growth and rescues the Time mutation in mice. Development. 2001; 128:1881-7.

Yadav PS, Kues WA, Herrmann D, Carnwath JW, Niemann H. Bovine ICM derived cells express the Oct4 ortholog. Mol Reprod Dev 2005; 72:182-90.

Yamauchi N, Sasada H, Soloy E, Dominko T, Kikuchi K, Naga T. Effects of hormones and osmolarity in the culture medium on germinal vesicle breakdown of porcine oocytes. Theriogenology 1999; 52:153-62.

Yanagimachi R, Chang MC. Fertilization of hamster eggs in vitro. Nature. 1963; 200:281-2.

Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science. 1997; 275:1129-32.

Yang X, Kubota C, Suzuki H, Taneja M, Bols PEJ, Presicce GA. Control of oocyte maturation in cows - Biological factors. Theriogenology 1998; 49:471-82.

Yaseen MA, Wrenzycki C, Herrmann D, Carnwath JW, Niemann H. Changes in the relative abundance of mRNA transcripts for insulin-like growth factor (IGF-I and IGF-II) ligands and their receptors (IGF-IR/IGF-IIR) in preimplantation bovine embryos derived from different in vitro systems. Reproduction. 2001; 122:601-10.

Yazawa S, Aoyagi Y, Konishi M, Takedomi T. Characterization and cytogenetic analysis of Japanese Black calves produced by nuclear transfer. Theriogenology. 1997 ;48:641-50.

Young LE, Sinclair KD, Wilmut I. Large offspring syndrome in cattle and sheep. Rev Reprod. 1998; 3:155-63.

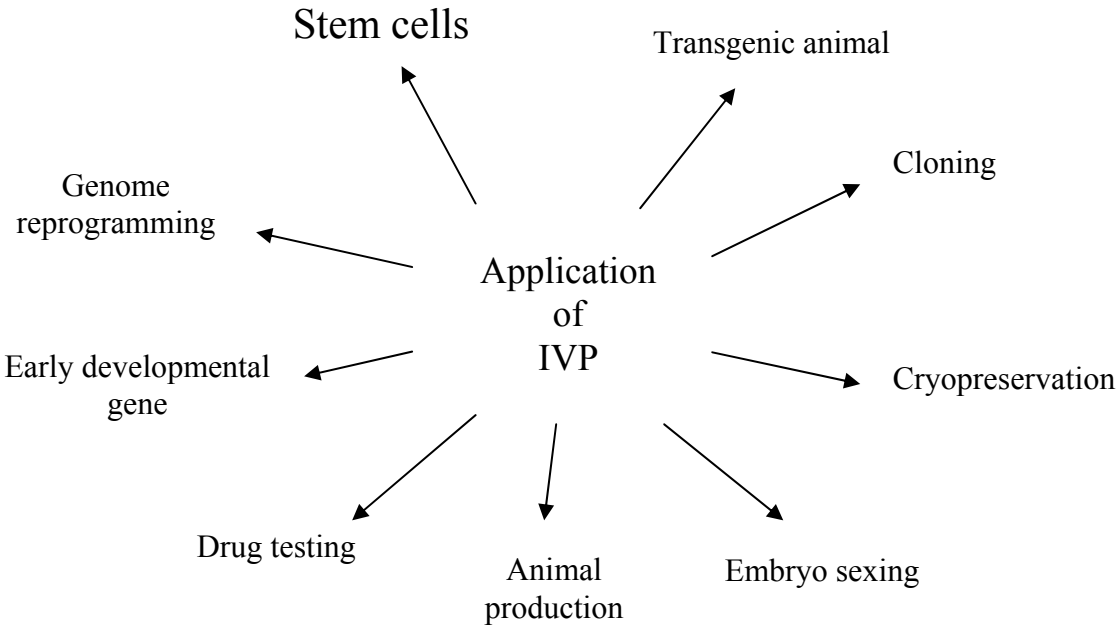
Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I, Sinclair KD. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. Nat Genet 2001; 27:153-4.

Younis AI, Brackett BG, Fayrer-Hosken RA. Influence of serum and hormones on bovine oocyte maturation and fertilization in vitro. Gamete Res. 1989; 23:189-201.

APPENDIX

Why an appendix about ovine embryonic stem cells?

In this thesis the author studied just a few of the several applications of IVP, trying to define the quality of the embryo produced in vitro by pregnancy rate, lambing rate, birth weight and gene expression of some genes responsable for embryo welfare. In our laboratory there was the possilility to consider another IVP application, in fact sheep blastocysts produced in vitro were used as a potential source to produce and characterize embryonic stem-like cells.



1. Summary

Gene expression of staminality markers in in vitro produced sheep blastocysts and embryonic stem-like cells with emphasis on *OCT4* role.

Embryonic Stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocysts with the potential to maintain an undifferentiated state indefinitely. Currently, the most widely tested and validated panel of pluripotent markers includes: alkaline phosphatase (PA), stage specific embryonic surface antigens (SSEAs), transcription factors as *OCT4, NANOG, SOX2* and *STAT3* (Reubinoff et al., 2000).

Up today in sheep, the characterization of embryonic stem (ES)-like cells has been tested immunocytochemically using AP activity and SSEA-1, SSEA-3, SSEA-4 (Dattena et al., 2006), but this system is not longer sufficient to fully verify stem cell pluripotency. Thus in order to strengthen their identity, the transcription factors involved in maintenance of pluripotency, *OCT4, NANOG, SOX2* and *STAT3,* were tested by PCR in this study. Moreover, this study was conducted to quantitatively detect *OCT4* gene expression in in vitro produced sheep blastocysts and in ES-like cells at the undifferentiated stage and during differentiation.

Ovaries of Sarda sheep were collected from slaughterhouse. Oocytes were matured in TCM-199 supplemented with 4 mg/ml BSA for 24 h then fertilized with fresh ram semen. Zygotes were cultured in SOFaa supplemented with 8 mg/ml fatty acid free BSA for 6/7 or 8 days. A total of 144 blastocysts were used for this study: 10 were used for PCR and sequencing of *OCT4, NANOG, SOX2* and *STAT3*, 30 at 6/7 (BL6/7) days and 30 at 8 days (BL8) were used to detect *OCT4* expression by quantitative Real Time PCR (qRTPCR). The remaining 74 blastocysts at 6/7 days were divided as follows: 30

were biopsied in order to obtain ICM and TB, 20 to evaluate the number of cells, 24 were destined for ES-like and ECD (ES-like cells in the course of differentiation) cells.

The cDNA produced by Reverse Transcription (RT)-PCR was synthesized and amplified by PCR, the sequencing revealed 98%, 95%, 98% homology to the bovine sequence of *OCT4, NANOG, STAT3* respectively. Using the ovine sequence of 290 bp, quantitative expression of *OCT4* in blastocysts, inner cell mass, trophoblast and embryonic stem-like cells was performed by qRTPCR.

PCR data showed that the sheep ES-like cells expressed *OCT4, NANOG, SOX2* and *STAT3* strengthening the characterization of sheep ES-like cells. Moreover, *OCT4* was expressed in blastocysts, inner cell mass, trophoblast and embryonic stem-like cells. The level of *OCT4* expression in BL8 was significantly higher than in BL6/7, in inner cell mass was significantly higher than in trophoblast. This might be useful in defining the quality of embryos produced and it makes possible to use *OCT4* to detect cell pluripotency. In addition, the different levels of *OCT4* expression between undifferentiated and differentiating embryonic stem-like cell cultures make possible to use this gene as staminality marker.

2. Materials and methods

The experiments were done at the Agris – DIRPA (Dipartimento della Ricerca nelle Produzioni Animali) – Servizio Zootecnico – Settore Scientifico Riproduzione S.S. 291 Km 18.6, 07100 Sassari (Italy) together with Neuroscienze PharmaNess Pula Scarl, Parco Scientifico Sardegna Ricerche, Loc. Piscinamanna, Pula (CA).

2.1. Production of embryos

The same IVM, IVF, IVC protocol of B(BSA_8) group (Table 1) used in first experiment was used also in this experiment.

2.2. Destination of blastocysts

A total of 114 blastocysts at 6/7 days and 30 blastocysts at 8 days were used for this study. Ten blastocysts, in groups of 2, were used for PCR and sequencing of *OCT4, NANOG* and *STAT3*. Thirty blastocysts at 6/7 (BL6/7) day and 30 at 8 (BL8) days were used to detect *OCT4* expression by qRTPCR. The remaining 74 were divided as follows: 50 blastocysts were biopsied in order to obtain ICM and TB, 30 (3 pools of 10 respectively) were used for *OCT4* detection in qRTPCR and 20 to evaluate the number of cells. Twenty-four blastocysts were destined for ES-like and ECD (ES-like cells in the course of differentiation) cells. All samples were placed into RNase-free tubes in 3-5 µl of PBS using fine glass capillary pipettes and were snap frozen in liquid N_2 for 3-5 minutes. Samples were stored at -80°C and then used for gene expression analysis.

2.3. Isolation of ICM and TB

At 6-7 days after fertilization, high-quality embryos at the expanded blastocyst stage were washed in calcium-free PBS with BSA and biopsied. Micromanipulation procedures were carried out at room temperature (23°C). The embryos were put individually into a 200 µl drop of calcium-free PBS without BSA in a 60 mm Petri dish (Corning Incorporated) to prevent slippage of the embryo during cutting. The ICMs and TBs were slashed with a vertical microblade movement under an inverted microscope (Labovert) using a micromanipulator (Leitz Labovert, Heidelberg). After each biopsy, microblade and glass capillary pipettes were changed to avoid DNA contamination by debris attached to the blade. After cutting, the holding medium [(H-TCM 199 supplemented with 10% (v/v) FBS (Fetal Bovine Serum; Gibco-Life Technologies BRL, Germany)] was added into the dish to neutralize the attraction of the cells to the metal blade and to the plastic dish bottom.

Twenty ICMs and TBs were stained with 10 µg/ml bisbenzimide (Hoechst 33342) and exposed to ultraviolet light for less than 2 sec to evaluate the number of cells, which were about 30/50 and ~90 for ICM and TB respectively.

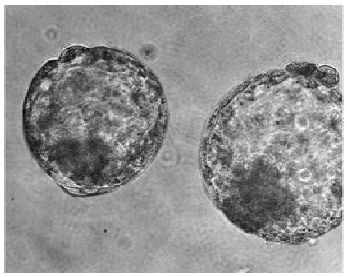
2.4. Production of ES-like and ECD cells

2.4.1. Immunosurgery and culture

Twenty-four blastocysts were used to produce ES-like and ECD cells according to the methods of Dattena et al. (2006). Briefly, the zona pellucida was removed by pronase treatment (3 mg/ml) followed by washing in enriched PBS with 10% (v/v) FBS (Fig. 12).

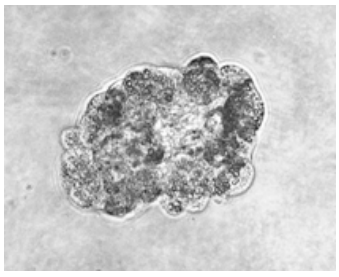
The embryos underwent immunosurgical complement mediated lysis of TB cells to isolate ICMs (Fig. 13). Blastocysts were incubated in a humidified atmosphere of air with 5% CO_2 at 38.5°C for 40 min in heat-inactivated rabbit anti-sheep whole serum diluted at 1:16 with enriched PBS with 0.4 % BSA. They were then washed three times for 10 min in enriched PBS with 0.4% BSA and incubated for 10 min at 38.5°C with guinea pig complement diluted 1:5 in enriched PBS with 0.4% BSA. After washing, ICMs were released from the TBs by gentle pipetting with a fine pulled glass capillary pipette, disaggregated in groups of 3–4 cells and cultured on a feeder layer STO (MIF) in high glucose DMEM (Dulbecco's modified Eagle's medium) supplemented with 2mM L-glutamine, 1mM sodium-pyruvate, 0.1 mM 2-mercaptoethanol, 0.1 mM non-.essential amino acids, 10 ng/ml LIF (Leukaemia inhibitoy factor), 20 μg/ml insulin, 10^3 i.u./ml penicillin, and 10 μg/ml streptomycin. From 24 blastocysts, 12 ES-like colonies (50%) were obtained.

Fig. 12.



7 day blastocysts after pronase treatment

Fig. 13.



Inner Cell Mass after immunosurgery

2.4.2. Collection of ES-like and ECD cells

ES like colonies were divided as follow: 9 were isolated from the feeder layer after 5-7 days of culture to obtain ES-like cells and 3 were isolated after ~10 days to obtain ECD cells. These were used to show how *OCT4* expression undergoes downregulation in the course of differentiation. Colonies were mechanically isolated and

washed in a drop of calcium-free PBS to remove residual serum and plunged into 500 µl trypsin (0.25%). The cells were incubated for 5 min at 38°C, and disaggregated cells were centrifuged at 5500xg for 3 min. They were then resuspended in calcium-free PBS +10% serum to inactivate trypsin, were recentrifuged at 5500xg for 3 min, resuspended in calcium-free PBS and were collected in RNase-free tubes in pools of 200 cells. We used 6 replicates to detect expression of *OCT4, NANOG SOX2* and *STAT3* in RT-PCR and PCR (see below) in order to better characterize ES-like cells. We used 3 replicates of ES-like and ECD cells to detect *OCT4* by qRTPCR analysis.

2.5. Granulosa cell collection

Granulosa cells (GR) were used as negative control for PCR or for qRTPCR. They were obtained from matured sheep oocytes by pipetting with hyaluronidase in H-TCM+BSA. After collection, GR were centrifuged at 200xg for 5 min at room temperature and the pellet was resuspended in DMEM with 10% FBS. GR were cultured in DMEM with 10% (v/v) FBS at 38° C in a humidified atmosphere with 5% CO_2 until approx. 70% confluency was reached. Cells used as negative controls in PCR and qRTPCR were disaggregated by incubation in 0.25% (w/v) trypsin solution for 7 min at 38°C, and stored in aliquots of 200 cells at -80°C.

2.6. Gene expression analysis

The same mRNA isolation and cDNA synthesis protocols of experiment 1 were used for blastocysts (6/7 and 8 days), ICM, TB, ES, ECD and GR cells in this experiment.

2.6.1. PCR primers

The ovine genomic sequences of *OCT4, NANOG* and *STAT3* were not found in online databases and the PCR primers utilized were designed with bovine genomic sequence, whereas for *SOX2* the pair of primers were designed with an ovine genomic sequence (GeneBank® accession number X96997) using Custom Primers software by Invitrogen. They are shown in Table 5.

2.6.2. PCR program

Only 10% of the cDNA produced from blastocysts, was used for PCR. The final PCR reaction was carried out in 50 µl of 1X PCR buffer [200 mM Tris/HCl (pH 8.4) and 500 mM KCl], 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 units of Taq DNA polymerase (Invitrogen) and 0.2 µM of each primer, using a thermal cycler (Eppendorf Mastercycler gradient). The *OCT4* PCR program was as follows: an initial step at 94°C for 2 min followed by 35 cycles of 30 sec each at 94°C for DNA denaturation, 1 min at 52°C for annealing of primers, and 1 min at 72°C for primer extension. The PCRs of *NANOG, SOX2* and *STAT3* were under following conditions: 94°C for 5 min, 94°C for 30 sec, 53°C (*NANOG*) and 53.5°C (*SOX2* and *STAT3*) for 30 sec, 72°C for 30 sec for 35 cycles. The last cycle of PCRs was followed by a 7 min final elongation at 72°C and cooling to 4°C. Tubes prepared without cDNA were used as negative control. Amplification products (10 µl) were subjected to electrophoresis on 2% agarose gel in 1X TAE buffer, stained with ethidium bromide (EtdBr) and evaluated under UV light. An image of the gel was recorded using a digital colour camera with the DigiDoc-It Imaging System (UVP, Inc., Upland, California).

Table 5. Primers and conditions used for PCR.

Gene	Primer Sequence (5'- 3') Forward and Reverse	Fragment size (bp)	Annealing temperature (°C) x cycle number	Gene Bank® Accession No.
OCT4	(F) CAATTTGCCAAGCTCCTAAA (R) TTGCCTCTCACCTTGTTCTC	290	52 x 35	AY 490804
NANOG	(F) TTCCTTCCTCCATGGATCTG (R) AGGAGTGGTTGCTCCAAGAC	501	53 x 35	NM 001025344
SOX2	(F) TGATACGGTAGGAGCTTTGC (R) GGTCTCTAAAGGGGCAAAAG	362	53.5 x 35	X 96997
STAT3	(F) TGGACAACATCATTGACCTG (R) CTGCTGCTTGGTGTAAGGTT	239	53.5 x 35	NM 001012671

2.6.3. Sequencing of OCT4, NANOG and STAT3

Amplified cDNA by PCR was directly sequenced in both directions (forward and reverse) by an automated capillary sequencer (model ABI3100; Applied Biosystems, Foster City, CA, USA). Sequencing reactions were carried out using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Biosystem, Foster City, CA, USA). Alignments with the published genomic sequences were performed using NCBI-Blast.

2.6.4. Quantitative Real Time PCR

Quantitative expression of *OCT4* gene was analysed by qRTPCR in BL6/7, BL8, ICM, TB, ES-like, ECD and GR cells with an ABI PRISM 7000 (Applied Biosystems,

Foster City, CA, USA) sequence detector and software system. TaqMan MGB probes (FAM dye-labelled) and primers for ovine *OCT4* and *GADPH* (glyceraldehyde-3-phosphate dehydrogenase, - Genebank® accession number DQ386891) were ordered from Applied Biosystems assays-on-demand (22× assay mixes) using the File Builder program. qRTPCR was performed with 4 µl of cDNA, 12.5 µl of TaqMan Universal PCR master mixture (Applied Biosystems), 1.25× assay-on-demand mixes of primers and TaqMan MGB probes, *GADPH* was used as endogenous control. All samples were analyzed in triplicate and PCR was performed in optical 96-well microtitre plates (Applied Biosystems). After an initial denaturation step at 95°C for 10 min, the cDNA products were amplified with 40 PCR cycles consisting of a denaturation step at 95°C for 15s and an extension step at 60°C for 1 min. The ΔΔCt method was used to evaluate the relative expression of the samples.

2.6.5. Statistical analysis

qRTPCR data are given as mean arbitrary units ± S.E.M. from three separate experiments, and were statistically analysed by one-way ANOVA, followed by Newman Keuls post hoc test using Graph Pad Prism (San Diego, CA). Differences with $P<0.05$ were considered statistically significant.

3. Results

3.1. Sequencing of ovine *OCT4, NANOG* and *STAT3*

We designed and used bovine primers to obtain amplification by PCR. Since the sequence of ovine *OCT4*, N*ANOG* and *STAT3* were not known at the time we starded these studies, it was necessary to sequence amplified PCR product. The sequences obtained were composed like this: *OCT4* of 290 bp (Fig.14A), N*ANOG* of 501 bp (Fig 14B) and *STAT3* of 239 bp (Fig.14C) and were deposited into the GenBank database under accession number FJ970649, FJ970651, FJ970650 respectively.

Fig. 14A. Ovine *OCT4* sequence of 290 bp (accession number FJ970649)

```
CAATTTGCCAAGCTCCTAAAGCAGAAGAGGATCACCCTAGGATATACCCAGGCCGATGTGGGGCTCACCC
TGGGGGTTCTCTTTGGAAAGGTGTTCAGCCAAACGACTATCTGCCGTTTTGAGGCTTTGCAGCTCAGTTT
CAAGAACATGTGTAAGCTGCGGCCCCTGCTGCAGAAGTGGGTGGAGGAAGCTGACAACAACGAGAATCTG
CAGGAGATATGCAAGGCAGAGACCCTTGTGCAGGCCCGAAAGAGAAAGCGGACGAGTATCGAGAACAAGG
TGAGAGGCAA
```

Fig. 14B. Ovine *NANOG* sequence of 501 bp (accession number FJ970651)

```
TTCCTTCCTCCATGGATCTGCTTATTCAGGACAGTCCTGATTCTTCCACAAGCCCCAGAGTGAAACCATT
GTCCCCATCTGCAGAGGAGAGCACAGAGAAGGAAGAGAAGGTCCCCGTCAAGAAACAAAAGATCAGAACT
GTGTTCTCACAGACCCAGTTGTGTGTGCTCAATGACAGATTTCAGAGGCAGAAATACCTCAGTCTCCAGC
AAATGCAAGAACTTTCCAACATCTTGAACCTCAGCTACAAGCAGGTGAAGACCTGGTTCCAGAATCAGAG
AATGAAATGTAAGAAATGGCAGAAAAACAACTGGCCGAGGAATAGCAATGGTGTGCCTCAGGGCCCAGCA
ACGGCAGAATACCCGGGCTTCTATTCCTACCATCAGGGGTGTCTTGGTGAACTCTCCTGGAAACCTGCCC
ATGTGGGGGTAACCAGACCTGGAATAACCCCCACGTGGAGCAATCAGAGCTGGAACAGTCAGTCTTGGAG
CAACCACTCCT
```

Fig. 14C**.** Ovine *STAT3* sequence of 239 bp (accession number FJ970650)

```
TGGACAACATCATTGACCTGGTGAAAAAGTACATCCTGGCCCTTTGGAACGAAGGGTATATAATGGGCTT
CATCAGCAAGGAGAGGGAACGGGCCATCTTGAGCACTAAGCCCCCAGGCACCTTCCTGTTGAGATTCAGT
GAAAGCAGCAAAGAAGGAGGAGTCACCTTCACCTGGGTGGAGAAGGACATCAGCGGCAAGACCCAGATCC
AGTCAGTGGAACCTTACACCAAGCAGCAG
```

3.2. Sequence alignment

Ovine sequences were subsequently analyzed and aligned to the bovine sequence using NCBI-Blast. A high similarity was notice: *OCT4* (98%) (Fig. 15A), *NANOG* (95%) (Fig. 15B) and *STAT3* (98%) (Fig. 15C).

Fig. 15A. Alignment of ovine (accession n° FJ970649) and bovine (accession n° AY490804) *OCT4* sequence.

```
Ovine    1  CAATTTGCCAAGCTCCTAAAGCAGAAGAGGATCACCCTAGGATATACCCAGGCCGATGTG  60
            |||||||||||||||||||||||||||||||||| |||||||||||||||| ||||||
Bovine  37  CAATTTGCCAAGCTCCTAAAGCAGAAGAGGATCACACTAGGATATACCCAGGCTGATGTG  96

Ovine   61  GGGCTCACCCTGGGGGTTCTCTTTGGAAAGGTGTTCAGCCAAACGACTATCTGCCGTTTT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Bovine  97  GGGCTCACCCTGGGGGTTCTCTTTGGAAAGGTGTTCAGCCAAACGACTATCTGCCATTTT  156

Ovine  121  GAGGCTTTGCAGCTCAGTTTCAAGAACATGTGTAAGCTGCGGCCCCTGCTGCAGAAGTGG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bovine 157  GAGGCTTTGCAGCTCAGTTTCAAGAACATGTGTAAGCTGCGGCCCCTGCTGCAGAAGTGG  216

Ovine  181  GTGGAGGAAGCTGACAACAACGAGAATCTGCAGGAGATATGCAAGGCAGAGACCCTTGTG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Bovine 217  GTGGAGGAAGCTGACAACAACGAGAATCTGCAGGAGATATGCAAGGCAGAGACCCTTGTG  276

Ovine  241  CAGGCCCGAAAGAGAAAGCGGACGAGTATCGAGAACAAGGTGAGAGGCAA  290
            |||||||||||||||||||||||||||||||||| | ||||||||||||
Bovine 277  CAGGCCCGAAAGAGAAAGCGGACGAGTATCGAGAACCAAGTGAGAGGCAA  326
```

Fig. 15B. Alignment of ovine (accession n° FJ970651) and bovine (accession n° NM 001025344) *NANOG* sequence.

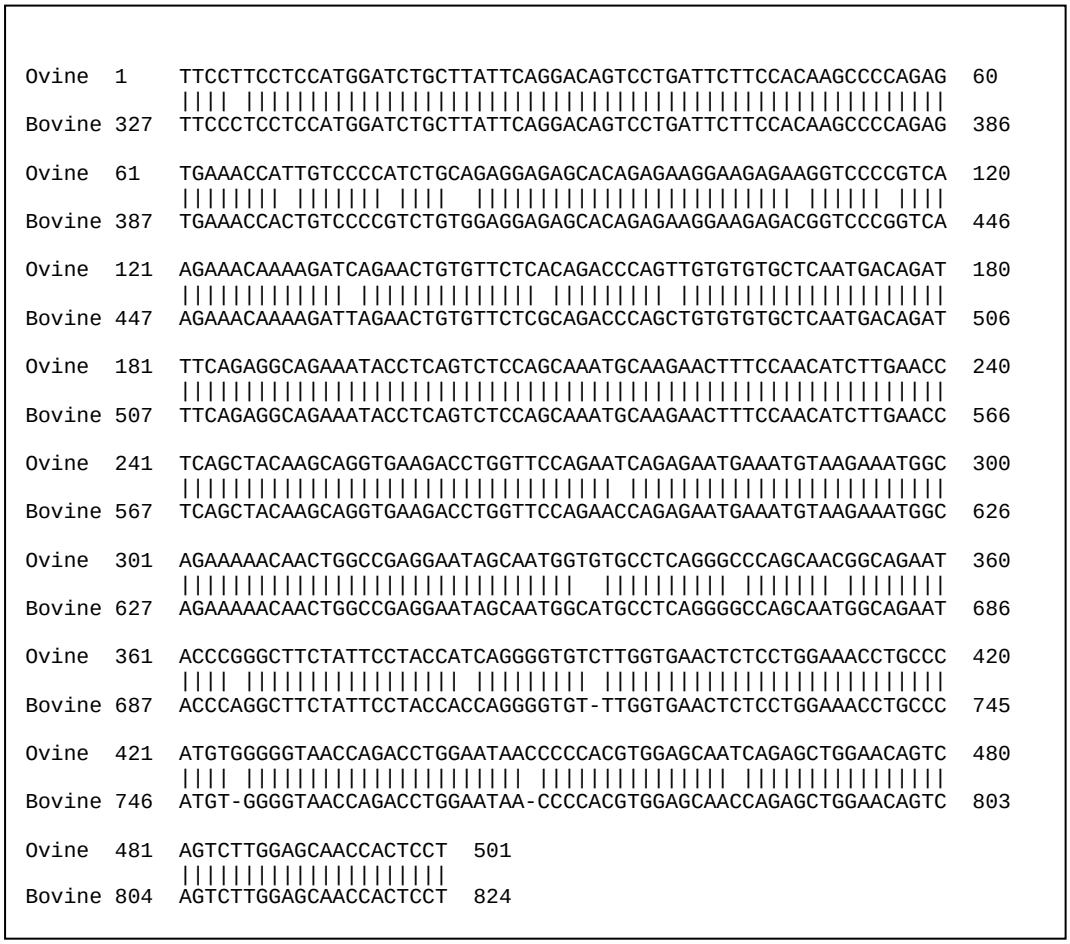
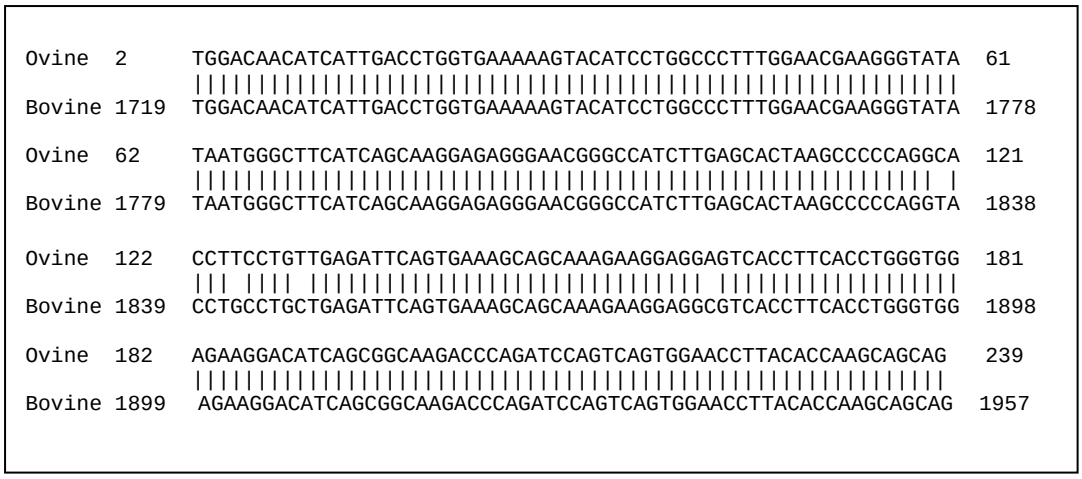


Fig. 15C. Alignment of ovine (accession n° FJ970650) and bovine (accession n° NM 001012671) *STAT3* sequence by NCBI-blast.



3.3. Characterization of ES-like and ECD cells

ES like colonies were isolated from the feeder layer after 5 to 7 days of culture (Fig. 16) and were characterized. The morphology of ECD colonies was characterised by cells resembling epithelium, cells with vacuoles in cytoplasm or elongated cells (Fig. 17), which the low *OCT4* gene expression later confirmed (see below). To examine in ES-like cells the expression of *OCT4, NANOG, SOX2* and *STAT3* a representative gel photo shows following results: ovine blastocysts and ES-like cells were positive, GR cells were negative for *OCT4, NANOG, SOX2* and positive for *STAT3* as expected (Fig. 18).

Fig. 16. ES-like cells colony at 7 days of culture

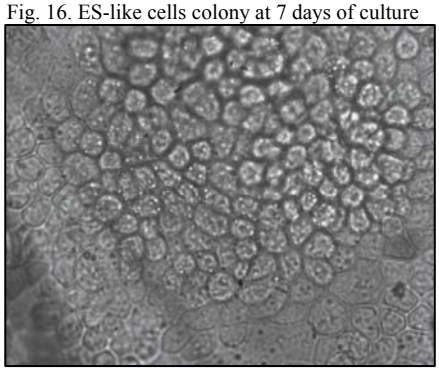


Fig. 17. ES-like cells colony at 10 days of culture

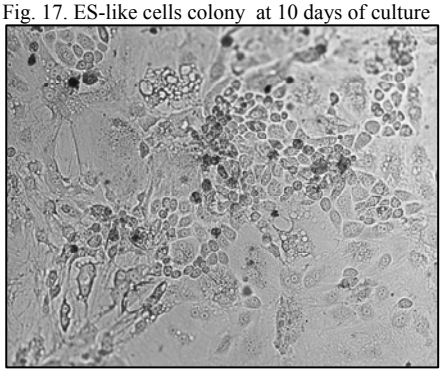
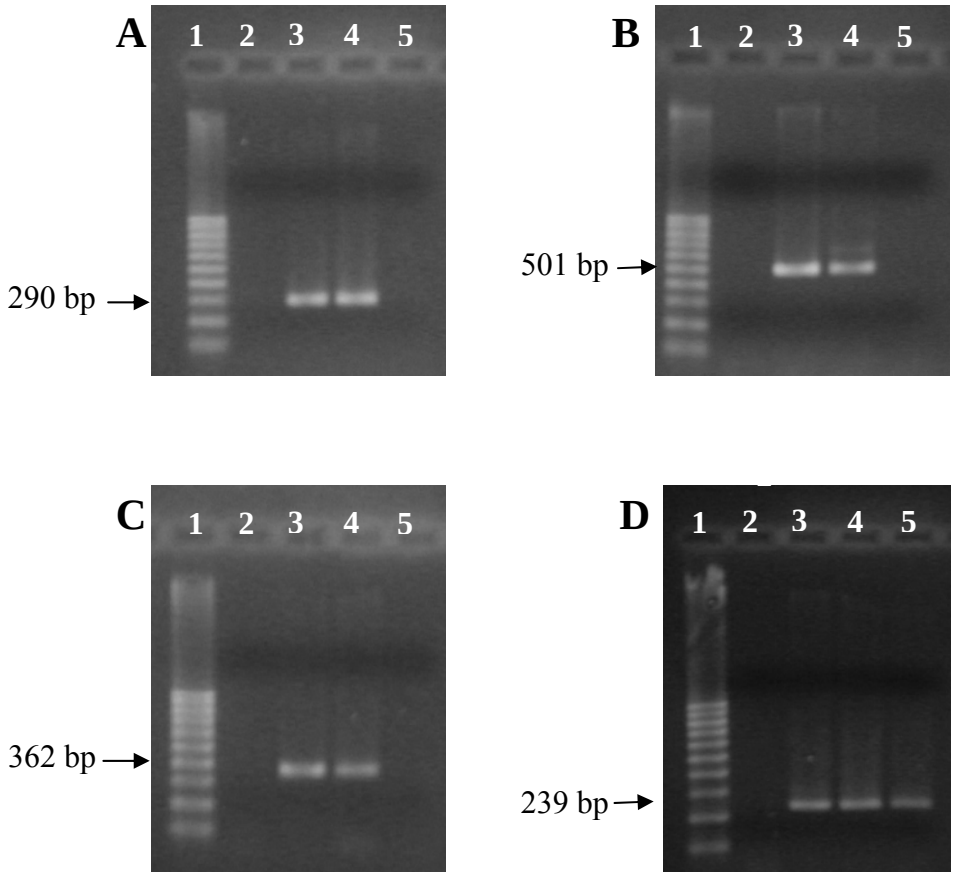


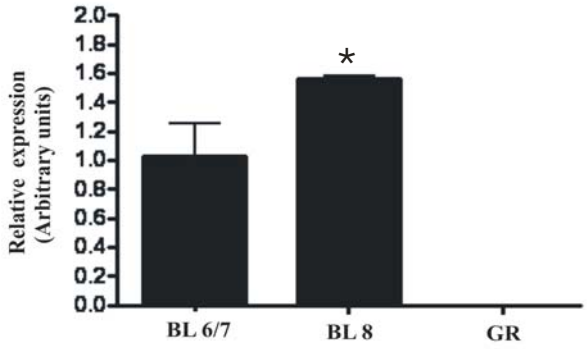
Fig. 18. Representative gel photos of cDNA expression in vitro produced ovine blastocysts and ES like-cells. (**A)** *OCT4*; (**B)** *NANOG*; **(C)** *SOX2;* ***D*** *(STAT3).* Lane 1, DNA marker (100 bd ladder); Lane 2, negative control (no CDNA); Lane 3, sheep blastocysts; Lane 4, ES-like cells; Lane 5, GR cells.



3.4. *OCT4* gene expression in BL6/7 and in BL8

The expression level of *OCT4* gene transcript was studied in blastocysts at 6/7 day and at 8 days by qRTPCR. The relative abundance of gene was markedly higher in BL8 compared with BL6/7. The comparison was statistically different ($P<0.05$) between groups as demonstrated in Figure 19. Transcript of *OCT4* was not express in GR cells (negative control).

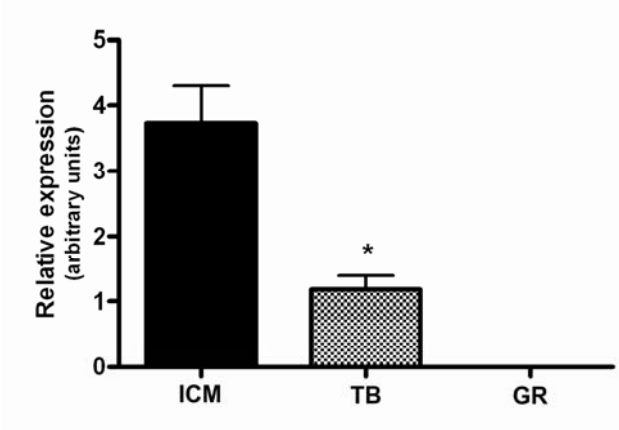
Fig.19. Relative expression of ovine *OCT4* gene in *in vitro* blastocysts at day 6/7 (BL6/7), at day 8 (BL 8) and Granulosa cells (GR) (negative control). Significant difference (P<0.05) was indicated by an asterisk (*).



3.5. *OCT4* gene expression in ICM and in TB

The expression level of *OCT4* gene transcript was studied in ICMs and TBs by qRTPCR. The relative abundance of gene was markedly higher in ICM compared with TB. The comparison was statistically different (P<0.05) between groups as demonstrated in Figure 20. Transcript of *OCT4* was not express in GR cells (negative control).

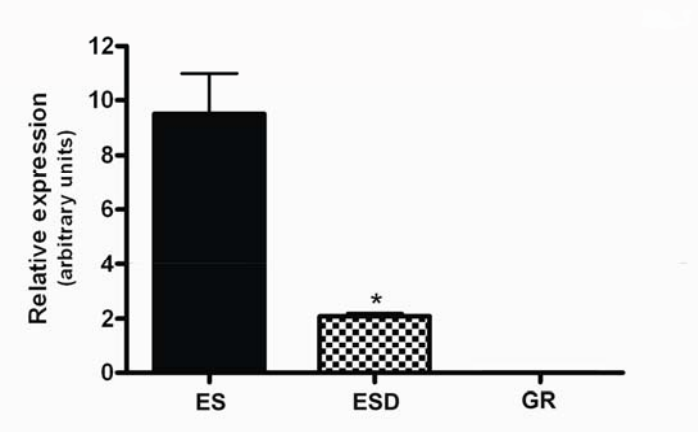
Fig. 20. Relative expression of ovine *OCT4* gene in the Inner Cell Mass (ICM), Trophoblast (TB) and Granulosa cells (GR) (negative control) of *in vitro* cultured blastocysts. Significant difference (P<0.05) was indicated by an asterisk (*).



3.6. *OCT4* gene expression in ES-like and ECD cells

The relative expression of *OCT4* in ES-like and ECD cells was compared in undifferentiated and in differentiating states respectively. The results showed a significant difference among the two groups ($P<0.05$) (see Fig. 12). Again, *OCT4* was not expressed in GR cells (negative control).

Fig. 21. Relative expression of ovine *OCT4* gene in the Embryonic Stem-like cells (ES), Embryonic Stem-like cells in course of differentiation (ECD) and Granulosa cells (GR) (negative control). Significant difference ($P<0.05$) was indicated by an asterisk (*).



4. Discussion and Conclusion

The transcription factors *OCT4, NANOG, SOX2* and a signalling molecule *STAT3* are known to have important role on embryo welfare during early embryonic development and to play a critical role in the maintenance of pluripotency of ICM, staminality and self-renewal of ES cells (Niwa et al., 1998; Chambers et al., 2003; Avilion et al., 2003; HE et al., 2005; Yadav et al., 2005).

The present study is probably the first report of the ovine *OCT4, NANOG, SOX2* and *STAT3* gene detected in blastocysts, ICM, TB, undifferentiated and differentiating ES-like cells in *in vitro* culture.

We have detected the presence of these key factors in ovine blastocysts and in ES-like cells. Bovine primers were successfully used in PCR. Thus, it was essential to amplify and sequence the PCR products for *NANOG* and *STAT3, OCT4,* because the ovine genomic sequences for these genes were not found in online databases, excepted for *SOX2* (Payen et al., 1997), whose sequence is known. Comparisons by NCBI-Blast in several species (mouse, human, equine, rhesus monkey, porcine and bovine) showing variations between 88% and 98%, suggest that these sequences are highly conserved among species in accordance with previous studies (Takeda et al., 1992; Pesce and Schöler, 2000; Kurosaka et al., 2004). In fact, ovine embryos showed gene expression similar to other mammals The homology between ovine and bovine fragments confirm that also in ovine these sequences are highly conserved.

The presence of these genes might be used to define blastocyst quality, and it is well known that their expression may influence several genes involved in early development (Ezashi et al., 2001; Niwa, 2007a; Mitsui et al. 2003; Li et al., 2007; Antczak and Van Blerkom, 1997). This is especially true for the *in vitro* produced embryos that can easily undergo modification of gene expression according to the culture

system used (Rizos et al., 2002a; Lazzari et al. 2002; Lonergan et al., 2003). In addition, qualitative study by PCR indicated that under our experimental conditions, the sheep ES-like cells cultured in a feeder layer until day 7, expressed these key markers suggesting pluripotency.

The *OCT4* gene expression was detected quantitatively by qRTPCR. Interestingly, the *OCT4* gene was upregulated in 8 days old embryos when compared with day 7 embryos. It was previously reported in mice (Palmieri et al., 1994), that *OCT4* is transiently increased in the primitive endodermal cells of the blastocyst before final downregulation. Handyside et al. (1987) reported that the delamination of endoderm had already occurred at day 7 in *in vivo* derived sheep embryos. On the contrary, Dattena et al. (2006) did not observe endoderm cells at this stage in *in vitro* produced embryos, explaining that this may have been due to different ages of differentiation in the *in vitro* vs *in vivo* cultured embryos. This could explain the high *OCT4* expression noted in our experiment at day 8. However, further testing would be needed to confirm this. The exact physiological processes that cause variations in timing of *OCT4* downregulation are still unknown, but several hypotheses may be offered, including differences in differentiation times among species. For example, in pigs and in cattle downregulation first appears at 14-16 days after fertilization and several authors (van Eijk et al., 1999; Kurosaka et al., 2004) suggested that delayed downregulation of *OCT4* could reflect this prolonged period of preimplantation development.

In the present study, ovine *OCT4* expression in ICM was higher than in TB, suggesting a relationship between *OCT4* and pluripotency. This finding, obtained by qRTPCR makes *OCT4* a suitable marker for identification of pluripotent cell populations in sheep. Similar results were reported for other mammals including mouse (Palmieri et

al., 1994), rhesus monkey (Mitalipov et al., 2003), horse (Choi et al., 2007) and human (Hansis et al., 2000).

Under our experimental conditions, the ES-like cells cultured in a feeder layer expressed *OCT4* until day 8 of culture, suggesting staminality. On the contrary, when ECD cells were tested for *OCT4* expression, the level of the gene was significantly lower indicating the *OCT4* downregulation typical of cells undergoing differentiation. This is similar to the findings of Dattena et al. (2006) who reported that the embryonic cell lines differentiated after the second passage. The author is aware of the possibility that improving on the media culture with some factors such as bFGF or Activin A the differentiation might happen later. According to Niwa (2007b) the transcription factor *OCT4* is regarded as pivotal because its loss of function abolishes self-renewal and induces differentiation. Thus *OCT4* can be considered as a key feature of ES cells that have not undergone differentiation.

In conclusion, the present study confirms that the presence of *OCT4, NANOG, SOX2*, *STAT3* in sheep blastocysts and ES-like cells, the different expression of *OCT4* in the ICM compared with TB suggest pluripotency and they can be used as a complementary method for embyo quality and to improve the characterization of sheep ES-like cells.