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CENTRO DE TECNOLOGÍA ANIMAL

"FACTORS AFFECTING THE *IN VITRO* EMBRYO PRODUCTION IN CATTLE ASSOCIATED TO OVUM PICK UP SYSTEM"

DOCTORAL THESIS

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Certificate

Dr. Alberto Cebrian Serrano (identity card number: 73566670-C) has completed an internship in the above named institute from 4th of May to to 15th of October 2009 in the Department of Biotechnology. He has very skillfully assisted in several ongoing scientific experiments with main emphasis on sperm sexing techniques, in vitro cell culture systems and evaluation, culture, and preservation of gametes and embryos from large farm animals. We enjoyed the time having Mr. Cebrian in our group and wish him all the best for his carrier.

Prof. Dr. med.vet.habil. Detlef Rath
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Cuando emprendas el viaje hacia Itaca

ruega que sea largo el camino,

lleno de aventuras, lleno de experiencias.

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TABLE OF CONTENTS

ABSTRACTS 5

1. English 5
2. Castellano 7
3. Valencià 9

GENERAL INTRODUCTION 13

1. Economic importance of the cattle production 13
1.1. European Union 13
1.2. Spain 14
1.3. Community of Valencia 16

2. Ovum pick up -*In vitro* fertilization: characteristics, current status and progress 17
2.1. Characteristics of the Ovum pick up -*In vitro* fertilization technology: historical perspective and application 17
2.2. Current status of *in vitro* production of embryo in cattle 18
2.3. Progress achieved 20
References 24

OBJECTIVES 29

EXPERIMENTS 31

Experiment 1:
Effect of the Bovine Oviductal Fluid on *In Vitro* Fertilization, Development, and Gene Expression of *In Vitro*-Produced Bovine Blastocysts 31
Abstract 33
Introduction 33

Materials and Methods 35
Results 42
Discussion 46
Acknowledgments 50

Experiment 2:
Effect of number of oocytes and embryos on *in vitro* oocyte maturation, fertilization and embryo development in bovine 57

Abstract 59
Resumen 59
Introduction 60
Material and methods 61
Results 65
Discussion 69
Acknowledgments 73
References 74

Experiment 3:
Beneficial Effect of Two Culture Systems with Small Groups of Embryos on the Development and Quality of *In Vitro*-Produced Bovine Embryos 79

Summary 81
Introduction 81
Materials and methods 84
Results 88
Discussion 91
Acknowledgments 95
References 96

Experiment 4:

Beneficial Effect of Melatonin on Blastocyst *In Vitro* Production from Heat Stressed Bovine Oocytes 103

Abstract.. 105

Introduction 105

Materials and Methods 108

Results 111

Discussion.. 117

Acknowledgments 123

References 124

Experiment 5:

Effect of Sex-sorted Sperm on Development and Quality of *In Vitro*-Produced Bovine Embryos Derived from Ovum Pick Up Oocytes .. 131

Abstract.. 133

Introduction 133

Materials and Methods 136

Results and discussion 140

Acknowledgments 144

References 145

GENERAL DISCUSSION 151

CONCLUSIONS 155

ABSTRACTS

1. English

"Factors Affecting the *In Vitro* Embryo Production in Cattle Associated to Ovum Pick Up System"

Production of embryos through the collection of immature oocytes by ovum pick up (OPU), and the following maturation, fertilization and culture in the *in vitro* laboratory, offers many benefits to optimize reproductive potential. Moreover, *in vitro* production of embryos (IVPE) with OPU oocytes supposes an alternative method with several advantages over the conventional hormonal treatment of superovulation and *in vivo* embryo recovery. However, currently, the IVPE is still not as efficient, or can produce embryos of similar quality to *in vivo* embryos, which has limited their widely application. This thesis was performed with the aim to optimize the IVPE technology in cattle, conditioned by the specific characteristics and deficiencies of the IVPE when OPU oocytes are used. To this end, five experiments were performed. Firstly, we studied the effect of the bovine oviductal fluid (bOF) on embryo development and quality (Experiment 1). The steps of the IVPE process at which the culture of a single or low number of oocytes/embryos could impair their further development to the blastocyst stage or/and their quality were studied in the Experiment 2. In the Experiment 3, we tested whether the development and quality of the embryos *in vitro* cultured in low number can be improved by the addition of a mixture of epidermal growth factor, insulin, transferrin, and selenium (EGF-ITS) or by the system of embryo culture known as 'Well of Well' (WOW). The protective role of the melatonin regarding the oxidative stress damage subsequent to the IVPE conditions or subsequent to the heat stress during oocyte maturation were assessed in the Experiment 4. Finally, in our last experiment (Experiment 5), we used oocytes collected by OPU

to test the effect of sex-sorted sperm in the *in vitro* fertilization and their further embryo development and quality. The results of the Experiment 1 showed that a short bOF treatment in oocytes had no effect on the embryo development until blastocyst stage and neither on the quality attending their blastocyst morphology. However, the study of the genetic expression suggested that bOF treatment could be useful to mitigate some lacks on the lower quality blastocysts produced *in vitro.* Regarding the Experiment 2, we observed that the period of culture after the fertilization, especially between the days 3 and 8, seems to be the most important stage for embryo development on single and/or low number (5-10) of embryos culture. In the Experiment 3, the addition of EGF-ITS increased the quality of the embryos cultured in low number, whereas culture embryos in WOW improved significantly the development rates. In the fourth experiment (Experiment 4), the addition of melatonin (10^{-4} M) to heat-stressed bovine oocytes enhanced the further development to blastocyst stage. However, compared to the control, none of the melatonin concentration tested (10^{-12}, 10^{-9} and 10^{-4} M) showed any significant effect when oocytes were matured under conventional *in vitro* conditions. Concerning the results of the Experiment 5, zygotes fertilized by sex-sorted sperm showed a lower cleavage rate in comparison with those fertilized with unsorted sperm, but similar blastocyst rate, as well as similar morphological quality and timing of blastocyst formation.

2. Castellano

"Factores que Afectan a la Producción *In Vitro* de Embriones Bovinos Asociado a un Sistema de *Ovum Pick Up*"

La producción de embriones mediante la recuperación de ovocitos inmaduros por *ovum pick up* (OPU), y su posterior maduración, fecundación y cultivo en el laboratorio *in vitro,* presenta numerosos beneficios para optimizar el potencial reproductivo, tanto de hembras como de machos. Además, frente a la superovulación convencional mediante tratamiento hormonal y la recogida de embriones *in vivo,* la producción *in vitro* de embriones (PIVE) con ovocitos de OPU ofrece considerables ventajas. Sin embargo, actualmente la PIVE continua siendo ineficiente e incapaz de producir embriones de calidad similar a los *in vivo*, lo cual ha limitado una aplicación más amplia de esta tecnología. Así pues, el objetivo de esta tesis fue la optimización de la PIVE en ganado vacuno, condicionado por las peculiaridades y deficiencias de la PIVE cuando los ovocitos son recuperados por la técnica de OPU. Con este fin, cinco experimento se llevaron a cabo en esta tesis. En el primero de ellos se estudió el efecto del fluido oviductal bovino (FOb) sobre el desarrollo y la calidad embrionaria (Experimento 1). Las fases del proceso de PIVE en las cuales el cultivo de ovocitos/embriones, bien individualmente o bien en número reducido, pudiera perjudicar el posterior desarrollo hasta el estadio de blastocisto y/o a su calidad, se estudiaron en el Experimento 2. En el Experimento 3 se testó si el desarrollo y la calidad de embriones cultivados *in vitro* en número reducido podría ser mejorada con la adición conjunta de factor de crecimiento epidérmico, insulina, transferrina y selenio (FCE-ITS) o por el sistema de cultivo de embriones llamado *well of well* (WOW). Las propiedades protectoras de la melatonina frente a los daños causados por el estrés oxidativo, subsecuentes de las condiciones de PIVE o de un estrés térmico durante la maduración ovocitaria, fueron evaluadas en el Experimento 4. Por último, en el Experimento 5 usamos ovocitos recolectados por OPU para

evaluar el efecto del semen sexado sobre la fecundación *in vitro* y su posterior desarrollo y calidad embrionaria. Los resultados del Experimento 1 evidenciaron que un tratamiento breve con el FOb sobre ovocitos no produjo ningún efecto sobre el desarrollo embrionario hasta el estadio de blastocisto y tampoco en la calidad atendiendo a su morfología blastocitaria. Sin embargo, el estudio de la expresión genética sugirió que el tratamiento con FOb podría ser útil para mitigar algunas de las carencias de los blastocistos producidos *in vitro* de peor calidad. En los resultados del Experimento 2, observamos que el periodo de cultivo posterior a la fecundación, especialmente entre los días 3 y 8, podría ser el más importante para el desarrollo embrionario cuando se cultivan embriones individualmente y/o en grupos reducidos (5-10 embriones). En el Experimento 3, la adición de FCE-ITS incrementó la calidad de los embriones cultivados en grupos reducido, mientras que el cultivo de embriones en WOW mejoró las tasas de desarrollo significativamente. En la cuarta experiencia (Experimento 4), la adición de melatonina (10^{-4} M) a ovocitos bovinos estresados por calor durante la maduración mejoró su posterior desarrollo a estadio de blastocisto. Sin embargo, comparado con el control, ninguna de las concentraciones testadas (10^{-12}, 10^{-9} y 10^{-4} M) mostró efecto alguno cuando los ovocitos fueron madurados bajo las condiciones *in vitro* convencionales. En lo que respecta al Experimento 5, los ovocitos fecundados con semen sexado mostraron una menor tasa de división que aquellos fecundados con semen no sexado, aunque similar tasa de blastocistos, calidad morfológica y momento de formación blastocitaria.

3. Valencià

"Factors que Afecten la Producció *In Vitro* d'Embrions Bovins Associat a un Sistema d'*Ovum Pick Up*"

La producció d'embrions per mitjà de la recuperació d'ovòcits immadurs per *ovum pick up* (OPU), i la seua posterior maduració, fecundació i cultiu en el laboratori *in vitro*, presenta nombrosos beneficis per a optimar el potencial reproductiu, tant de femelles com de mascles. A més, enfront de la superovulació convencional per mitjà de tractament hormonal i el recull d'embrions *in vivo*, la producció *in vitro* d'embrions (PIVE) amb ovòcits d'OPU ofereix considerables avantatges. No obstant això, actualment la PIVE continua sent ineficient i incapaç de produir embrions de qualitat semblant als *in vivo*, la qual cosa ha limitat una aplicació més àmplia d'aquesta tecnologia. Així doncs, l'objectiu d'aquesta tesi és l'optimació de la PIVE en bestiar boví, condicionat per les peculiaritats i deficiències de la PIVE quan els ovòcits són recuperats per la tècnica d'OPU. Amb aquesta finalitat, cinc experiment es van dur a terme en aquesta tesi. En el primer es va estudiar l'efecte del fluid oviductal boví (FOb) sobre el desenvolupament i la qualitat embrionària (Experiment 1). En l'Experiment 2, se estudiaren les fases del procés de PIVE en les quals el cultiu d'ovòcits/embrions, bé individualment o bé en nombre reduït, poguera perjudicar el posterior desenvolupament fins l'estat de blastòcit o a la seua qualitat. En l'Experiment 3 vam testar si el desenvolupament i la qualitat dels embrions cultivats *in vitro* en nombre reduït podrien millorar-se amb l'addicció de factor de creixement epidèrmic, insulina, transferrina i seleni (FCE-ITS) i pel sistema de cultiu d'embrions anomenat *well of well* (WOW). Les propietats protectores de la melatonina enfront dels danys causats per l'estrès oxidatiu, subsegüent a les condicions de PIVE o per un estrès tèrmic durant la maduració dels ovòcits, es van avaluar en l'Experiment 4. Finalment, en l'Experiment 5 hem utilitzat ovòcits recol·lectats per OPU per a avaluar l'efecte del semen sexat en la fecundació *in vitro* i el

posterior desenvolupament i qualitat embrionària. Els resultats de l'Experiment 1 van evidenciar que un tractament breu amb el FOb sobre ovòcits no va produir cap efecte sobre el desenvolupament embrionari fins a l'estat de blastòcit i tampoc en la qualitat atenent a la seua morfologia blastocitaria. No obstant això, l'estudi de l'expressió genètica va suggerir que el tractament amb FOb podria ser útil per a mitigar algunes de les mancances dels blastòcits produïts *in vitro* de pitjor qualitat. En els resultats de l'Experiment 2, observem que el període de cultiu posterior a la fecundació, especialment entre els dies 3 i 8, podria ser el més important per al desenvolupament embrionari quan es cultiven embrions individualment i/o en grups reduïts (5-10 embrions). En l'Experiment 3, l'addició del FCE-ITS va incrementar la qualitat dels embrions cultivats en grups reduïts, mentre que el cultiu d'embrions en WOW va millorar les taxes de desenvolupament significativament. En la quarta experiència (Experiment 4), l'addició de melatonina (10^{-4} M) a ovòcits bovins estressats per calor durant la maduració en va millorar el posterior desenvolupament a l'estat de blastòcit. No obstant això, comparat amb el control, cap de les concentracions testades (10^{-12}, 10^{-9} i 10^{-4} M) va mostrar cap efecte quan els ovòcits van ser madurats en condicions *in vitro* convencionals. Pel que fa a l'Experiment 5, els ovòcits fecundats amb semen sexat van mostrar una menor taxa de divisió, encara que una semblant taxa de blastòcits, qualitat morfològica i moment de formació dels blastòcits.

GENERAL INTRODUCTION

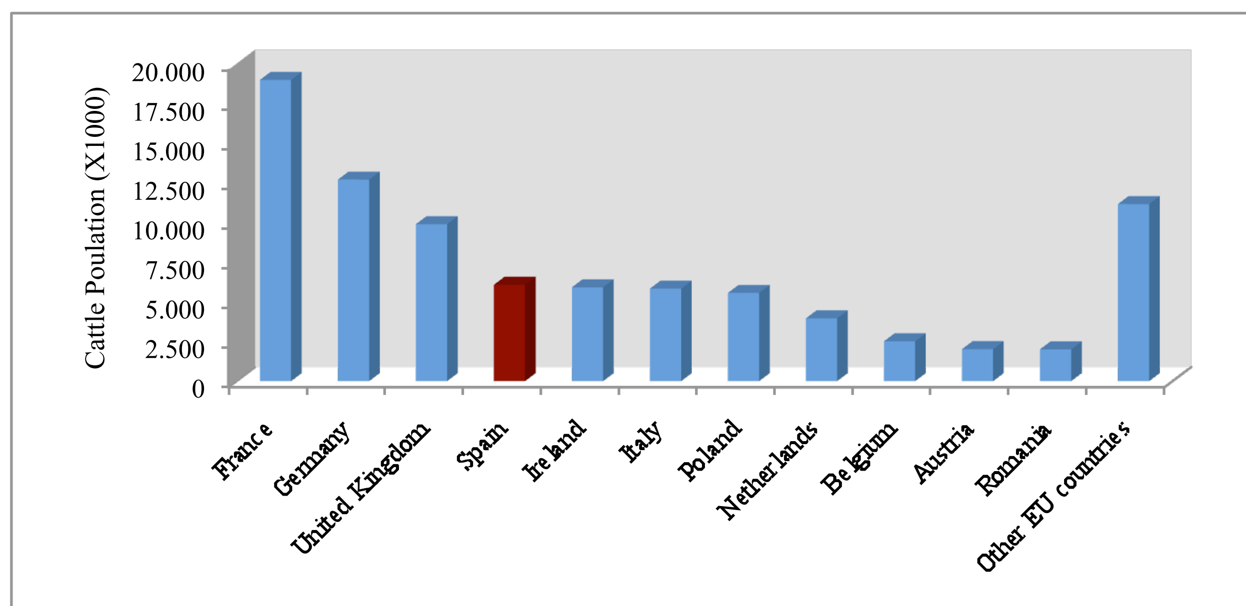
1. Economic importance of the cattle production

1.1. European Union

The cattle production is considered one of the most profitable industries in the European Union (EU) agriculture. The recent data published by the EU showed that cattle production represented the 20.8% of the total agricultural production of Europe. Within this overall percentage, approximately 60% was represented by the dairy cattle industry (European Commission and Eurostat, 2012).

Concerning the number of cattle at the EU countries, France represented the greatest population, with approximately 19 million of heads, followed by Germany, United Kingdom, and Spain (rates of 22, 15, 11, and 7% respectively, from the total cattle population, Figure 1) (European Commission and Eurostat, 2010a).

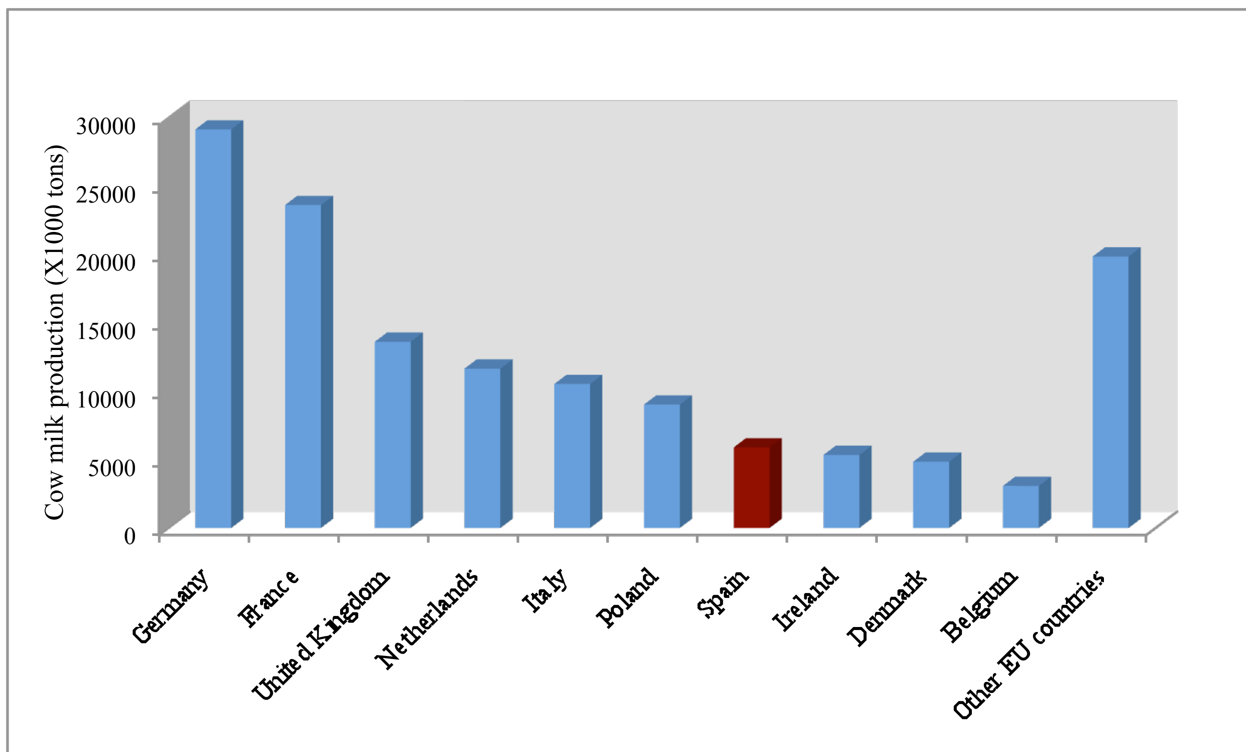
Figure 1. Total of cattle population in the European Union (x1000).



Source: European Commission, Eurostat, 2010a.

As regards milk production, the EU is the largest dairy producer of the world, generating over 135 millions of tons in 2010 (United States Department of Agriculture and Foreign Agricultural Service, 2012). The three largest dairy cattle-producing countries of the EU were Germany, France, and United Kingdom (21, 17, and 10% respectively, over the EU total output, Figure 2), while Spain remained in seventh position of this ranking (4% over the EU total output) (European Commission and Eurostat, 2010b).

Figure 2. Cow milk production in the European Union (x1000 tons).



Source: European Commission, Eurostat, 2010b.

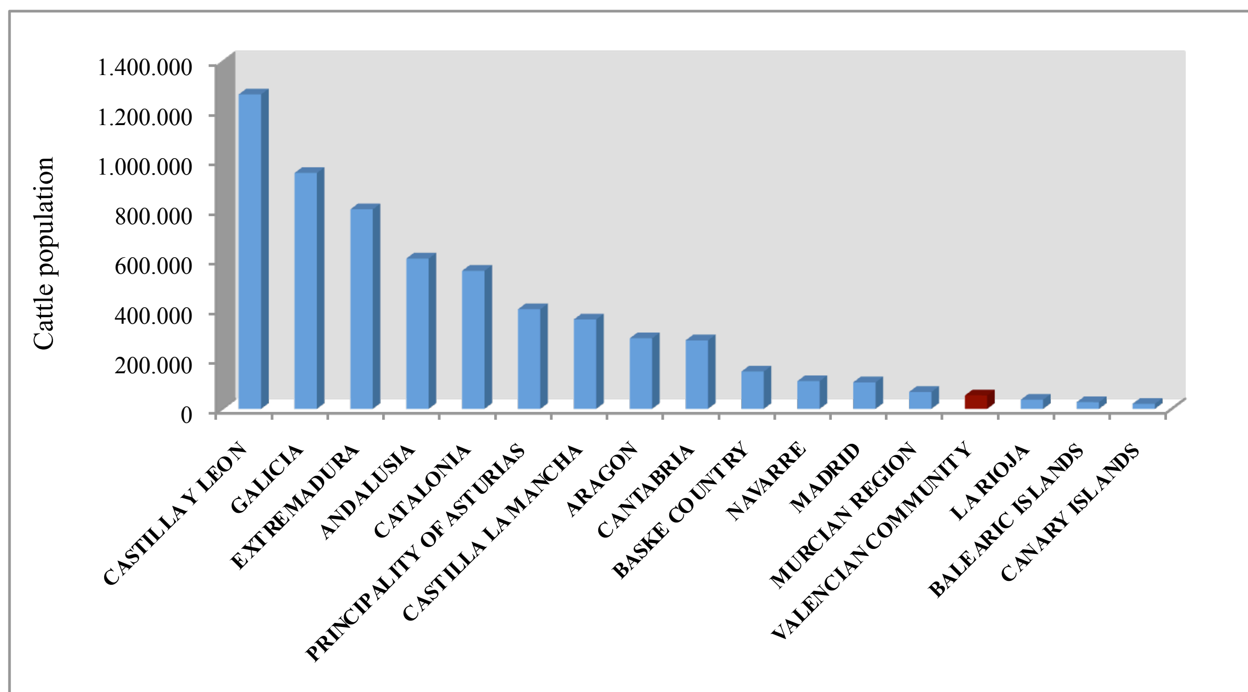
1.2. Spain

In 2010, the bovine industry accounted for approximately 32% of the total livestock production in Spain (Ministerio de Medio Ambiente y Medio Rural y Marino, 2011). In addition, this industry is considered the second more important animal production sector, behind the porcine. Indeed, the Spanish beef and dairy cattle industry generated 2,181 and 2,117 millions of

euros in 2010, respectively. In 2011, Spain produced 604,112 tones of beef (11% of the total meat production) and 6,299 millions of liters of cow milk (87% of the total milk production), which is a significant contribution for the food and agricultural sector (Ministerio de Medio Ambiente y Medio Rural y Marino, 2011).

In Spain, it was confirmed the existence of more than six million cattle. Castilla-Leon was the first region in number of bovine heads, followed by Galicia and Extremadura (21, 16, and 13% respectively, over the total output, Figure 3) (Ministerio de Medio Ambiente y Medio Rural y Marino, 2010a).

Figure 3. Total of cattle population in the Autonomous Communities of Spain.



Source: Ministerio de Medio Ambiente y Medio Rural y Marino, 2010a.

Geographically, farms of dairy cattle are mostly situated in the Northwest of Spain. In fact, the regions of Galicia, Castilla-Leon, and Asturias are among the largest milk producers of the country (38, 14, and 9% respectively, over the total output of Spain) (Ministerio de Medio Ambiente y Medio Rural y Marino, 2010b). On the other hand, beef cattle sector in Spain presents two differentiated structures production: the suckled cows and the beef cattle. The subsector of the suckled cows is mainly localized in the North and South-West of Spain. Furthermore, beef cattle are localized near the places of cereal production and beef consumption (Madrid, Catalonia, Aragon, Castilla-Leon, Castilla-La Mancha), which have the most important slaughterhouses.

1.3. Community of Valencia

In 2011, the Valencia Community produced 51,238 tones of cow milk and 25,218 tones of beef, representing the 79% and the 6.6% of the total milk and meat production, respectively, of this region (Conselleria de Agricultura, Pesca, Alimentacion y Agua, Comunidad Valenciana, 2011). Focusing in the cattle distribution, approximately half of the total heads were located in Valencia province, followed by Castellon and Alicante in 2010 (50, 40, and 10%, respectively) (Ministerio de Medio Ambiente y Medio Rural y Marino, 2010a).

2. Ovum pick up -*In vitro* fertilization: characteristics, current status and progress

2.1. Characteristics of the Ovum pick up -*In vitro* fertilization technology: historical perspective and application

In vitro production of embryos (IVPE) in domestic animals is applied with the aim to maximize genetic improvement, increase fertility and optimize genetic breeding schemes. Moreover, bovine *in vitro* fertilization (IVF) has been used for the acquirement of in-depth knowledge of the molecular mechanisms of the *in vivo* reproduction processes, for studying interaction gametes and embryos, and for allowing the manipulation of early development in farm animals (Hansen, 2006; Galli and Lazzari, 2008). Furthermore, IVPE has allowed the development of other technologies such us gamete/embryo cryopreservation.

The rabbit was the first mammalian species in which live offspring was produced by IVF (Chang, 1959). Since this data, IVF has been applied progressively to a large amount of species (Bavister *et al.,* 2002). The ultrasound-guided transvaginal oocyte aspiration or ovum pick up (OPU) was first developed in humans and then adapted in bovine species (Pieterse *et al*., 1991). The OPU technology provides immature oocytes that through the IVPE processes (*in vitro* oocyte maturation, fertilization, and embryo culture) develop into blastocysts that could either be transferred into a recipient or frozen.

The technology of OPU-IVF meant a new way to produce embryos *in vitro* and offered several advantages to optimized donors reproductive potential in comparison to conventional hormonal superovulation treatment and *in vivo* embryo recovery (MOET; multiovulation and embryo transfer). Eleven years ago, Galli *et al*. (2001) extensively reviewed the advantages of OPU-IVF compared to MOET; higher number of embryos could be produced and from almost

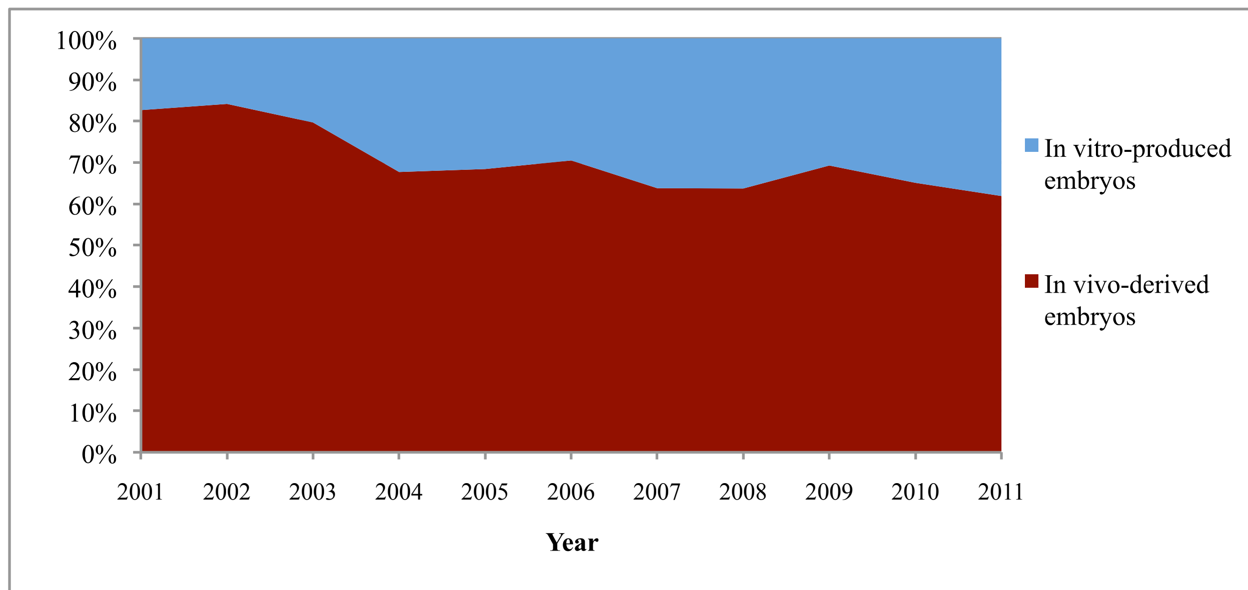
every female, regardless of their physiological status. In addition, oocytes could be retrieved since two-months-old calves to adult cows, giving a chance to shorten the generation interval if embryos are produced from juvenile or pre-pubertal heifers. This versatility, together with the establishment of safe and repeatable IVPE technique in the last years, turned OPU-IVF into a routine procedure in assisted bovine reproduction and in breeding-improvement schemes.

Moreover, OPU technology has no adverse impact on cattle welfare or fertility (Chastant-Maillard *et al*., 2003). Quite the opposite, OPU showed even a therapeutic application as certain infertility cases could be treated through this technique (Bols *et al*., 1997; Hasler *et al*., 1995). Ovum pick up technology has been successfully applied in other large species such as buffalo (Boni *et al*., 1996) and horses (Galli *et al*., 2007). As in the case of bovine, in buffalo species, OPU in combination with IVF has produced more transferable embryos than MOET (2 vs. 0.6 embryos per month, respectively) (Gasparrini, 2002).

2.2. Current status of *in vitro* production of embryo in cattle

In 2010, one triad of the worldwide bovine embryos produced, approximately 451,000, were *in vitro*-produced, while the number of those transferred was nearly 340,000 (Stroud, 2011). Compared to the 42,000 *in vitro*-produced embryos transferred ten years ago, the progress in IVPE technologies is considerably driving cattle production worldwide (Figure 4) (http://www.iets.org, accessed 24/02/2012). Brazil has been the clear global leader of the IVPE during the recent years; since 2005, this country is producing approximately half or more of the *in vitro*-produced bovine embryos around the world. In the case of the last data published by the International Embryo Transfer Society (IETS), in 2010, Brazil produced and transferred 59% and 77%, respectively, of the *in vitro*-produced embryos worldwide.

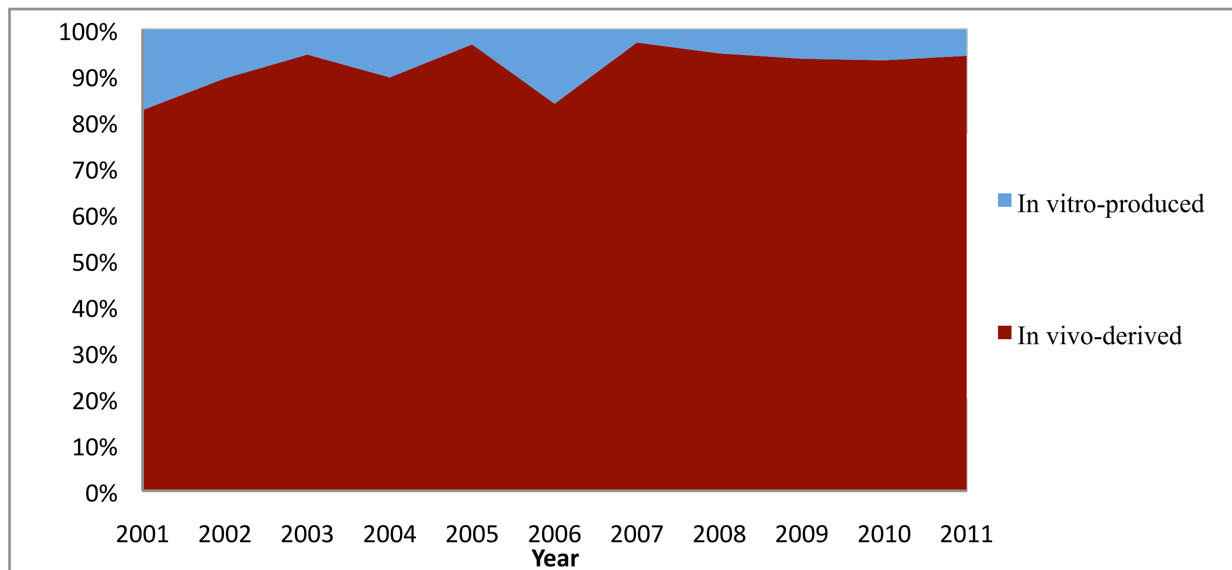
Figure 4. Percentage of transferrable *in vitro*-produced and *in vivo*-derived bovine embryos collected/derived worldwide (vertical axis) from 2000 to 2010.



Source: International Embryo Transfer Society, http://www.iets.org.

Regarding the embryo activity reported in Europe, the distance between the number of *in vivo*-derived and *in vitro*-produced embryos is still large than in worldwide. It seems that since 2007 the number of transferrable *in vitro*-produced bovine embryos remained stable from 7,000 to 8,000, while those transferred ranged from 5,600 to 5,800 (Figure 5) (http://www.aete.eu, accessed 24/02/2012). Regarding the country distribution, the Netherlands topped both list of transferrable and transferred *in vitro*-produced bovine embryos, with approximately a half of the activity in the recent years, and followed by Germany. In this regard, Spain did not report any activity of *in vitro*-produced bovine embryos since 2007, although certanly, based on studies published in the topic by Spanish teams during last years, *in vitro*-produced bovine embryos were obtanied but not reported to the European Embryo Transfer Association (AETE). On the other hand, in 2010, over 2,500 *in vivo*-derived bovine embryos were reported in Spain to the AETE.

Figure 5. Percentage of transferrable *in vitro*-produced and *in vivo*-derived bovine embryos collected/derived in Europe (vertical axis) from 2000 to 2010.



Source: Association Europeenne de Transfert Embryonnaire*, http://www.aete.eu.*

Regarding the economic cost to produce one embryo, *in vitro*-produced embryo derived from OPU oocytes could cost approximately two (*Bos taurus)* or one-and-a-half-fold (*Bos indicus)* higher than *in vivo*-derived embryos produced by MOET (van Wagtendonk-de Leeuw, 2006; Pontes *et al*., 2009).

2.3. Progress achieved

2.3.1.In OPU technology

The OPU technology allows the possibility of exploiting the large pool of growing oocytes in the ovaries. Since the first report describing the OPU technique in bovine species twenty years ago (Pieterse *et al*., 1991), enhancing the number and the quality of the recovered oocytes has been the motivation of researchers in reproductive biology and animal production (Merton *et al*., 2003; van Wagtendonk-de Leeuw, 2006). Thus, a large number of scientific papers have been published focused on methods for improving OPU process efficiency and

mainly, they have been focused on the factors affecting the future competence and recovery rate of the oocytes.

Regarding to the mechanical factors affecting the final efficiency of the OPU-IVF technology (in terms of embryos produced per OPU session), vacuum pressure, needle thickness, and bevel angle have shown a significant impact (Bols *et al*., 1997; Fry *et al*., 1997). One of the most considerable advances achieved in this regard was the design of an OPU system with dispensable needle, cheaper, easily replaceable, and sterile than the original 50 cm stainless steel needle (Bols *et al*., 1995). Furthermore, the influence of the biological factors on the OPU-IVF technology has widely been studied in the last years. On this subject, the estrous stage of the donor, the interval between OPU sessions (Merton *et al*., 2003; Chaubal *et al*., 2006), the use of a hormonal pre-treatment (Lacaze *et al*., 1997; Sirard *et al*., 1999; De Roover *et al*., 2005) and the donor productive status (Kendrick *et al.,* 1999) have shown to affect the quantity and quality of the oocytes recovered by OPU. Intrinsic oocyte developmental competence has been associated with the stage of follicular wave and the size of the antral follicle (Lonergan *et al*., 1994; Hagemann *et al*., 1999). However, when OPU session is performed in a repetitive way every three or four days, the quality of the cumulus-oocyte-complexes (COCs) recovered remains high and quite homogeneous between sessions. The explanation could be related with the induction of a new follicular wave in the ovary through each OPU session (Merton *et al*., 2003), which has shown to be favourable for the oocyte competence (Merton *et al*., 2003).

On the other hand, some authors have studied the effect of different hormonal treatments prior to OPU session with the aim to induce a superstimulatory response in the donor ovary that would increase the follicle and oocyte recovery number. These ones included treatment with GnRH (Bordignon *et al*., 1997), eCG (Vos *et al*., 1994 ; van de Leemput *et al*., 1999), FSH (Bousquet *et al*., 1999; Sirard *et al*., 1999; van de Leemput *et al*., 1999), gonadotropin plus BST

(Hwang *et al*., 1997) and anti-inhibin immunisation (Konishi *et al*., 1996). However, in spite the advances introduced in the super-stimulation protocol, as the "coasting period" between FSH administration and OPU (Sirard *et al*., 1999), the results of these procedures did not show always a true improvement to justify the economic cost increase subsequently to the treatment (Merton *et al*., 2003; De Roover *et al*., 2005). Therefore, hormonal treatment prior to OPU session is a questionable option to improve OPU-IVF efficiency so far, besides to introduce a problem solved by the OPU technology; the large variability in the super-ovulatory response to gonadotropin treatments (Mapletoft *et al*., 2002).

2.3.2. In IVF technology

During the 90s and the last decade, the efforts of the embryologists were addressed mainly to increase the efficiency of the IVPE. In this line, the use of the semi-defined medium, used by Tervit *et al*. (1972) forty years ago, instead of co-culture cells and serum during *in vitro* culture of bovine embryos, improved the health of *in vitro*-produced calves born (van Wagtendonk-de Leeuw *et al*., 2000) and reduced considerably the incidence of the Large Offspring Syndrome (LOS) in sheep and cattle species following transfer of *in vitro*-produced embryos (Young *et al*., 1998). The LOS has been characterized by a multitude of pathologic changes, of which extended gestation length and increased birth weight are predominant features, and has been described in detail in sheep and cattle species (Lonergan and Fair, 2008).

The availability of new methods to evaluate the embryo quality (Hardy, 1999; Maddox-Hyttell *et al*., 2003; Wrenzycki *et al*., 2005) and the possibility of comparing *in vitro*-produced and *in vivo*-derived embryos (Lonergan *et al*., 2006; Rizos *et al*., 2008) have helped to understand the weaknesses of the *in vitro*-produced embryos and hence optimized their production. In addition, the development of the sex-sorted sperm technology has allowed the

production of sexed embryos by IVF that have had obvious advantages for breeding and enhance the final efficiency of the process (Rath *et al*., 2009). In this regard, a combination with different biotechnologies has been suggested in order to improve these efficiencies and to develop the commercial opportunities for sex-sorted sperm (Rath and Johnson, 2008).

Despite these advances, *in vitro*-produced embryos still show lower competence to develop into offspring than those *in vivo*-derived (Lonergan and Fair, 2008). Furthermore, the IVPE is still inefficient, as less than 40% oocytes that are *in vitro* fertilized reach the blastocyst stage (Rizos *et al*., 2002). These reasons have maybe reduced the use of *in vitro*-produced blastocyst for embryo transfer and the dissemination of the OPU-IVF technique into the cattle industry, mainly in Europe. The culture of small groups of embryos from an individual egg donor is an issue that OPU-IVF laboratories have to face. However, this question will be addressed in the introduction sections of the Experiment 2 and Experiment 3.

The combined use of OPU-IVF with other technologies would be important to satisfy the needs of genetic selection and comercial production in Europe and in the world, and would reinforce the use of *in vitro*-produced embryos. The fertility improvement of sex-sorted sperm, as well as the survival increase of oocytes and embryos after cryopreservation, would spread the application of several embryo based reproductive technologies.

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OBJECTIVES

The overall objective of this work was to optimize the *in vitro* production of embryos (IVPE) technology in cattle, conditioned by the specific characteristics and deficiencies of the IVPE when OPU oocytes are used. To this end, several more specific objectives were proposed:

1. To evaluate the effect of the bovine oviductal fluid on *in vitro* fertilization (IVF) parameters, embryo development and blastocyst quality, as assessed by morphological embryo quality and relative transcript abundance of several developmental genes in blastocysts (Experiment 1).

2. To study the effect of the number of oocytes/embryos in the IVPE as well their specific requirements when reduced number of embryos are in culture:
    a. Identifying the steps of the IVPE process at which the culture of a single or low number of oocytes/embryos could impair embryo development and blastocyst quality (Experiment 2).
    b. Testing whether the development and quality of the preimplantation embryos *in vitro* cultured with low number (5 embryos) can be improved by the addition of epidermal growth factor, insulin, transferrin, and selenium (EGF-ITS) or by the WOW system (Experiment 3).

3. To evaluate the effects of different concentrations of melatonin on embryo development and quality, after oocytes were submitted or not to heat stress treatment (Experiment 4).

4. To study the effects of sex-sorted spermatozoa on the IVF of OPU-derived oocytes and their further embryo development and quality (Experiment 5).

EXPERIMENTS

Experiment 1:

Effect of the Bovine Oviductal Fluid on *In Vitro* Fertilization, Development, and Gene Expression of *In Vitro*-Produced Bovine Blastocysts*

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Abstract

Oviductal microenvironment generally provides better conditions for early embryo development than the conventional *in vitro* system. In an attempt to simulate the oviduct conditions or the main potentially influencing factors, the effect was studied of a bovine oviductal fluid (bOF) treatment applied prior to *in vitro* fertilization (IVF) on 1) IVF parameters, 2) cleavage rate, 3) blastocyst yield, and 4) blastocyst quality. Embryo quality was assessed by morphological embryo quality and relative transcript abundance of several developmental genes in bovine blastocysts. Furthermore, in order to study the effect of bOF without the male effect and zona-sperm interaction, artificially activated metaphase II oocytes were also treated with bOF. *In vitro* matured bovine oocytes from abattoir ovaries were treated or untreated with bOF for 30 minutes and then washed prior to IVF or activation. Subsequently, *in vitro* fertilized and parthenogenetic embryos were *in vitro* cultured for seven to eight days. The bOF treatment had no effect on fertilization parameters, cleavage, blastocyst rates both on parthenogenetic and IVF bovine embryos and neither on morphological quality of IVF blastocysts. *G6PD* and *SOD2* genes from IVF blastocysts showed significant changes in their expression after a bOF treatment. Significant differences were found for the expression of *SCL2A1, GPX1*, *BAX*, *AKR1B1,* and *PLAC8* genes between excellent or good blastocysts (Grade 1) and fair blastocysts (Grade 2). To our knowledge, this is the first study that evaluates the effect of bOF oocyte treatment on fertilization parameters, development, and quality of bovine embryos.

Introduction

The approach of the *in vitro* production of embryo system to the physiological conditions should result in an improvement in the blastocyst quality and development rates. In particular, the oviduct, where a part of oocyte maturation, capacitation of spermatozoa, fertilization, and the first stages of embryonic development take place, has been described as decisive for proper

physiological gamete and early embryo development (Hunter, 1998). The oviductal microenvironment, even using oviducts from different mammal species, generally provides better conditions for early embryo development than conventional *in vitro* systems (reviewed by Rizos *et al.,* 2010). Nevertheless, *in vivo* temporary embryo culture in oviduct is not practical for welfare reasons as two laparotomies are required, along with the technical complexities that it entails.

Oviductal fluid (OF) composition is very complex. It is formed by numerous metabolites such as growth factors, hormones, proteases and inhibitors, antioxidants, defence agents, glycosidases and glycosyltransferases, glycosaminoglycans and proteoglycans, chaperones, and heat shock proteins (reviewed by Aviles *et al.,* 2010). It is known that OF growth factors could contribute to a more efficient embryo development (reviewed by Diaz-Cueto and Gerton, 2001, Hardy and Spanos. 2002) and that OF antioxidants could protect the gametes and embryos against reactive oxygen species (ROS) present in the microenvironment (reviewed by Guerin *et al.,* 2001).

Regarding embryo culture, with the attempt to simulate the oviductal conditions, *in vitro* co-culture with oviductal cells has been reported as beneficial for the embryo development (Xu *et al*., 2004). Nevertheless, co-culture with cells has several problems, such as the methodological complexity, lack of repeatability, and biosanitary risk. The effects of the different OF components have been studied previously. For example, iC3b, derived from the complement protein C3, has been shown to stimulate embryo development (Lee *et al*., 2004). Oviduct-specific glycoprotein (OVGP1) increased monospermy rates in *in vitro* fertilized porcine oocytes while maintaining high penetration rates (Coy *et al*., 2008a) and exerted an embryotrophic effect in porcine embryos (Kouba *et al*., 2000; McCauley *et al*., 2003). Moreover, OVGP1 induced hardening of the zona pellucida (ZP), suggesting that OVGP1 modulates the interaction with spermatozoa and contributes to the regulation of polyspermy (Coy *et al*., 2008a).

In the same way, Lloyd *et al.* (2009) observed an improvement of the cleavage rate after short exposure to bOF prior to fertilization in porcine oocytes. Furthermore, in the latter study, production and quality of the *in vitro*-produced blastocysts were also improved, showing that bOF contained factors that could act positively on porcine oocytes, promoting embryo development and quality.

Analysis of gene expression is a useful tool to assess the normality of embryos and optimise the assisted reproduction technology (Wrenzycki *et al*., 2005). Lloyd *et al.* (2009) also showed that short treatment of porcine oocytes with bOF affected the gene expression of the blastocysts produced, promoting a better quality of embryos. Nevertheless, in cattle, no information was found about the effect of the OF on fertilization parameters and embryo development at once.

Therefore, the aim of this study was to examine the effect of the presence or absence of a bOF treatment applied prior to IVF on 1) IVF parameters, 2) cleavage rate, 3) blastocyst yield, and 4) blastocyst quality, as assessed by morphological embryo quality and relative transcript abundance of several developmental genes in bovine blastocysts.

Materials and Methods

Culture media

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich Quimica SA (Madrid, Spain). Handling medium (HM199) consisted of Hepes Medium 199 (M7528) supplemented with 7.4% foetal bovine serum (FBS; Invitrogen-10108-157, Alcobendas, Madrid, Spain). To avoid the formation of blood clots during oocyte collection, 2.2 mg/mL heparin (H9399) was added to HM199. Maturation medium (MM199) consisted of medium 199 (M4530) supplemented with 10% FBS, 10 ng/mL EGF (E1257), 0.1 UI/mL FSH and 0.1 UI/mL LH. The IVF medium was Fert-TALP (Parrish *et al*., 1988) with 10 μg/mL

heparin and without epinephrine, hypotaurine, and glucose. Culture medium (CM) used was modified synthetic oviductal fluid amino acids supplemented following Holm *et al*. (1999) recommendations with some modifications (mSOFaaci): 4.2 mM sodium lactate (L4263), 0.73 mM sodium pyruvate (P4562), 30 µL/mL BME amino acids (B6766), 10 µL/mL MEM amino acids (M7145), 1 µg/mL phenol-red (P0290), and 5% FBS. On day 5 of culture (day 0 = IVF), the medium was supplemented with FBS until a concentration of 10% was reached. Seventy-five µg/mL potassium penicillin G (P3032) and 50 µg/mL streptomycin sulphate (S6051) were added in all culture media as antibiotics. All culture media containing bicarbonate were covered with mineral oil (M8410) and equilibrated overnight in culture conditions (see below). Further *in vitro* maturation (IVM), IVF, and *in vitro* culture (IVC) were performed in four-well dishes with 500 µl of medium.

In vitro maturation

Cumulus–oocyte-complexes (COCs) were aspirated from 2 to 8 mm diameter follicles of slaughterhouse ovaries from heifers younger than one year-old. Oocytes with several layers of cumulus cells, morphologically bright and uniform cytoplasm were washed thrice in HM199 and *in vitro* matured for 22 to 24 h in MM199 in humidified atmosphere at 38.5 ºC and 5% CO_2 in air. Groups of approximately 50 COCs were matured per well.

Obtaining and processing bOF

Genital tracts from 14 to 20 month-old heifers (Charolais, Limousine and Simmental) were obtained at the abattoir and transported to the laboratory on ice. Once in the laboratory, the oestrous cycle stage (early follicular, late follicular, early luteal and late luteal) was assessed on the basis of ovarian morphology (Carrasco *et al*., 2008) and only those coming from animals at the late follicular phase were used because it has been demonstrated that this type of fluid consistently shows beneficial effects on porcine IVF (Coy et al. 2008a). Once classified, 25 bovine oviducts were separated from the tracts and quickly washed once with 0.4% v/v cetrimide

solution, twice in saline and transferred to Petri dishes on ice and dissected. Once dissected, the bOF from the whole oviduct was collected by aspiration with an automatic pipette using a tip for a maximum 200 µL volume as previously described (Carrasco *et al*., 2008). Bovine oviductal fluid was centrifuged (7000 × *g*, 10 min, 4ºC) to remove cellular debris and the supernatant immediately stored at -80 ºC until use. Frozen samples were lyophilized by freeze drying (ALPHA 1–2 LD plus, Christ, 6.59 kPa).

Bovine oviductal fluid treatment of oocytes

Lyophilised bOF was reconstituted with 800 µL of water (W3500). After IVM and before IVF or artificial activation, oocytes were incubated in reconstituted bOF for 30 min, in microdrops of 10 µL (10 oocytes/microdrop) covered with mineral oil in humidified atmosphere at 38.5 ºC and 5% CO_2 in air. Control group oocytes (without bOF treatment) were incubated in HM199 medium under the same conditions as above.

Evaluation of zona pellucida hardening after bOF treatment

In order to assess the hardening of the oocyte ZP after bOF treatment, a pronase test was performed using some oocytes per group in each experimental session. Briefly, *in vitro* matured decumulated oocytes were transferred into PBS, washed by pipetting, and introduced into 50 µL of 0.5% (w/v) pronase solution in PBS (Coy *et al*., 2002). Zona pellucida was continuously observed for dissolution under a stereomicroscope equipped with a warm plate at 37ºC. The dissolution time of the ZP in control oocytes was always around 3 min, whereas for treated oocytes, the dissolution time was approximately longer than one hour.

Artificial activation of oocytes

Furthermore, in order to study the effect of bOF without the male effect and zona-sperm interaction, parthenogenetic embryos were produced as follows. *In vitro* matured oocytes were denuded of cumulus cells with a hyaluronidase (H4272) treatment (1 mg/mL in HM199, exposure of no longer than 1 min) and gently pipetted with a stretched glass Pasteur pipette.

Oocytes showing one visible polar body under the stereomicroscope were considered as metaphase II (MII) oocytes. Then, these MII oocytes were artificially activated by exposure to 5 μM ionomycin (I0634) for 5 min, washed twice and incubated in 2 mM 6-dimethylaminopurine (D2629) for 3.5 h. After that, oocytes were washed and *in vitro* cultured for 8 days as detailed below in the IVC embryos conditions.

Sperm preparation and IVF

Two frozen semen straws of 0.25 mL from two bulls were thawed at 37 ºC in a water bath for 1 min and centrifuged for 20 min at 700 × *g* through a gradient of 2 mL of 45% Percoll and 2 mL of 90% Percoll, in 15 mL centrifuge tubes. The Percoll 90% was made according to the protocol described by Parrish *et al.* (1995). In order to prepare the Percoll 45%, the Percoll 90% was mixed 1:1 with HM199. The sperm pellet was isolated and washed in 5 mL of HM199 by centrifugation at 350 × *g* for 5 min. The remaining pellet was diluted with approximately 100 μL of HM199. Then, the sperm concentration was determined and a final concentration of 1×10^6 sperm/mL was adjusted for IVF. Oocytes were washed thrice in fertilization medium and after that were incubated with spermatozoa for 18 to 20 h in 5% CO_2 in air at 38.5 ºC.

Assessment of nuclear status of presumptive zygotes

After IVF, putative zygotes (see experimental design) were fixed and stained for 15 min with ethanol and Hoechst 33342 (25 μg/mL) (B2261) and mounted on glass slides. Mounted slides were examined under an epifluorescence microscope at X 400 and X 600 magnification and classified into four groups according to the morphological criteria in order to evaluate the fertilization process: 1) no fertilized oocytes (no pronuclear formation), 2) fertilized oocytes (formation of two pronuclei), 3) polyspermic fertilized oocytes (with more than two pronuclei) and 4) oocytes with other abnormal fertilization (oocytes with other nuclear structures).

In vitro culture of embryos

Presumptive *in vitro* fertilized zygotes were denuded from surrounding expanded cumulus cells in HM199 by repeated pipetting using a pulled glass Pasteur pipette, washed three times in CM and incubated at 38.5 ºC in a humidified atmosphere of 5% CO_2 and 5% O_2 in air. Culture was performed in groups from 20 to 30 and 30 to 40 for *in vitro* fertilized and parthenogenetic embryos, respectively. Cleavage was recorded at day 2, and blastocyst formation rate was recorded at day 7 and day 8, both under a stereomicroscope. *In vitro* fertilized blastocysts were classified according to their morphological quality as: grade 1 (G1), excellent or good blastocysts (code 1 on the International Embryo Transfer Society [IETS] morphological classification [Robertson and Nelson, 1998]) and grade 2 (G2), fair blastocysts (code 2 on the IETS morphological classification [Robertson and Nelson, 1998]). For the later gene expression analysis, four blastocysts groups were considered: G1 and G2 blastocysts untreated with bOF (CG1 and CG2, respectively) and G1 and G2 blastocysts treated with bOF (bOFG1 and bOFG2, respectively). Finally, blastocysts were washed with PBS, placed in PCR Eppendorf tubes, frozen by immersion in liquid nitrogen, and kept at –80ºC.

RNA extraction, reverse transcription (RT), and quantification of mRNA transcript abundance

We studied expression analysis of genes involved in glucose metabolism (*AKR1B1*), placenta formation (*PLAC8*), apoptosis (*BAX*), de novo methylation (*DNMT3A*), mitochondrial activity (*SOD2*), ROS detoxification (*GPX1*), metabolism (*SCL2A1*) and oxidative stress (OS) (*G6PD*). Molecular biology procedures were carried out as described previously (Bermejo-Alvarez *et al*., 2010b). Poly(A) RNA was extracted from pools of ten blastocysts following the manufacturer's instructions using the Dynabeads mRNA Direct Extraction KIT (Dynal Biotech, Oslo, Norway) with minor modifications: lysis was achieved in 100 µL lysis buffer for 10 min with occasional gently pipetting and then the fluid lysate was hybridised with 20 µL prewashed beads for 5 min

with gently shaking. After hybridisation, two washes in 100 µL washing buffer A and two more in washing buffer B were performed. Finally, beads were eluted in 30 µL Tris–HCl. Immediately after extraction, the RT reaction was carried out following the manufacturer's instructions (Bioline, Ecogen, Madrid, Spain) using poly(T) primer, random primers, and Moloney murine leukaemia virus reverse transcriptase enzyme in a total volume of 40 µL to prime the RT reaction to produce cDNA. Tubes were heated to 70ºC for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 100 units of reverse transcriptase. They were then incubated at 42ºC for 60 min to allow the RT of RNA, followed by 70ºC for 10 min to denature the RT enzyme. The quantification of all mRNA transcripts was carried by real-time quantitative RT (pRT)-polymerase chain reaction (PCR) in three independent experiments. Experiments were conducted to contrast relative levels of each transcript and histone *H2AFZ* (*H2AFZ*) in every sample (Table 1). Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 1. For quantification, qRT-PCR was performed as described previously (Bermejo-Alvarez *et al.*, 2010a). PCR conditions were tested to achieve efficiencies close to 1 and then the comparative cycle threshold (C_t) method was used to quantify expression levels. Quantification was normalised 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 C_t method, the DCT value was determined by subtracting the *H2AFZ* CT value for each sample from each gene C_t value of the sample. Calculation of $\Delta\Delta C_t$ involved using the highest sample ΔC_t value (i.e. the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔC_t sample values. Fold changes in

the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta Ct}$. The forward is the first primer in the Table 1 while the reverse is the second one.

Table 1. Details of primers used for real-time quantitative reverse transcription-polymerase chain reaction.

Gene	NCBI official name	Primer sequence (5–3´)	Fragment size (bp)	GenBank accession number
H2AFZ	*H2A histone family, member Z*	**AGGACGACTAGCCATG GACGTGTGCCACCA CCAGCAATTGTAGC CTTG**	209	NM_174809.2
AKR1B1	*Aldo-keto reductase family 1, member B1*	**CGTGATCCCCAAGTCA GTGAAATCCCTGTGGG AGGCACA**	152	M314631
PLAC8	*Placenta-specific 8*	**CGGTGTTCCAGAGGTT TTTCCAAGATGCCAGT CTGCCAGTCA**	166	NM_0010253 25.1
BAX	*BCL2-associated X protein*	**CTACTTTGCCAGCAAA CTGGTCCCAAAGTAGG AGAGGA**	158	NM_173894.1
DNMT3A	*DNA methyltransferase 3A*	**CTGGTGCTGAAGGACT TGGGCCAGAAGAAGG GGCGGTCATC**	317	AY271299
SOD2	*Superoxide dismutase 2, mitochondrial*	**GCTTACAGATTGCTGC TTGTAAGGTAATAAGC ATGCTCCC**	101	S67818.1
GPX1	*Glutathione peroxidase 1*	**GCAACCAGTTTGGGCA TCACTCGCACTTTTCG AAGAGCATA**	116	NM_174076.3
SCL2A1	*Solute carrier family 2 (facilitated glucose transporter), member 1*	**CTGATCCTGGGTCGCT TCATACGTACATGGGC ACAAAACCA**	168	NM_174602.2
G6PD	*Glucose-6-phosphate dehydrogenase*	**CGCTGGGACGGGGTG CCCTTCATCCGCCAGG CCTCCCGCAGTTCATC A**	347	XM_583628.4

Experimental design

To evaluate the effect of a short oocyte treatment with bOF prior to fertilization, several IVF and parthenogenetic activation sessions were carried out. After IVM, oocytes were treated for 30 min with bOF or non-treated, as described above, and divided into three groups:

1) In six replicates, 334 presumptive zygotes were fixed after IVF and nuclear status were assessed as described previously.

2) In eight replicates, 744 presumptive zygotes were *in vitro* cultured. Cleavage and blastocyst rates were recorded as embryo development parameters. Embryos reaching blastocyst stage were assessed with morphological quality parameters and used to analyse the relative mRNA expression of the genes above mentioned.

3) Two hundred and fifteen oocytes were artificially activated and *in vitro* cultured in three replicates. Cleavage and blastocyst rates were recorded as embryo development parameters.

Statistical analysis

Results of pronuclear formation, cleavage, blastocyst and morphological embryo quality rates were analysed using the chi-square test. $P<0.05$ was considered statistically different. Results of gene transcriptions were analysed using the SigmaStat (Jandel Scientific, San Rafael, CA, USA) software package. Relative mRNA abundance differences among groups were analysed by one-way ANOVA.

Results

Results of pronuclear formation after IVF are shown in Table 2. Oocytes treated with bOF did not show significant differences in normal fertilization, non-fertilization, polyspermic fertilization or other abnormal fertilization rates in comparison with the control group.

Table 2. Effect of bovine oviductal fluid (bOF) on IVF parameters of bovine oocytes.

Treatment	Replicates	Total number of oocytes	Percentage of non fertilization[2]	Percentage of normal fertilization[3]	Percentage of polyspermic fertilization[4]	Percentage of other abnormal fertilization[5]
Control	6	167	9.58	70.06	14.37	5.99
bOF[1]	6	167	9.58	75.45	11.98	2.99

[1]Treatment prior to fertilization, 30 min of incubation.[2]Oocytes without pronuclear formation. [3]Oocytes with two pronuclei formation. [4]Oocytes with more than two pronuclei formation. [5]Oocytes with other nuclear structures).

Regarding embryo development (Table 3), no differences were found between control and bOF groups in cleavage or blastocyst rates. The effect of bOF on the morphological classification of the blastocysts produced by IVF is shown in Table 4. No significant differences were found between control and bOF group in the morphological classification.

Table 3. Effect of bovine oviductal fluid (bOF) on the development rates of *in vitro* bovine embryos.

Treatment	Replicates	Total number of oocytes	Percentage of cleavage	Percentage of blastocyst from cleavage embryos
Control	8	369	80.49	34.68
bOF[1]	8	375	81.07	31.58

[1]Treatment prior to fertilization, 30 min of incubation.

Table 4. Effect of bovine oviductal fluid (bOF) on the morphological quality of the *in vitro* bovine blastocysts produced by IVF.

Treatment	Replicates	n	Percentage of grade 1 blastocyst[2]	Percentage of grade 2 blastocyst[3]
Control	7	77	61.04	38.96
bOF[1]	7	74	60.81	39.19

[1]Treatment prior to fertilization, 30 min of incubation.[2]Code 1 on the International Embryo Transfer Society (IETS) morphological classification, excellent or good blastocysts. [3]Code 2 on the IETS morphological classification, fair blastocysts. Robertson and Nelson (1998).

Table 5 shows the effects of bOF on parthenogenetic embryo development. Neither group, without (control) or with a bOF treatment (bOF), showed any differences in cleavage or blastocyst rates.

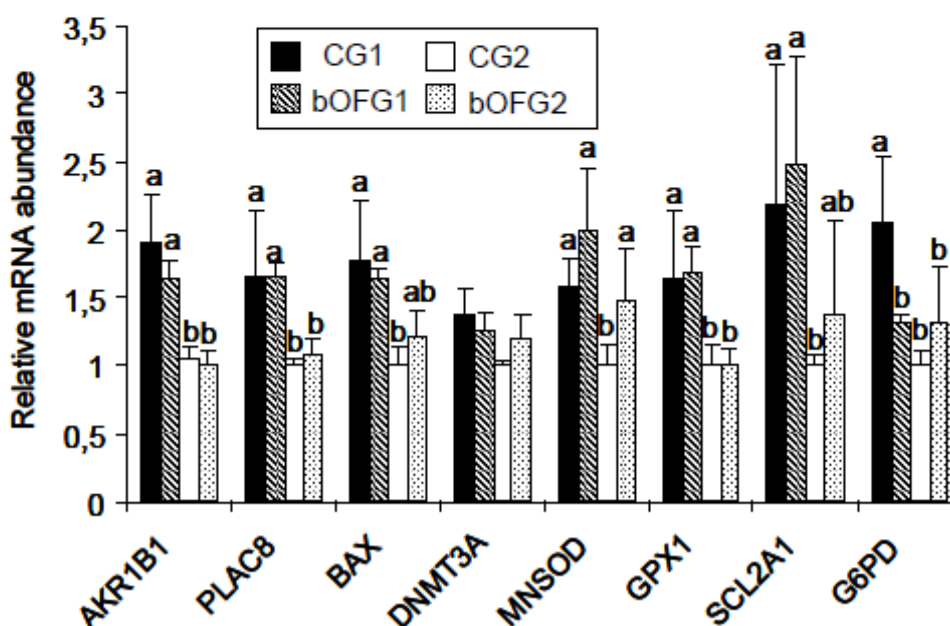
Table 5. Effect of bovine oviductal fluid (bOF) on the development rates of *in vitro* bovine parthenogenetic embryos.

Treatment	Replicates	Total number of oocytes	Percentage of cleavage	Percentage of blastocyst from cleavage embryos
Control	3	93	88.17	23.17
bOF[1]	3	122	84.43	19.42

[1]Treatment prior to activation, 30 min of incubation.

Figure 1 shows the relative mRNA expression of the genes above mentioned. No significant effect of bOF was found on gene expression in the G1 blastocysts, except for *G6PD* gene (P<0.05). Similarly, no significant effect of bOF was found on gene expression in the G2 blastocysts, except for *SOD2* gene (P<0.05). The genes *AKR1B1*, *PLAC8* and *GPX1* showed higher (P<0.05) abundance of transcript in G1 blastocysts than in G2 ones regardless of bOF treatment. *BAX* and *SCL2A1* showed a higher transcription (P<0.05) in the groups CG1 and bOFG1 than CG2, but not higher than bOFG2.

Figure 1. Effect of bovine oviductal fluid (bOF) on relative mRNA expression of eight genes in grade 1 and grade 2 *in vitro* bovine blastocysts produced by IVF.



CG1, Control Grade 1 blastocysts. **CG2**, Control Grade 2 blastocysts. **bOFG1**, bOF treatment Grade 1 blastocysts. **bOFG2**, bOF treatment Grade 2 blastocysts. Different letters (a, b) in each gene denote significant differences (P<0.05) among groups. Error bars in Figure 1 represent the SEM for the average $2^{-\Delta\Delta CT}$ values.

Discussion

The hardening of ZP subsequent to sperm fertilization is a widely studied process and has been considered an important event to avoid the penetration of additional spermatozoa (Ducibella *et al*., 1990; Vincent *et al*., 1990; Ducibella *et al*., 1993). However, between ovulation and fertilization, ZP of oocytes also undergoes hardening during its journey inside the oviduct (Coy *et al*., 2008b), and this fact has been less studied. The pre-fertilization ZP hardening, as described by Coy *et al.* (2008a), is produced by OVGP1 presents in the OF but it is absent during IVF in conventional *in vitro* systems (Coy *et al*., 2002; Coy *et al*., 2005). It has been suggested that the ZP hardening has a direct correlation with the polyspermy block (Coy *et al*., 2008b). To date, the environmental conditions of the oviduct have not been completely reproduced in *in vitro* systems. In an attempt to simulate these conditions or the main factors that could be influencing them, we proposed the study of the effect of bOF on bovine oocytes in IVF parameters, embryo development, blastocyst quality, and gene expression.

The bOF effect on the hardening of the oocyte ZP was verified through a pronase test (Coy *et al*., 2005) before IVF. So, the dissolution time of the ZP in control oocytes was around 3 min, whereas for treated oocytes, the dissolution time was usually longer than 1 hour. Despite this modification of the ZP characteristics in the present work, the bOF oocyte treatment did not affect fertilization. Also in bovine oocytes and after a bOF treatment similar to our study (for 30 min prior to IVF), Coy *et al.* (2008a) observed that the mean number of spermatozoa per oocyte as well as the number of spermatozoa bound to the ZP remained invariable. In contrast, in the same study, oocytes treated with bOF showed an increase in the percentage of monospermy, but it was reduced the percentage of penetration. Also, no significant effect was found for ampullary or isthmic fluids on IVF rate of bovine oocytes (Suriyasathaporn *et al*., 1997). In other study, but

in pig oocytes, the percentages of penetration and monospermy in bOF treated and untreated groups of oocytes were similar between them (bOF treatment for 30 min prior to IVF) (Lloyd *et al*., 2009). The method to collect the bOF in this study as well as in the Coy *et al.* (2008a) work were similar to ours.

It is well known that polyspermy in bovine zygotes after IVF is not as high as in pigs (Coy *et al*., 2008b; Canovas *et al*., 2009). Therefore, the role of the OF in IVF and sperm-oocyte interaction as well as the pre-fertilization hardening of ZP could become more important in porcine than in bovine species, probably due to different ways of blocking the polyspermy, apparently more dependent on the cortical granule exudates in bovine species (Coy *et al*., 2008b).

Concerning embryo development, the bOF oocyte pre-treatment did not affect preimplantational embryo development in our work. Incubation of bovine embryos in oviductal glycoproteins had no effect on blastocyst development rates (Hill *et al*., 1997; Vansteenbrugge *et al*., 1997) or only partially (at day 6, but not at day 7 of *in vitro* development) (Martus *et al*., 1997). However, other authors observed that the percentage of cleaved porcine embryos and blastocysts obtained from bOF-treated oocytes were higher than from untreated oocytes (Lloyd *et al*., 2009).

On the other hand, we studied the effect of bOF oocyte treatment, but without the male effect and zona-sperm interaction, using artificially activated MII oocytes (Gupta *et al*., 2008; Alfonso *et al*., 2009; Gomez *et al.,* 2009). In our case, bovine OF treatment on bovine parthenogenetic embryos showed no effect on cleavage or blastocyst rate. To our knowledge, no other work in the literature has studied this effect on parthenogenote oocytes to compare these results.

Regarding embryo quality, the treatment of bOF did not affect the morphological quality of the IVF blastocysts in our work. Anyway, it is convenient to note that a visual assessment of

embryos continues being a subjective evaluation and it is not an exact science as IETS indicated. In agreement with us, Lloyd *et al.* (2009) did not find differences with the bOF treatment in the morphological quality of the porcine blastocysts.

Concerning gene expression, no difference between G1 blastocysts (from oocytes treated or not with bOF) was found, except for *G6PD* gen in which the bOF treated group presented lower gene expression than control group, and at similar levels to G2 blastocysts. The gene *G6PD* is a potent indicator of the pentose phosphate pathway activity (Guerin *et al*., 2001), so consequently is a sentinel for ROS, leading rapidly to the generation of NADPH for maintenance of the cellular redox state (Lonergan *et al*., 2003). Oviductal proteins such as superoxide dismutase, glutathione reductase, and thioredoxin families act for controlling ROS generation and are regulated by gametes in the oviduct (Georgiou *et al*., 2005), therefore bOF could play an antioxidant role. Lopes *et al.* (2007) observed that IVF cattle embryos with poor quality showed a low expression of *G6PD* gene, although only the joint effect of embryo morphological quality and stage of development statistically significantly affected the gene expression. However, this link between embryo quality and gene expression is not evident, since other authors observed a significantly higher *G6PD* expression in *in vitro*-produced blastocysts than in others cultured *in vivo* in ewe oviduct (Lonergan *et al*., 2003) or obtained *in vivo* (Wrenzycki *et al*., 2002; Balasubramanian *et al*., 2007). Moreover, the expression of this gene can also be influenced *in vitro* by other factors such as sex of embryo, origin of embryo (nuclear transfer, parthenogenetic or IVF) or respiration rate (Gutierrez-Adan *et al*., 2000; Wrenzycki *et al*., 2002; Lopes *et al*., 2007).

In G2 blastocysts, there was no difference in gene expression between groups treated or not with bOF, except for *SOD2* gene, in which the embryos previously treated with bOF showed a higher expression than non-bOF treated blastocysts, reaching levels similar to G1 embryos. The transcription of *SOD2* is performed by cells to neutralise the ROS and is located in the

matrix of the mitochondria (Holley *et al*., 2010). Therefore, expression of *SOD2* in embryos could be indicative of mitochondrial activity (Rizos *et al*., 2002) and subsequently to good quality embryos (Ramalho-Santos *et al*., 2009). This would be consistent with the results of Lloyd *et al.* (2009), who suggested lower mitochondrial activity in the untreated oocytes in comparison with bOF treated oocytes. Furthermore, the expression of this gene in bovine embryos obtained *in vivo* was higher than *in vitro*-produced embryos (Rizos *et al*., 2002). In this way, Lonergan *et al* . (2003) observed that bovine blastocysts at day 7 *in vivo* cultured in ewe oviduct showed a higher level expression of this gene than those *in vitro* cultured. Moreover, in a study of embryo culture media, bovine blastocysts cultured in medium with the highest embryo survival rates after vitrification (higher quality) showed a significant increase in the gene expression of *SOD2* (Rizos *et al*., 2004). However, it seems that the relationship between gene expression and embryo quality change with the embryo stage, since several authors observed a higher gene expression of *SOD2* at day 3 in bovine embryos *in vitro* cultured than in *in vivo* (ewe oviduct) cultured (Lonergan *et al*., 2003; Gutierrez-Adan *et al*., 2004).

Observing the genetic expression of embryos with different morphological grades, in the control group blastocysts from G1 and G2 showed different genetic expression in the majority of the studied genes (seven of the eight genes). In contrast, in the bOF group, blastocysts from G1 and G2 were only different in the expression of three genes. In the work of Lloyd *et al.* (2009), differences in gene expression between good quality and poor quality bOF treated embryos were found in six of the eight genes analysed. However, in the control groups all the gene expression was different between good- and poor-embryos. Lloyd *et al*. (2009) hypothesized that the ZP modifications, subsequent to bOF treatment, lead to select the better sperm to fertilized oocytes in comparison with the untreated oocytes. In this regard, Suriyasathaporn *et al*. (1997) observed that the proportion of abnormally fertilized bovine oocytes was lower in presence of isthmic fluid than in the controls. In our experiment, it is possible that bOF mitigate some lacks on the

poor quality blastocysts. Lloyd *et al.* (2009) suggested that low-quality blastocysts would be more susceptible to being improved by the bOF effect because they are more sensitive to the stresses imposed by the IVC conditions.

On the other hand, also it could occur that bOF effects were shown after blastocyst stage. In this regard, Libik *et al.* (2002) did not obtain differences neither in penetration nor monospermy rates in IVM sheep oocytes after a long exposure to ovine OF (during the final period of the oocyte maturation process) but obtained a higher pregnancy rate those embryos previously treated. In this study, OF were collected with permanent canullae inserted to the oviduct of adult sheep during the breeding season. We have not evaluated the pregnancy rates in the present study.

To our knowledge, this is the first study that evaluates the effect of bOF oocyte treatment on fertilization parameters, development and quality of bovine embryos. In conclusion, we showed here that a short bOF oocyte treatment had no effect on fertilization parameters, cleavage, blastocyst rates both on parthenogenetic or IVF bovine embryos and either on morphological quality of IVF blastocysts. However, *G6PD* and *SOD2* genes from IVF blastocysts showed significant changes in their expression after a bOF treatment. Significant differences were also found for the expression of *SCL2A1, GPX1*, *BAX*, *AKR1B1* and *PLAC8* genes between excellent or good blastocysts and fair blastocysts.

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Experiment 2:

Effect of Number of Oocytes and Embryos on *In Vitro* Oocyte Maturation, Fertilization and Embryo Development in Bovine*

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Abstract

The aim of this study was to identify the *in vitro* development stage at which the culture of a single or low number (n = 5 or 10) of oocytes/embryos could impair development in comparison with culture in group (n = 50). In the Experiment 1, it was confirmed that single *in vitro* embryo production yielded lower cleavage and blastocyst rates than in group (49.4 vs. 83.0%; 0% vs. 37.8%, respectively; P < 0.05). In Experiment 2 and 3, it was observed no effect on embryo development of culturing single or low number of oocytes during maturation and fertilization, respectively. In Experiment 4, it was observed a detrimental effect on blastocyst rate when cultured single or low number of embryos during post-fertilization *in vitro* culture (2.9; 10.2-10.8; 33.2%, in single, low number of embryos [5-10], and control-grouped [50], respectively; P < 0.05). In Experiment 5, it was observed that the last part of the culture period (day 3 onwards) seemed to be more affected by the low number of embryos placed in culture. In conclusion, post-fertilization culture, especially on days 3 to 8 after fertilization, seems to be the most important stage for embryo development on single and/or low number (5-10) of embryos culture.

Resumen

Efecto del número de ovocitos y embriones en la maduración, fecundación y desarrollo *in vitro* en vacuno

El objetivo de este trabajo fue identificar la etapa del proceso de producción de embriones *in vitro* en la cual, el cultivo individualizado o con bajo número de oocitos/embriones (n = 5 ó 10) penaliza al desarrollo embrionario respecto del cultivo en grupo (n = 50). En el Experimento 1, se confirmó que la producción individualizada de embriones *in vitro* resulta en una menor tasa de división embrionaria y de blastocistos que las obtenidas con el cultivo en grupo (49.4 vs. 83.0%; 0% vs. 37.8%, respectivamente; P < 0.05). En los Experimentos 2 y 3, no

se observó efecto sobre el desarrollo embrionario del cultivo individualizado o de bajo número de embriones en las etapas de maduración y fecundación, respectivamente. En el Experimento 4, se observó un efecto negativo sobre la tasa de blastocistos durante el cultivo *in vitro* post-inseminación cuando se cultivaron los embriones individualmente o con bajo número (2.9; 10.2-10.8; 33.2%, en el cultivo individual, con bajo número de embriones [5-10], y grupo control [50], respectivamente; P < 0,05). En el Experimento 5, se observó que en la última parte del cultivo (día 3 post-inseminación en adelante) es donde más parece que se manifiesta el efecto negativo del cultivo con número reducido de embriones. Como conclusión, el cultivo post-inseminación, especialmente entre los días 3 y 8 de cultivo, parece ser la etapa más determinante del proceso de producción de embriones *in vitro* para el desarrollo embrionario en el cultivo individualizado y/o en número reducido de embriones (5-10).

Introduction

The *in vitro* production of embryos (IVPE) in cattle using single or low numbers of oocytes and embryos during culture is increasing among researchers linked to the production of embryos from oocytes collected by ovum pick-up (OPU) (see Carolan *et al.,* 1996; Vajta *et al.,* 2000; Ward *et al*., 2000; Vajta *et al*. 2008; Goovaerts *et al.,* 2009).

For experimental purposes in bovine, IVPE from oocytes collected from abattoir ovaries is in general quite efficient when a high number of oocytes are cultured together, although the quality of these *in vitro*-produced embryos continually lags behind that of embryos produced *in vivo* (Lonergan *et al.,* 2003). These *in vitro* systems usually culture approximately 40-50 oocytes/embryos in 400-500 µL of medium, obtaining blastocyst rates around 30-40% (Holm *et al.,* 1999; Gordon, 2003). However, for commercial purposes such as use in OPU, where it is usually necessary just to keep the immature oocytes from one donor together, a small number of oocytes/embryos can be cultured (approximately three to six oocytes recovered per non-

stimulated cow; Rizos *et al.,* 2005; Chaubal *et al.,* 2006). It has been widely reported that *in vitro* embryo development in bovine and other mammalian species tends to be suppressed in cultures with a single or low number of embryos (in mouse: Paria and Dey, 1990; Canseco *et al.,* 1992; Lane and Gardner, 1992; Kato and Tsunoda, 1994; in bovine: Palma *et al.,* 1992; Ferry *et al.,* 1994; Keefer *et al.,* 1994; Blondin and Sirard, 1995; Carolan *et al.,* 1996; Donnay *et al.,* 1997; O`Doherty *et al.,* 1997; Ward *et al*., 2000; Oyamada and Fukui, 2004; Pereira *et al.,* 2005; Fujita *et al.,* 2006). It seems that the culture systems developed to culture embryos in groups could be unsuitable and/or incomplete for individual embryo culture, and there must be some kind of limiting factor or condition in single embryo *in vitro* culture systems which remain still without knowing (Nagao *et al.,* 2008).

Even though several reports have addressed single embryo IVP with varying levels of success (Carolan *et al.,* 1996; Hagemann *et al.,* 1998; Oyamada *et al.,* 2004), there is still lack of repeatability and high variability in the results among laboratories.

The aim of this work was to identify the stage of the *in vitro* process at which single and low number of embryo development could be impaired, in order to be able to optimise and develop an effective bovine oocyte culture system. To this end, it was studied the effect of single embryo and/or small number embryo culture during the whole *in vitro* process (maturation, fertilization and culture), and also in each process separately, on the bovine embryo development and quality.

Material and methods

In vitro maturation

Cumulus–oocyte-complexes (COCs) were aspirated from 2-8 mm follicles of slaughterhouse ovaries from heifers, and washed three times in holding medium (HM199) based on Hepes TCM199 (Sigma M7528) supplemented with 7% foetal bovine serum (FBS; GIBCO®,

Invitrogen) and 75 µg mL^{-1} potassium penicillin G (P3032) and 50 µg mL^{-1} streptomycin sulphate (S6051) as antibiotics. COCs with more than three surrounding layers of cumulus cells and uniform cytoplasm were selected, washed three times in HM199, and *in vitro* matured (IVM) for 22-24 h in TCM199 medium (Sigma M4530) supplemented with 10% FBS, 10 ng mL^{-1} of epidermal growth factor (EGF), 0.1 IU mL^{-1} of recombinant human (rh)-FSH (Gonal-F 75, Serono, Madrid, Spain) and 0.1 IU mL^{-1} of rh-LH (Luveris, Serono). In the Experiment 2a (see below in experimental design), hormonal supplementation was not used. COCs were matured in humidified atmosphere at 38.5ºC and 5% CO_2 in air.

Sperm preparation, in vitro fertilization and artificial activation

Two straws with 0.25 mL of frozen semen from two bulls were thawed at 37ºC in a water bath for 1 min and centrifuged for 20 min at 700 × *g* through a Percoll gradient of 2 mL 90%, 2 mL 45% Percoll, in 15 mL centrifuge tubes. The Percoll 90 was made according to the protocol described by Parrish *et al.* (1995). To prepare the Percoll 45, the Percol 90 was mixed 1:1 with HM199. The sperm pellet was isolated and washed in 5 mL of HM199 by centrifugation at 350 × g for 5 min. Approximately 50 µL of semen pellet remained after the final centrifugation and was diluted with approximately 100 µL of HM199. Final concentration of 1×10^6 sperm mL^{-1} was used for *in vitro* fertilization (IVF). After IVM, COCs were washed three times in fertilization Fert-Talp medium (Parrish *et al.,* 1988) with 10 µg mL^{-1} of heparin (Sigma H9399), and co-incubated with the spermatozoa for 18-20 h in 5% CO_2 at 38.5ºC under mineral oil (Sigma M8410).

To study a complete effect on cytoplasmic maturation and to separate the sperm interaction, artificial activated oocytes were used. In order to obtain parthenogenetic embryos, *in vitro* matured oocytes were denuded of cumulus cells and oocytes with one visible polar body were considered as metaphase II (MII) oocytes. Then, the MII oocytes were activated by exposure to 5 µM ionomycin (Sigma I0634) for 5 min, washed twice, and incubated in 2 mM 6-

dimethylaminopurine (DMAP; Sigma D2629) for 3 h. After that, 6-DMAP was washed off, and the oocytes were *in vitro* cultured.

In vitro culture of embryos

Culture medium used was Synthetic Oviductal Fluid (Holm *et al.,* 1999) with the following modifications (mSOFaaci): 4.2 mM sodium lactate (Sigma L4263), 0.73 mM sodium pyruvate (Sigma P4562), 30 µL mL^{-1} BME amino acids (Sigma B6766), 10 µL mL^{-1} of MEM amino acids (Sigma M7145), 75 µg mL^{-1} penicillin G (Sigma P3032), 50 µg mL^{-1} streptomycin (Sigma S6501), 1 µg mL^{-1} phenol-red (Sigma P0290) and 5 % FBS.

After IVF or parthenogenetic activation, oocytes were washed three times in culture medium, and cultured at 38.5ºC in a humidified atmosphere with 5% CO_2 and 5% O_2, under mineral oil, for the *in vitro* culture (IVC). After IVF, oocytes were previously denuded from surrounding cumulus cells in HM199 before IVC. In Experiments 1 to 4, at day 5 of culture (day 0=IVF), IVC medium was supplemented with FBS to reach 10% concentration. In Experiment 5, this FBS supplementation was done at day 3, when changing of volume culture conditions was done (see below in experimental design), in order to reduce handling of embryos. Cleavage and blastocyst formation rates were recorded at day 2, and at day 7-8 of culture, respectively. Blastocysts were fixed and stained in ethanol with 25 µg mL^{-1} of bisbenzimide (Hoechst 33342, Sigma B2261), and the total number of cells was counted under an epifluorescence microscope.

Experimental design

Experimental design is summarised in Table 1. Experiment 1 was performed to study the effect of single embryo culture in the process of *in vitro* embryo production. Subsequently, a series of four experiments were carried out to determine in which step, or steps, of the *in vitro* embryo production the embryo viability was affected by single or low culture number (IVM, IVF, IVC, in Experiment 2, 3, 4 respectively). In the Experiment 2, *in vitro* matured oocytes were either *in*

vitro fertilised (Experiment 2a) or artificially activated (Experiment 2b). Finally, in Experiment 5, the effect of changing from low number embryo (n = 5) culture to control-grouped embryo culture (n = 50), and vice-versa, at day 3 of culture after fertilization, was studied. The same conditioned medium in which embryos were cultured previously was used.

Table 1. Experimental design.

Experiment	Treatments	Nºoocytes or embryos / vol. medium			
		IVM[1]	IVF[2]	IVC[3]	
Exp. 1	sIVP	1/ 10µL	1/ 10µL	1/ 10µL	
	gIVP	50/500µL	50/500µL	50/500µL	
Exp. 2	sIVM	1/10µL			
	r5IVM	5/50µL	50/500µL	50/500µL	
	r10IVM	10/100µL			
	gIVM	50/500µL			
Exp. 3	r5IVF	50/500µL	5/50µL	50/500µL	
	gIVF		50/500µL		
Exp. 4	sIVC	50/500µL	50/500µL	1/10µL	
	r5IVC			5/50µL	
	r10IVC			10/100µL	
	gIVC			50/500µL	
				Days 1-3	**Days 4-8**
Exp. 5	gIVC	50/500µL	50/500µL	50/500µL	50/500µL
	r5IVC			5/50µL	5/50µL
	r5IVC+gIVC			5/50µL	50/500µL
	gIVC+r5IVC			50/50µL	5/50µL

[1]IVM: *in vitro* maturation; [2]IVF: *in vitro* fertilization; [3]IVC: *in vitro* culture.

Statistical analysis

At least, three to six replicates were performed in each experiment. Results of maturation, cleavage, and blastocyst rates were analysed using the χ^2 test. When a single degree of freedom was involved, the Yates' correction for continuity was carried out. Results of blastocyst cell number were analysed using analysis of variance (ANOVA). A probability of $P < 0.05$ was considered to be statistically different.

Results

In Experiment 1 (Table 2), it was observed that when the whole embryo production process is performed maintaining individually maturation, fertilization and culture (sIVP), a significantly lower cleavage and blastocyst rates are yielded compared to grouped culture (gIVP).

Table 2. Single *in vitro* bovine embryo production (individual maturation, fertilization and culture) vs. group *in vitro* embryo production (Experiment 1).

Treatment[1]	No. COCs[2]	% Cleavage (No.)	% Blastocyst (No.)[3]	LSMean of Cells/Blastocyst (±SEM)
sIVP	164	49.4 (81)[a]	0 (0)[a]	_
gIVP	188	83.0 (156)[b]	37.8 (59)[b]	93 (±4.8)

[1]See Table 1. [2]COCs: cumulus–oocyte-complexes. [3]Percentage of blastocyst is calculated from cleaved embryos.

[a,b]: Different superscripts in the same column indicate a statistical difference ($P < 0.05$). No statistical analysis of blastocyst cells was performed.

In Experiment 2a (Table 3), no difference was observed among single maturation (sIVM), low number maturation (r5IVM, r10IVM) and control-grouped maturation (gIVM) in either nuclear maturation (ranging from 76.2 to 83.5%), cleavage rate (68.1-76.2%), blastocyst yield (30.2-46.5%) and blastocyst cell number (105-117 cells/blastocyst). In Experiment 2b (Table 3), using chemically activated parthenogenote embryos, no significant differences were observed among treatments in any of the characters studied: nuclear maturation (78.6-87.3%), rate of cleavage of MII oocytes after chemical activation (73.2-81.7%), rate of blastocyst from cleaved zygotes (20.9-25.4%) and blastocyst cell number (53-65 cells/blastocyst).

Table 3. Effect of different *in vitro* maturation (IVM) conditions, varying the number of oocytes, on *in vitro* development of a) *in vitro* fertilised bovine embryos (Experiment 2a) and b) parthenogenetic bovine embryos (Experiment 2b).

Treatment[1]	% MII[2] (No.)	No. COCs[3]	% Cleavage (No.)	% Blastocyst (No.)[4]	LSMean of Cells/Blastocyst (±SEM)
Experiment 2a					
sIVM	76.2 (59/77)	176	68.1 (120)	38.3 (46)	116 (±5.3)
r5IVM	77.3 (58/75)	190	73.2 (139)	30.2 (42)	117 (±5.6)
r10IVM	83.5 (66/79)	193	76.2 (147)	33.3 (49)	113 (±5.4)
gIVM	81.9 (95/116)	192	75.0 (144)	46.5 (67)	105 (±4.4)
Experiment 2b					
sIVM	78.6 (88)	112	81.7 (67/82)	20.9 (14)	55 (±4.3)
r5IVM	87.3 (76)	87	80.8 (59/73)	25.4 (15)	59 (±5.8)
r10IVM	84.7 (72)	85	73.2 (52/71)	25.0 (13)	65 (±6.7)
gIVM	86.6 (97)	112	79.5 (70/88)	24.3 (17)	53 (±5.1)

[1]See Table 1. [2]MII: metaphase II oocytes. [3]COCs: cumulus–oocyte-complexes. [4]Percentage of blastocyst is calculated from cleaved embryos.

In Experiment 3 (Table 4), no significant difference was observed in cleavage, blastocyst rate or number of cells per blastocyst, between low number fertilization (r5IVF) and control-grouped fertilization (gIVF).

In Experiment 4 (Table 4), it was observed that embryos cultured in control-grouped (gIVC) conditions showed significant higher cleavage rate than those cultured single (sIVC) or in low number n = 5 (r5IVC) conditions ($P < 0.05$), although no difference reached statistical significance when compared to low number n = 10 (r10IVC) conditions. Regarding blastocyst yield, sIVC embryos showed the lowest blastocyst rates (2.9%; $P < 0.05$), whereas gIVC embryos yielded the highest blastocyst rates (33.2%; $P < 0.05$). No differences on number of cells per blastocyst were observed.

In Experiment 5 (Table 4), it was again observed that culturing embryos in r5IVC resulted in a significant lower cleavage than culturing in gIVC, and gIVC resulted in a higher blastocyst yield than any other group tested ($P < 0.05$). When variation of the culture conditions took place, embryos cultured under low number (n=5) conditions from day 1 to day 3 and changed to control-grouped conditions day 4 onwards (r5IVC+gIVC) showed higher blastocyst yield and number of cells per blastocyst, than when variation of the culture density conditions was done vice versa (gIVC+r5IVC) ($P < 0.05$). Regarding the blastocyst quality, gIVC+r5IVC group showed a significant lower number of cells per blastocyst than the other experimental groups tested ($P < 0.05$).

Table 4. Effect of different *in vitro* culture conditions, varying the number of oocytes in fertilization (IVF) (Experiment 3), or zygotes in culture (IVC) (Experiment 4) and using conditioned medium in IVC (Experiment 5), on *in vitro* development of bovine embryos.

Treatment[1]	No. COCs[2]	% Cleavage (No.)	% Blastocyst (No.)[3]	LSMean of Cells/Blastocyst (±SEM)
Experiment 3				
r5IVF	120	75.8 (91)	30.8 (28)	86 (±6.1)
gIVF	153	85.6 (131)	29.7 (39)	82 (±2.7)
Experiment 4				
sIVC	292	80.0 (234)[a]	2.9 (7)[a]	69 (±25.5)
r5IVC	285	82.1 (234)[a]	10.2 (24)[b]	72 (±15.2)
r10IVC	310	83.5 (259)[ab]	10.8 (28)[b]	108 (±9.9)
gIVC	359	88.0 (316)[b]	33.2 (105)[c]	97 (±5.0)
Experiment 5				
gIVC	300	86.0 (258)[a]	29.4 (76)[a]	82 (±2.7)[a]
r5IVC	284	76.0 (216)[b]	6.0 (13)[b]	83 (±8.6)[a]
r5IVC + gIVC	298	79.5 (237)[b]	10.5 (25)[b]	83 (±3.2)[a]
gIVC + r5IVC	311	85.8 (267)[a]	1.8 (5)[c]	56 (±6.6)[b]

[1]See Table 1. [2]COCs: cumulus–oocyte-complexes. [3]Percentage of blastocyst is calculated from cleaved embryos.

[a,b]: Different superscripts in the same column indicate a statistical difference ($P < 0.05$).

Discussion

This work was planned to try to identify the main step of the *in vitro* process at which single or low number embryo development could be impaired.

In Experiment 1, it was observed that when the embryo production process is performed maintaining individually maturation, fertilization and culture, a significant lower cleavage and blastocyst rate was obtained, as it was observed in other works (Keefer *et al.,* 1994; Ward *et al.*, 2000; Fujita *et al.,* 2006).

Oocyte maturation environment has been demonstrated to be essential for high *in vitro* blastocyst yields (Sirard and Blondin, 1996; Jewgenow *et al.,* 1999; Rizos *et al.,* 2002). To investigate this, it was studied the effect of varying the number of oocytes placed in culture during IVM, but maintaining the ratio oocytes/volume of IVM medium, on their *in vitro* development of bovine embryos after IVF or parthenogenetic activation. Blastocyst yield after single, low number (n = 5-10) or control (n = 50) IVM was no significantly different. Results obtained here are in agreement with those reported by other authors (Carolan *et al.,* 1996; Ward *et al.*, 2000); however, it can be found some contradictory results in the bibliography, where it is also observed that single oocyte maturation could reduce the developmental capacity of oocytes compared with group maturation (Blondin and Sirard, 1995; Jewgenow *et al.,* 1999) even maintaining the same oocyte/volume ratio (1 oocyte in 10 μL *vs*. 10 oocytes in 100 μL) (Feng *et al.,* 2007). By other hand, it seems that developmental potential of oocytes following IVM, IVF and IVC could greatly depend on the presence and morphology of the surrounding cumulus cells and follicular environment (Araki *et al.,* 1998; Gordon, 2003; Feng *et al.,* 2007). Araki *et al.* (1998) observed that the tight layer of cumulus cells enhanced the developmental capacity of individual cultured oocytes, achieving higher rates of blastocyst when oocytes with

homogeneous ooplasm and surrounding with more than four layers of cumulus cells were individually cultured through IVM to IVC, compared to that with two to three layers. Furthermore, Feng *et al.* (2007) showed that blastocyst rates of bovine oocytes were highly correlated with the degree of cumulus expansion. In the present work, oocytes with more than three layers of surrounding cumulus cells and homogeneous cytoplasm were used, so it could be thought that the high intrinsic quality of oocytes used could explain the fact that individual IVM did not impair blastocyst yield during IVF and culture in group.

Regarding IVF process, it was observed that when five oocytes were fertilised in a drop of 50 µL (r5IVF), no significant effect was observed either in cleavage rate, blastocyst yield or blastocyst cells number compared with control-grouped fertilization when in the remaining steps (IVM, IVC) they were cultured in group. Although Carolan *et al.* (1996) observed that maintaining the control in-group oocyte/volume ratio for individual IVF (1 embryo in 10 µL) seriously compromised subsequent embryo development, these authors observed that individual oocytes could be successfully fertilised by increasing the volume of IVF to 50-100 µL, with no differences in terms of embryo development compared to in-group.

In the conditions of the present work, the post-fertilization culture conditions seem to be the most important step in the whole process of *in vitro* embryo production concerning to number of oocytes/embryos. This effect has also been observed by Ward *et al.* (2000), but furthermore, in the present work it was detected that the last part of embryo culture, day 3 to day 8, is the stage when is more noticeable the impairment of small number of embryos in culture on blastocyst formation.

It was observed that culture of embryos in control-grouped conditions resulted in a higher cleavage and blastocyst rate than individual or low embryo number ($n < 10$) culture conditions. Single embryo culture results obtained here are very low, but this level is also observed in other

works culturing embryos individually (sIVC), and even regrouped for the remaining steps (gIVM, gIVF) (Carolan *et al.,* 1996; Ward et *al.*, 2000; Goovaerts *et al.,* 2009). It is suggested in several animal species (mice, bovine, porcine) that suppression of early development of embryos cultured individually might be caused by a deficiency of cooperative interaction among embryos (Paria and Dey, 1990; Keefer *et al.,* 1994; Stokes *et al.,* 2005). It seems that several growth factors could act as possible embryotrophic factors reciprocally stimulating embryo development, in a paracrine/autocrine fashion, when embryos are cultured *in vitro* in groups (Paria and Dey, 1990; Palma and Brem, 1995; Thibodeaux *et al.,* 1995; Lim and Hansel, 1996; Stokes *et al.,* 2005; Contramaestre *et al.,* 2008). In addition, Nagao *et al.* (2008) concluded that the cooperative interaction among bovine early embryos during *in vitro* culture may be also mediated by the reduction of toxic factors, so at low embryo density, reduced oxygen tension or the exclusion of inorganic phosphate from the medium enhances blastocyst development. Moreover, volume of medium also seems to be an important factor for individual embryo culture. Very small volumes (< 10 µL) may accumulate toxic metabolites (Lane and Gardner, 1992; Carolan *et al.,* 1996), in contrast, larger volumes of medium or renewal of the media may dilute, or replace, autocrine embryotrophic factors (Paria and Dey, 1990). However, the mechanisms negatively affecting *in vitro* individual incubation of bovine embryos are still not understood in depth, as there is still high variability in the results among laboratories. The lower limit number of embryos from which reduced efficiency appears is not conclusive, and the comparison with other works is sometimes not easy since, apart from different culture conditions, the ratio oocyte/volume of medium may be changed even in the same work. In the present work, significant differences were observed when 10 embryos in 100 µL (1/10 oocyte μL^{-1}) were *in vitro* cultured. In this sense, Hoelker *et al.* (2009) detected significant differences in embryo development with 16 embryos (1/32 oocyte μL^{-1}) in comparison with the control

group (n = 50, 1/10 oocyte μL^{-1}). However, other authors (Fujita *et al.,* 2006) detected differences only when a lower number of embryos (n = 3, 1/5 oocyte μL^{-1}) were cultured.

Some studies have addressed the use of conditioned medium to investigate the potential beneficial effect of the growth factors secreted in the media, by embryos cultured in groups or cell co-culture, on the promotion of single embryo development (Fujita *et al.,* 2006; Goovaerts *et al.,* 2009). However, to our knowledge, not in the same way as tested in the present work, in which possible depletion, renewal or dilution of media was avoided, and only the effect of number of embryos could be studied. Here, it was tried to investigate the effect of changing from low number embryo culture to grouped embryo culture and vice-versa using the same conditioned media in which embryos were previously cultured, and maintaining the control embryo/volume of medium ratio. It was observed that the most deleterious change for embryo development, in terms of blastocyst formation, is when group-cultured embryos are placed in low number embryo culture conditions at day 3 and onwards. From the results obtained here, it could be summarised that the last part of embryo culture is likely to be a crucial step when deficiency of cooperative interaction among embryos could arise, impairing blastocyst formation. Although the comparison with porcine embryo development should be cautiously considered, since pig are multiparous while bovine is monoparous, in porcine embryos, it was observed that the beneficial effect of grouped embryo culture, at an optimum distance of 81-160 µm separation among embryos, rose at 48-96 h after insemination and became more evident towards the last part of the culture period, for 96-144 h which corresponded to day 5-6 of culture (Stokes *et al.,* 2005). These latter authors suggested that group culture confers a greater advantage on development post genome activation, and even more so at the morula and blastocyst stage. In bovine embryos, two stages seem to be crucial for embryo development: 8- to 16-cell stage, at days 2-3 of culture after fertilization, in which developmental arrest could

occur in the called “8-cell block” (Eyestone and First, 1991); and compaction and cavitation for blastocyst formation from morula, which occurs around the 32-cell stage approximately after day 5 of *IVC* (Van Soom *et al.,* 1997). It was shown in the literature that a serum supplementation to the culture medium at day 5 after fertilization increased the proportion of oocytes that developed to blastocyst (De Moraes and Hansen, 1997; Paula-Lopes *et al.,* 1998; Hagemann *et al.,* 1998), and as it is widely known, growth factors, hormones and other active substances are present in FBS (Gordon, 2003). Taking these data together, it seems likely that some important autocrine/paracrine growth factor(s) and/or other components secreted to the culture media by the embryos could become more important in later stages of culture.

It could be concluded that, in the conditions tested here, post-fertilization culture conditions, regarding number of embryos in culture, seem to be the most important step determining blastocyst yield. Culture of single and/or a low number of embryos (< 10 embryos) drastically impaired the efficiency of blastocyst yield. Moreover, it seems that the final part of the culture (day 3 to day 8) could be crucial for the manifestation of this deleterious effect with a low number of embryos. More research is needed to elucidate the factors negatively affecting individual and low number embryo *in vitro* culture, and to overcome its lower efficiency.

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Experiment 3:

Beneficial Effect of Two Culture Systems with Small Groups of Embryos on the Development and Quality of *In Vitro*-Produced Bovine Embryos*

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Summary

Currently, *in vitro*-produced embryos derived by ovum pick up (OPU) and *in vitro* fertilization (IVF) technologies represent approximately one third of the embryos worldwide in cattle. Nevertheless, the culture of small groups of embryos from an individual egg donor is an issue that OPU-IVF laboratories have to face. In this work, we tested whether the development and quality of the preimplantation embryos *in vitro* cultured in low numbers (five embryos) could be improved by the addition of epidermal growth factor, insulin, transferrin and selenium (EGF-ITS) or by the WOW system. With this aim, immature oocytes recovered from slaughtered heifers were *in vitro* matured and *in vitro* fertilized. Presumptive zygotes were then randomly cultured in four culture conditions: one large group (LG) (50 embryos/500 µL medium) and three smaller groups (five embryos/50 µL medium without [Control] or with EGF-ITS [EGF-ITS], and five embryos per microwell in the WOW system [WOW]). Embryos cultured in LG showed a greater ability to develop to blastocyst stage than embryos cultured in smaller groups, while the blastocyst rate of WOW group was significantly higher than in Control. The number of cells/blastocyst in LG was higher than Control or WOW, whereas the apoptosis rate per blastocyst was lower. On the other hand, the addition of EGF-ITS significantly improved both parameters compared to the Control and resulted in similar embryo quality to LG. In conclusion, the WOW system improved embryo development while the addition of EGF-ITS improved the embryo quality when smaller groups of embryos were cultured.

Introduction

Ovum pick up technology in combination with IVF is the most widely-used way to obtain *in vitro*-produced embryo from known donors in bovine as well as human species (van

Wagtendonk-de Leeuw, 2006). Indeed, in 2010 one third of the bovine embryos derived worldwide were *in vitro*-produced (Stroud, 2011).

Despite the several breakthroughs achieved in OPU technology over the last two decades, the average number of immature oocytes recovered per OPU session from an individual egg donor in *Bos taurus* or buffalo rarely exceeds eight (Gasparrini, 2002; Merton *et al*., 2003), which poses a problem for the IVF laboratory. *In vitro* culture (IVC) with small numbers of embryos may have a negative effect on the pregnancy rate after embryo transfer, as a lower embryo development, a lower number of cell per blastocyst, lower embryo viability and lower interferon-tau production have been reported by several authors when small groups of embryos were *in vitro* cultured (Canseco *et al*., 1992; Lane and Gardner, 1992; Palma *et al*., 1994; Ferry *et al*., 1994; Blondin and Sirard, 1995; Carolan *et al*., 1996; Larson and Kubisch, 1999; Oyamada and Fukui, 2004; Fujita *et al*., 2006; Salvador *et al*., 2011).

Several strategies have been developed in an effort to overcome the disadvantages of culturing small batches of embryos. Supplementation with different growth factors (GFs) – such as epidermal growth factor (EGF), insulin growth factor, transforming growth factor, platelet-derived growth factor, basic fibroblast growth factor – have been tested to this end in mouse (Paria and Dey, 1990; Brison and Schultz, 1997) and bovine (Carolan *et al*., 1996; Lim and Hansel, 2000; Mizushima and Fukui, 2001). In fact, GFs and other substances such as antioxidants are secreted by the maternal oviduct and have shown to maintain a proper embryo development (Aviles *et al*., 2010), so the external addition of these substances may act in a similar way during the embryo IVC (Diaz-Cueto and Gerton, 2001; Hardy and Spanos, 2002). Nevertheless, the results in this regard do not always agree and the use of GF and antioxidants in *in vitro* production of embryos (IVPE) when culturing small numbers of embryos is still an issue of study.

The reported mitogenic effect of EGF on preimplantation embryos makes it one of the most interesting and used GFs (Paria and Dey, 1990; Lonergan *et al*., 1996; Sirisathien and Brackett, 2003). Moreover, the combination of insulin, transferrin, and selenium (ITS) has shown to improve embryo development in rat (Zhang and Armstrong, 1990), bovine (Jaakma *et al*., 1997; Bowles and Lishman, 1998; Lim and Hansel, 2000; George *et al*., 2008; Cordova *et al*., 2010 in presence of acid ascorbic) and porcine (Jeong *et al.,* 2008). Together, EGF plus ITS have been used as supplements in defined medium to derived buffalo (Raghu *et al*., 2002) and rabbit (Carney and Foote, 1991) *in vitro*-produced embryos. Separately, insulin has shown a mitogenic and anti-apoptotic effect on the *in vitro* development of mammalian preimplantation embryos (Augustin *et al.*, 2003). Moreover, the positive action of transferrin is likely to prevent toxic hydroxyl radical production and their free-radical-mediated damage activity (Nasr-Esfahani and Johnson, 1992). In addition, it seems that transferrin acts as a GF (Sanders, 1986; Shapiro and Wagner, 1989) and helps transport iron into the cell (Alberts *et al*., 2004). On the other hand, selenium may prevent oxidative damage through glutathione peroxidase regulation (Margis *et al*., 2008).

Vajta and collaborators proposed an alternative approach to reduce the harmful effects of culture with small groups of embryos (Vajta *et al*,. 2000). In this system, called 'Well of the Well' (WOW), oocytes and embryos are cultured individually or in small groups in a handmade micro-well in the bottom of the 500 µL-well. The main advantage of this system is the combination of the benefits provided by the microenvironment culture – accumulation of autocrine and paracrine factors, because the volume of the WOW for culture is approximately 0.2 µL (Taka *et al*., 2005) – along with the benefits of a large volume culture – nutrition and dilution diffusion of metabolised toxic products (Bavister, 1995). However, few studies have

been focused on the effect of WOW system on embryo quality, particularly in apoptosis, and this was one of the goals of this work.

The main aim of an IVF laboratory is usually to produce high quality embryos for transfer; high quality embryos would better resist the cryopreservation process and be able to reach a higher pregnancy rate after embryo transfer than low quality embryos (Gardner *et al*., 2000; Gomez *et al*., 2008). Characteristic features of apoptosis, including cytoplasmic, nuclear and DNA fragmentation, appear to be linked with embryo arrest and abnormal development (Hardy, 1999), so detection of nuclear fragmentation in blastocysts is often used to provide information on the quality of *in vitro*-produced embryos (Schwarz *et al*., 2008). In addition, the average number of cells/blastocyst has been considered a rapid and simple assay to evaluate embryo quality (Knijn *et al*., 2003).

The aim of this work was to study whether the development and quality of *in vitro*-produced embryos cultured in small groups could be improved by the addition of a mixture of EGF plus ITS or by the WOW system.

Materials and methods

Culture media

Unless otherwise indicated, all chemical products were purchased from Sigma-Aldrich® Quimica SA (Madrid, Spain). Handling medium (HM199) consisted of Hepes Medium 199 (M7528) supplemented with 7.4% (v/v) of foetal bovine serum (Invitrogen®-10108-157) (FBS). Maturation medium (MM199) consisted of medium 199 (M4530) supplemented with 10% FBS and 10 ng/mL EGF (E9644). The IVF medium was the Fert-TALP (Parrish *et al.,* 1988) with 10 μg/mL of heparin (H9399) and without epinephrine, hypotaurine, and glucose. Culture medium (CM) used was modified synthetic oviductal fluid amino acids supplemented following recommendations by Holm *et al.* (1999) with some modifications (mSOFaaci) and previously

used in the laboratory (Salvador *et al.,* 2011): 4.2 mM sodium lactate (L4263), 0.73 mM sodium pyruvate (P4562), 30 µL/mL BME amino acids (B6766), 10 µL/mL MEM amino acids (M7145), 1 µg/mL phenol-red (P0290) and 3 mg/mL of BSA (Sigma-A7030). Seventy-five µg/mL potassium penicillin G (P3032) and 50 µg/mL streptomycin sulphate (S6051) were added to all culture media as antibiotics. All culture media containing bicarbonate were covered with mineral oil (M8410) and equilibrated overnight in culture conditions.

In vitro maturation

Cumulus–oocyte-complexes (COCs) were aspirated from 2 to 8 mm diameter follicles of slaughterhouse ovaries from heifers less than one year old. Oocytes with several layers of cumulus cells, morphologically bright and uniform cytoplasm were washed three times in HM199 and *in vitro* matured for 22 to 24 h in MM199 in humidified atmosphere at 38.5ºC and 5% CO_2 in air.

Sperm preparation and IVF

Three frozen semen straws of 0.25 mL from three bulls were thawed in a water bath at 37ºC for 1 min and centrifuged at 350 × *g* for 20 min through a gradient of BoviPure® Top and Bottom Layer (2 mL × 2) (Nidacon, Barcelon, Spain). The sperm pellet was isolated and washed in 10 mL of HM199 by centrifugation at 300 × *g* for 10 min. The remaining pellet was diluted with approximately 100 µL of HM199. Then, the sperm concentration was calculated and a final concentration of 1×10^6 sperm/mL was adjusted for IVF. Oocytes were washed thrice in fertilization medium and then incubated with spermatozoa for 18 to 20 h in 5% CO2 in humidified air at 38.5ºC.

In vitro culture

After IVF, presumptive zygotes were denuded in HM199 by repeated pipetting with a stretched glass Pasteur pipette, washed thrice in CM and incubated at 38.5 ºC, 5% CO_2 and 5% O_2 in

humidified air. Cleavage was evaluated on day 2 (day 0 =IVF) and blastocyst rate was evaluated at day 7, day 8 and day 9, under a stereomicroscope in both cases.

Production of WOW

Briefly, a steel rod was heated over a Bunsen burner for 4 to 6 s and gently pressed into the bottom of a Nunc® four-well dishes (176742), producing a micro-well. After approximately 40 s, the plastic was solidified and the rod was removed. Six to nine micro-wells were made in each 500 μL-well. Then, the well was rigorously flushed by pipetting with PBS with 5% FBS to remove the air bubbles and possible toxic materials, placed in CM again and incubated overnight in culture conditions. Five embryos were cultured per microwell and from twenty to thirty embryos per well.

Assessment of average number of cells and apoptosis rate per blastocyst

The TUNEL procedure, described by Schwarz *et al*. (2008) with some modifications, was followed to detect DNA fragmentation in embryo cell nuclei observed in late stages of apoptosis. The '*In situ Cell Death Detection Kit, Fluorescein'* (11684795910, Roche Diagnostics SL, Barcelona, Spain) was used for this purpose. Blastocysts from days 7 and 8 were fixed in 4% paraformaldehyde in phosphate-buffered saline solution (PBS) for at least 1 h at room temperature, then washed in PBS plus 1 mg/mL polyvinyl alcohol (P8136) (PBS-PVA) and finally processed immediately or kept at 4ºC overnight in PBS-PVA. Then, blastocysts were permeabilised in PBS containing 0.1% (v/v) Triton X-100 (T9284) and 1 mg/mL sodium citrate (S4641) at 37ºC in humified environment for 30 min. Next, embryos were divided into three groups: positive control, negative control, and experimental samples. The positive control blastocysts were treated in PBS-PVA with 50 IU/mL DNase (No. 04536282001, Roche Applied Science) at 37ºC during 30 min. After washing, positive control and also experimental samples were incubated in 0.2 mL PCR Eppendorf tube containing 30 μL TUNEL reagent from the kit,

composed of 10% enzyme solution (terminal deoxynucleotide transferase enzyme) and 90% staining solution (dUTP-fluorescein conjugate), at 37ºC in humidified atmosphere for 1 h. The negative control was incubated only in the staining solution without adding the enzyme solution. After washing, the embryos were fixed in ethanol and stained with 25 µg/mL Hoechst 33342 (B2261), mounted on poly-L-lysine-coated glass slides (Polysine® slides, Thermo Scientific) and evaluated by epifluorescence microscopy. Blastocysts cells were observed under an epifluorescence microscope (Nikon Eclipse E-400) using a FITC filter (excitation 340-380, dichroic mirror 505 and barrier filter 515-555) to examine the number of TUNEL positive nuclei and DAPI filter to determine the total number of nuclei. Cells with a green nucleus (Fluorescein) were considered TUNEL positive (apoptotic). Cells stained in blue (Hoechst) indicated cellular nuclei. Apoptosis rate was calculated as percentage of apoptotic cells per total cell number per each blastocyst. Day 7 and 8 blastocysts were stained with TUNEL and with Hoechst 33342, while day 9 blastocysts were only stained with Hoechst 33342.

Experimental design

A total of 1,757 *in vitro* fertilised presumptive zygotes were used in 9 replicates. After IVF, the presumptive zygotes were randomly allocated into four different culture systems, one with a large group of embryos and three with smaller groups of embryos:

- Large group (LG): fifty embryos cultured in 500 µL of CM using a Nunc® four-well dishes.
- Small groups: five embryos were cultured in 50 µL drops without (Control) or with EGF (10 ng/mL, Promega G502A) plus ITS (9 µg/mL insulin, 5 µg/mL transferrin, and 6 ng/mL selenium, from a commercial mixture, Gibco-51500 056) (EGF-ITS), or in a microwell under the WOW system (WOW) (Vajta *et al.* [2000] with slightly modifications).

At day 2, non-cleaved embryos were removed from culture, and except in LG, cleaved embryos were re-allocated within each group, keeping the same conditions as above mentioned.

Statistical analysis

Results of cleavage and blastocyst rate were analysed by chi-square test. Results of average number of cells and apoptotic rate per blastocyst were analysed using analysis of variance (ANOVA). The factors 'experimental group' and 'timing of blastocyst formation (days of culture)' and their interaction were analysed. Probabilities of $P<0.05$ and $P<0.001$ were considered statistically significant.

Results

Development rates of our four culture conditions systems are shown in Table 1. Embryos cultured under the WOW system obtained the highest cleavage rate and showed significant differences compared to the Control and EGF-ITS groups. The cleavage rate of the LG was also higher than the EGF-ITS. Regarding the blastocyst rate, embryos cultured in LG showed a higher ability to develop until blastocyst stage than embryos from the other groups. However, in the groups with a lower number of embryos, WOW reached a blastocyst rate higher than the Control group.

Table 1. Effect of large and small groups of embryos *in vitro* cultured on development rates.

	Treatment	Oocytes evaluated	Cleavage (%)	Blastocysts/Cleaved embryos (%)			Blastocysts/ Oocyte(%)
				At day 7	At day 8	At day 9	
	Large Group[1]	442	90.27^{ab}	12.53^{a}	29.32^{a}	36.59^{a}	33.03^{a}
Small Groups	Control[2]	334	86.83^{bc}	4.48^{b}	11.03^{c}	15.86^{c}	13.77^{c}
Small Groups	EGF-ITS[3]	444	84.46^{c}	7.20^{b}	15.47^{b}	19.20^{b}	16.22^{c}
Small Groups	WOW	378	91.53^{a}	12.43^{a}	21.68^{b}	26.00^{b}	23.81^{b}

[1]Fifty embryos/500 µL; [2]Five embryos/50 µl; [3]Five embryos/50 µl supplemented with 10 ng/mL epidermal growth factor, 9 µg/mL insulin, 5 µg/mL transferrin, and 6 ng/mL selenium. a-c Different superscripts in the same column indicate a statistical difference (P< 0.05). Day 0 = IVF.

The quality of the *in vitro*-produced embryos was assessed by the average number of cells/blastocyst and apoptosis rate. ‘Experimental group’ factor significantly affected average number of cells/blastocyst (P<0.001, Table 2). In this regard, the LG embryos showed a higher average number of cells/blastocyst than Control and WOW (98.48 vs. 77.16, 85.58 cells/blastocyst, respectively; P<0.001) (Figure 1). In addition, the average number of cells/blastocyst of EGF-ITS group (90.64 cells/blastocyst) reached higher values than Control (P<0.001) Similarly, apoptosis rate was also affected by ‘experimental group’ factor significantly (P<0.001). Blastocysts from LG showed a lower apoptosis rate than those of the Control and WOW groups (12.14 vs. 24.17 and 19.95%, respectively; P<0.001). Moreover, the

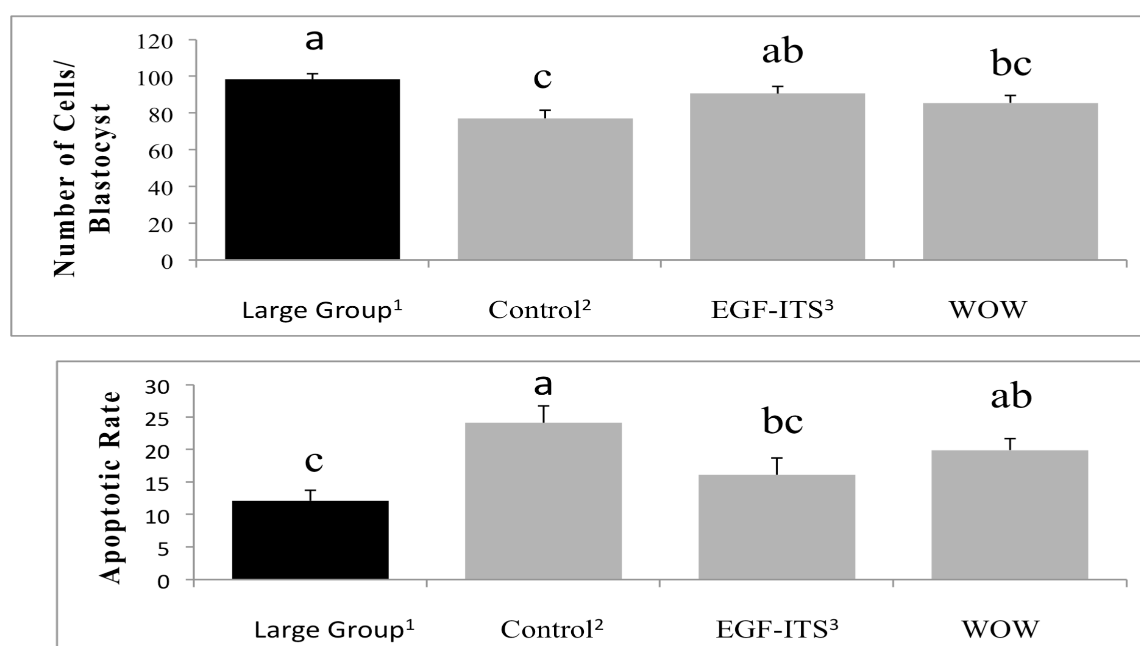
apoptosis rate of the EGF-ITS group (16.15%) was significantly lower than Control group (P<0.001).

Table 2. Statistic analysis of the model (Experimental groups: Large Group, Control, EGF-ITS and WOW; Days of culture: day 7, day 8 and day 9 for average number of cells/blastocyst; day 7 and day 8 for apoptosis rate) for average number of cells and apoptosis rate of IVF bovine blastocyst.

Variable	Factors		Interaction
	Experimental groups (G)	Day of culture (D)	G × D
Average number of cells/blastocyst	**	**	NS
Apoptosis rate	**	*	NS

*P<0.05. **P<0.001. NS, non-significant. Day 0 = IVF.

Figure 1. Effect of large (black bar) and small (grey bars) groups of embryos *in vitro* cultured on blastocyst quality.

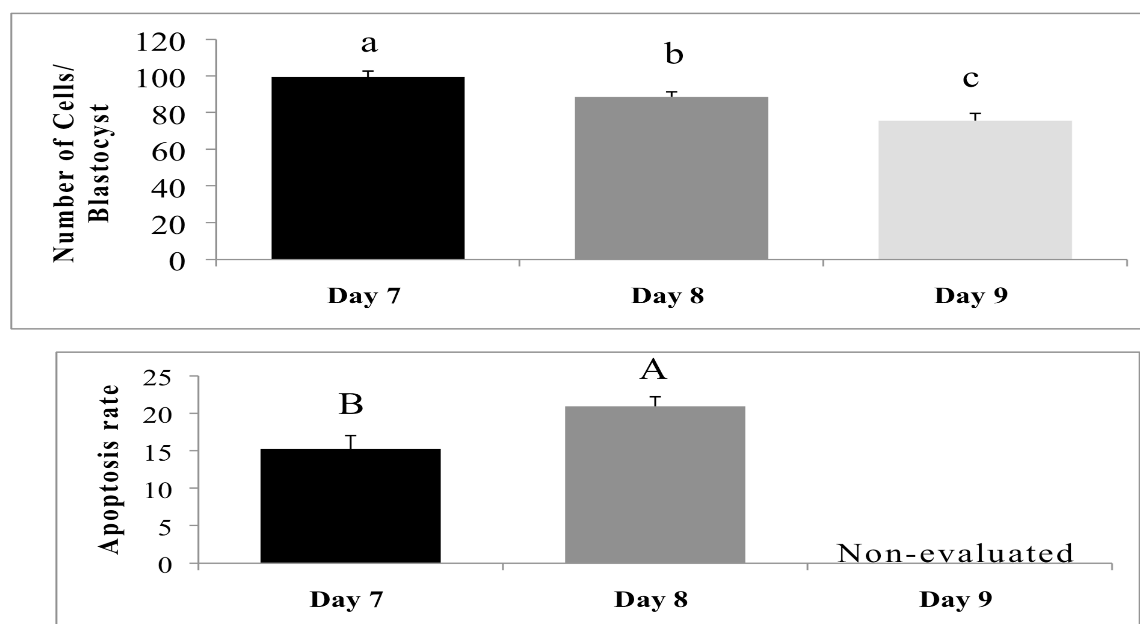


[1]Fifty embryos/500 µL; [2]Five embryos/50 µl; [3]Five embryos/50 µl supplemented with 10 ng/mL epidermal growth factor, 9 µg/mL insulin, 5 µg/mL transferrin, and 6 ng/mL selenium.

[a-c] Different letters in the same row indicate a statistical difference (P< 0.001).

The effect of the days in culture until reaching blastocyst stage on the embryo quality was also studied and results are shown in Figure 2. ‘Days of culture’ factor significantly affected average both number of cells/blastocyst and apoptosis rate (P<0.001 and P<0.05, respectively, Table 2). Blastocysts developed at day 7 showed a higher average number of cells/blastocyst than at day 8, and both showed a higher number of cells/blastocyst than at day 9 (99.49 vs. 88.69 vs. 75.71 cells/blastocyst, respectively; P<0.001). Concerning the apoptosis rate, blastocysts developed at day 7 showed a lower rate than blastocysts developed at day 8 of culture (15.26 vs. 20.95%, respectively; P<0.05).

Figure 2. Effect of day of the blastocyst formation on blastocyst quality.



Different letters in the same row indicate a statistical difference (a-c, P<0.001; A and B, P<0.05). Day 0 = IVF.

Discussion

In agreement with several authors (Paria and Dey, 1990; Lane and Gardner, 1992; Carolan *et al.*, 1996; O'Doherty *et al.*, 1997; Fujita *et al.*, 2006), a decrease in embryo development until blastocyst stage was observed when culturing single or small numbers of

embryos. To improve this efficiency, we enriched the culture medium with EGF plus ITS mixture. However, in our experiment, the addition of EGF plus ITS did not improve the embryo development. Similarly, EGF plus ITS was used by Carney and Foote (1991) with no significant differences in embryo development (from two to ten rabbit embryos in culture). However, in buffalo embryos, an increase in cleavage and blastocyst rates was reported with the addition of EGF plus ITS to the culture medium (Raghu *et al*., 2002) (from eight to twelve embryos in culture).

On the other hand, the culture of embryos in the WOW system improved their development in comparison with those cultured in conventional drops. However, blastocyst yield did not reach the development rate of the embryos cultured in large groups. No differences in the development of WOW- and control- *in vitro* cultured embryos were observed by other authors in porcine (Kamiya *et al*., 2006) and bovine (Pereira *et al*., 2005; Matoba *et al*., 2010; Somfai *et al*., 2010; Sugimura *et al*., 2010) species. Nevertheless, Vajta and collaborators tested the WOW system in bovine, porcine, murine, and human embryos in two different papers, and significant improvements were observed in all species except in murine (Vajta *et al*., 2000, 2008). Another study showed that embryos cultured singly in WOW system reached higher blastocyst rates than embryos cultured in groups of 16, but similar to those cultured in large groups (50 embryos) (Hoelker *et al*., 2009). Moreover, in two other studies, Hoelker *et al*. (2010) and Taka *et al*. (2005) achieved an improvement in embryo development only with certain WOW sizes, which suggests that the physical dimensions of the micro-well could have an influence on the development rates. In our conditions, the WOW culture system would provide a microenvironment for the embryos that would compensate the negative effects on development of low embryo densities, although this improvement was not sufficient to reach LG rates.

The conditions during the IVC period may have a significant impact on the quality of the resulting blastocyst (Lonergan and Fair, 2008). In this work, we recorded the embryo quality through the average number of cells and the apoptosis rate per blastocyst. In terms of the average number of cells/blastocyst, we observed that IVC with a smaller number of embryos decreased this parameter, which has been reported by other authors in bovine (Palma *et al.,* 1994; Larson and Kubisch, 1999; Nagao *et al*., 2008) and mouse (Paria and Dey, 1990; Brison and Schultz, 1997) species. Moreover, we observed that embryos cultured in small groups showed higher levels of apoptosis rate per blastocyst than in large groups. This finding agrees with the report by Brison and Schultz (1997).

In the present work, the addition of EGF plus ITS to the batches with small numbers of embryos significantly improved the embryo quality, as higher average numbers of cells/blastocyst and lower apoptosis rate were observed in comparison with those cultured in conventional drop without EGF-ITS supplement. In addition, EGF-ITS group showed similar average numbers of cells and apoptosis rate per blastocyst to those cultured in large group. In contrast, Carney and Foote (1991) added EGF and ITS to culture medium, combined and separately, and no effect on the average number of cells was reported. It has been suggested that the lack of growth factors and antioxidants may be the cause of the poor quality of the embryos cultured in small groups (Goovaerts *et al*., 2010). In our results, the negative effects on blastocyst quality of culturing small numbers of embryos were minimized with the supplementation of EGF plus ITS; therefore, our result could partially support the aforementioned hypothesis.

The exact mechanism whereby EGF and insulin could have a mitogenic and antiapoptotic effect on the IVPE remains unclear. It is likely that EGF might exert an antiapoptotic effect on embryo through the upregulation of *Bcl* gene expression in presence of BSA (Cui and Kim,

2003). Moreover, it seems that insulin in bovine species might have effects mediated by the IGF-I receptor (Matsui *et al.,* 1995). On the other hand, transferrin and selenium might improve the embryo development and quality by preventing oxidative damage (Nasr-Esfahani and Johnson, 1992; Uhm *et al*., 2007).

Regarding the quality of embryos produced under the WOW system, the average number of cells and the apoptosis rate recorded were similar to those of embryos cultured in small groups (with or without EGF-ITS addition). Comparing these results with the scientific literature available, some authors reported that blastocysts cultured in WOW did not differ in the average number of cells/blastocyst compared to their respective controls in bovine (Vajta *et al*., 2000; Pereira *et al*., 2005; Hoelker *et al*., 2009; Somfai *et al*., 2010; Sugimura *et al*., 2010) or in porcine (Taka *et al*., 2005; Kamiya *et al*., 2006) species. Moreover, apoptosis rate was studied by Hoelker *et al*. (2009) and they did not find differences between embryos cultured in WOW system and in groups of 16 or 50 embryos. However, the genetic expression of the blastocysts cultured under WOW conditions was different to those cultured in groups of 16 or 50 embryos and also to *in vivo*-derived blastocyst. On the other hand, Sugimura *et al*. (2010) observed a decrease in the blastocyst apoptosis rate when bovine embryos were cultured under individual WOW system. However, the morphological quality and inner cell mass, trophoectoderm and total cell numbers were not significantly different between the control and WOW in this work. Indeed, morphological embryo quality of WOW culture and control culture was similar at day 3 but not at day 5 in human species (Vajta *et al*., 2008). On the other hand, some effects of the WOW culture system may be evident after blastocyst stage. In this sense, WOW-derived blastocysts resulted in higher pregnancy rates than those derived from the control culture in bovine (Sugimura *et al*., 2010) and human (Vajta *et al.,* 2008) species.

The quality of the IVC blastocyst produced in our experiment was influenced meaningfully by the blastocyst formation day. We observed that the quality of the embryos decreased as the days of culture progressed. This negative correlation was previously noted by Larson and Kubisch (1999), who found a higher average number of cells per blastocyst at day 7 than at day 8 and day 9. In this sense, other authors also found that embryos reaching blastocyst stage later showed, in general, higher apoptosis rates (Sirisathien and Brackett, 2003; Van Soom *et al*., 2002). In addition, Lonergan *et al.* (2003) observed a higher gene expression of *SOX*, *G6PD* (oxidative stress), and *Bax* (apoptosis) in the embryos towards later stages of *in vitro* development.

In conclusion, embryo culture in the WOW system improved the development rates compared to the conventional culture drop with a small number of embryos. Furthermore, the addition of EGF plus ITS mitigated some of the harmful effects of low embryo numbers on embryo quality, increasing the average number of cells/blastocyst and decreasing the apoptosis rate.

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Experiment 4:

Beneficial Effect of Melatonin on Blastocyst *In Vitro* Production from Heat Stressed Bovine Oocytes*

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Abstract

Melatonin may play an important role in protecting gametes and embryos from the potential harmful effects of oxidative stress (OS). In this study, we first examined two different heat stress (HS) treatments for *in vitro* oocyte maturation (Experiment 1: 38.5 vs. 41.0ºC, during the first 20h; Experiment 2: 38.5 vs. 41.5ºC, during the entire period) on bovine oocyte maturation and embryo development. Second, we tested different melatonin concentrations added to the maturation and culture medium (Experiment 3: 0, 10^{-12}, 10^{-9}, 10^{-4} M; Experiment 4: 0, 10^{-3} M), both with, and without HS (38.5 or 41.5ºC, respectively). In Experiment 1, the HS treatment resulted in a lower maturation rate and number of cells/blastocyst and a higher blastocyst rate than that in the control group. In Experiment 2, oocytes/embryos from heat-stressed oocytes (HSO) had a lower maturation, cleavage, and blastocyst rates, as well as a lower cells/blastocyst compared to the control. In Experiment 3, in HSO groups, 10^{-4} M melatonin resulted in an increased blastocyst rate compared with 0 M melatonin, with a similar blastocyst rate to the non-HSO without melatonin. Melatonin did not have any effect in embryos from non-HSO groups compared with the control. In Experiment 4, 10^{-3} M melatonin produced lower cleavage and blastocyst rates in HSO and lower blastocyst rate in non-HSO when compared to melatonin untreated oocytes/embryos. In conclusion, 10^{-4} M melatonin was found to alleviate bovine oocytes from the harmful effects of HS.

Introduction

In vitro embryo production of embryos (IVPE) is increasingly applied. According to the most recent records, a third of the bovine embryos produced worldwide in 2010 were generated *in vitro* (Stroud, 2011), while approximately half a million of treatment cycles were performed in human clinics in Europe in 2007 (de Mouzon *et al.*, 2012). However, IVPE is still not as

efficient, or cannot produce embryos of similar quality to *in vivo* embryos (Lonergan *et al*., 2006) and less than half of *in vitro* fertilized embryos develop to the blastocyst stage (Lonergan and Fair, 2008). Oxidative stress, which embryos are exposed to during IVPE, is thought to contribute towards this defective embryo development (Guerin *et al*., 2001). Under optimum conditions, the balance between reactive oxygen species (ROS) and antioxidants is maintained. However, if the ROS production overcomes the antioxidant capacity of the cells, the subsequent imbalance results in OS (Combelles *et al*., 2009). Despite the high number of studies published on this topic, the problem of OS during the IVPE is still unsolved so far.

During IVPE, various enzymatic and non-enzymatic ROS scavengers are used to protect oocytes and embryos from harmful OS. Melatonin (N-acetyl-5-methoxytryptamine), a derivatef of tryptophan secreted by the pineal gland, is a ubiquitously acting antioxidant; it scavenges radicals and their derivatives, stimulates antioxidative enzymes, increases the levels of glutathione (GSH), and inhibits the pro-oxidative enzymes in cells and organs (Galano *et al*., 2011). In fact, melatonin has been found to be one of the most effective substances to reduce oxidative cell damage (Balao da Silva *et al*., 2011; Ortiz *et al*., 2011; Wang *et al*., 2011; Gao *et al*., 2012). Moreover, embryo development rates and blastocyst quality of *in vitro*-produced embryos from several species have been improved using melatonin treatments (murine [Tian *et al*., 2010], porcine [Choi *et al*., 2008], buffalo [Manjunatha *et al*., 2009]). Nevertheless, in cattle, the effect of melatonin on *in vitro* embryo development and quality has not been tested in detail.

During the hot season, the HS subsequent to the increase of temperature has been shown to be an important economic problem in livestock animals in largely part because of heat-induced reductions in fertility (bovine [Stott and Williams, 1962], ovine [Dutt, 1963], porcine [Tompkins *et al*., 1967]). In the case of bovine species, summer HS affects around 60% of the

world cattle population (Wolfenson *et al*., 2000) and is considered to be the main factor for low conception rates in high producing dairy cattle herds in warm areas worldwide (De Rensis and Scaramuzzi., 2003; Lopez-Gatius, 2003). Therefore, taking into account the current context of global warming and climate change, the need for solutions in this regard is necessary.

The oocyte is very susceptible to HS while preparing for fertilization: a HS episode during follicular growth had the potential to compromise the development of the resultant embryos (Hansen and Arechiga, 1999; Roth *et al*., 2001). Heat shock in the oocyte has also impaired both nuclear and cytoplasmic maturation and function of surrounding cumulus cells, induced apoptosis (Roth, 2008; Hansen, 2009) and reduced mitochondrial activity (Nabenishi *et al*., 2012b). Although underlying mechanisms remain unclear, ROS appeared to be implicated in the cell damage produced by HS in oocytes during the preovulatory period as the effects of HS both *in vivo* (Roth *et al*., 2008) and *in vitro* (Lawrence *et al*., 2004; Nabenishi *et al*., 2012a) were reduced by administration of antioxidants.

Although many publications have recognized the antioxidant function of melatonin (Galano *et al*., 2011), the potential application of melatonin to reduce heat-induced fertility problems has been only studied *in vitro* in pig (Rodriguez-Osorio *et al*., 2007) and *in vivo* in mouse and bovine (Matsuzuka *et al*., 2005; Garcia-Ispierto *et al*., 2012). In this regard, the aim of the present study was to examine the effects of different concentrations of melatonin added during oocyte maturation and embryo culture on blastocyst development rates and number of cells per blastocyst of *in vitro* bovine embryos, with or without induced HS treatment during *in vitro* maturation (IVM). To provide a test for our hypothesis, we also studied the effects of two different HS treatments during IVM on the subsequent embryo development and number of cells/blastocyst.

Materials and Methods

Culture media

Unless otherwise mentioned, all chemical products were obtained from Sigma-Aldrich® Quimica SA (Madrid, Spain). Handling medium (HM) was composed of medium 199 with 25 mM HEPES (M7528) supplemented with 7.4% (v/v) of foetal bovine serum (FBS: Invitrogen®-10108-157; Alcobendas, Madrid, Spain). Maturation medium consisted of medium 199 (M4530) supplemented with 10% FBS and 10 ng/mL of epidermal growth factor (E1257). The *in vitro* fertilization (IVF) medium was Fert-TALP (Parrish *et al*., 1988) containing 10 µg/mL of heparin (H9399) without glucose. Culture medium (CM) used in the experiments was modified synthetic oviductal fluid containing amino acids and 3 mg/mL of BSA (A7030), supplemented following recommendations by Holm *et al.* (1999) with some modifications (mSOFaaci) and previously used in this laboratory (Salvador *et al*., 2011): 4.2 mM sodium lactate (L4263), 0.73 mM sodium pyruvate (P4562), 30 µL ⁄ mL BME amino acids (B6766), 10 µL ⁄ mL MEM amino acids (M7145) and 1 µg ⁄ mL phenol-red (P0290). Seventy-five µg/mL potassium penicillin G (P3032) and 50 µg/mL streptomycin sulphate (S6051) were added to all culture media as antibiotics. All culture media containing bicarbonate were covered with mineral oil (M8410) and equilibrated overnight in varying culture conditions (see below). Further IVM and IVF were performed in four-well dishes in groups of 30 to 50 oocytes/500 µL medium, while *in vitro* culture (IVC) was performed in groups of 5 to 8 embryos per 30 µL medium drop placed in a Petri dish.

In vitro maturation

Ovaries from heifers younger than one year old were recovered from a slaughterhouse. Cumulus-oocyte complexes (COCs) were aspirated from follicles (approximately 2 to 8 mm diameter) using an 18 g needle on a syringe. Handling medium was used as oocyte collection medium ,and 2.2 mg/mL heparin (H9399) was added to the HM to prevent the formation of blood clots during

the process. After evaluation, only COCs with at least three layers of compact cumulus cells that were morphologically bright and had uniform ooplasm were selected for maturation. Cumulus-oocyte complexes were washed three times in HM and once in maturation medium and were cultured for approximately 24 h in an atmosphere of 5% CO_2 in humidified air throughout the experiments. *In vitro* oocyte maturation was performed at different temperatures (see section Experimental design). After IVM, and to score nuclear maturation (Experiment 1 and 2), a sample of *in vitro* matured oocytes were denuded of cumulus cells using hyaluronidase (H4272) (1 mg/mL HM, exposure of no longer than 1 min) and pipetted with help of a pulled glass Pasteur pipette. Oocytes containing a polar body, observed under a stereomicroscope, were considered as metaphase II (MII) oocytes. The number of oocytes evaluated per session varied from 20 to 30 and from 30 to 60 for Experiments 1 and 2, respectively.

Sperm preparation and IVF

Three 0.25 mL straws of frozen semen from three bulls were thawed in a water bath for 1 min at 37ºC. Thereafter, semen was pipetted on top of 1 mL BoviPure® Bottom Layer (Nidacon, Mölndal, Sweden) in an Eppendorf tube and centrifuged for 10 min at 300 × *g* for purifying the sample. The sperm pellet was isolated and then washed twice in 750 μL of Fert-TALP by centrifugation at 400 × *g* for 3 min. In the first wash, heparin-hypotaurine-epinephrine (HHE) was omitted and in the second wash, HHE mixture was included. Next, the sperm concentration was determined and a final concentration of 1×10^6 sperm/mL was used for IVF. Following IVM, COCs were washed three times in IVF medium and co-cultured with spermatozoa, for 18 to 20 h at 38.5ºC.

In vitro culture of embryos

Following IVF, presumptive zygotes were denuded in HM by repeated pipetting using a pulled glass Pasteur pipette, washed three times in CM, and *in vitro* cultured at 38.5ºC, in an

atmosphere of 5% O_2 and 5% CO_2. The cleavage rate was evaluated on day 2 (day 0=IVF). Blastocyst rate was evaluated on day 7, day 8 (embryos from day 7 plus day 8), and day 9 (all blastocysts from day 7 to day 9) of culture, in all cases using a stereomicroscope. An embryo was considered a blastocyst when a clear blastocoel was observed. The blastocysts obtained were fixed and stained in ethanol containing 25 µg/mL bisbenzimide (Hoechst 3342; B2261) and the total number of cells/blastocyst was counted under an epifluorescence microscope.

Experimental design

Experiments 1 and 2

The goal of Experiments 1 and 2 was to develop an *in vitro* model to test whether melatonin could mitigate the harmful effects of HS on embryos development from HSO. Rispoli et al. (2011) tested a temperature of 41.0 ºC during the first 12 h of the IVM, observing no effect on cleavage rate. With the aim to providing a more precise test of our hypothesis we tested more intense treatments. In the Experiment 1, COCs were matured at 41.0 ºC or 38.5 ºC for the first 20 h of IVM, while in the second experiment, COCs were matured at 41.5 ºC or 38.5 ºC during the entire IVM period (24 h). The effect of both treatments on nuclear maturation blastocyst development and average number of cells/blastocyst of *in vitro*-produced bovine embryo were assessed. The experiments were replicated six and fifteen times in Experiment 1 and 2, respectively.

Experiments 3 and 4

In these experiments, we tested our working hypothesis (above mentioned). Berthelot et al. (1990) showed the melatonin plasma concentration in cattle varied from 2.1×10^{-11} M to 3.87×10^{-9} M, although some studies indicated that follicles might contain a higher melatonin concentration than in plasma (Brzezinski et al. 1987; Rönnberg et al. 1990). In Experiment 3, we

tested four different melatonin concentrations: control, low, middle and high (0, 10^{-12}, 10^{-9}, and 10^{-4} M, respectively) (M5250). Cumulus-oocyte complexes were divided into eight experimental groups (4x2) combining melatonin concentration and temperature (38.5 ºC vs. 41.5ºC, during the entire IVM period). The effects of maturation temperature and melatonin added to the maturation and culture medium on the blastocyst development and the average number of cells/blastocyst of *in vitro*-produced bovine embryo were assessed. The experiment was replicated five times and a total number of 2,300 oocytes were used.

To test whether a higher concentration of melatonin could improve the competence of the heat-stressed or non-heat-stressed oocytes, we performed Experiment 4. The experimental design was similar to Experiment 3, except that only two melatonin levels were compared (0 and 10^{-3} M) (2x2) combining melatonin concentration and temperature. The experiment was replicated four times and a total number of 972 oocytes were used.

Statistical Analysis

Results of oocyte maturation, cleavage, and blastocyst rates were statistically analyzed by the chi-square test. Results of the average number of cells/blastocyst were statistically analyzed by analysis of variance (ANOVA). The factors experimental group, timing of blastocyst formation, and experimental session (see section Experimental design) were included in the model. A probability of $P<0.05$ was considered to be statistically significant.

Results

In Experiment 1, we observed that HS treatment (41.0ºC, during the first 20h of IVM) resulted in a lower maturation rate (66.67 vs. 83.69%; $P<0.05$) (Table 1) yet a higher blastocyst rate than in the group from non-HSO (32.04 vs. 23.16%, respectively; $P<0.05$), while cleavage did not differ between the groups. However, the number of cells per blastocyst was lower in the

embryos from HSO group than in the embryos from the control group (81.86±6.21 vs. 100.71±6.05 cells/blastocyst, respectively; P<0.05).

Table 1. Effect of heat stress (41.0ºC during the first 20h of *in vitro* maturation) on oocyte maturation, embryo development and average number of cells/blastocyst of bovine *in vitro* fertilized embryos.

Treatment	Oocyte maturation			Embryo development					
	Replicates	No. of oocytes examined	Maturation rate*	Replicates	No. of oocytes examined	Cleavage rate	Blastocyst rate from cleaved embryos (n)	Blastocyst rate from oocytes	Average number of cells/blastocyst (LS Mean ± SEM)(n)
38.5ºC	5	141	83.69[a]	6	494	74.29	23.16[b] (85)	17.21[b]	100.71[a] ±6.21 (75)
41.0ºC	5	138	66.67[b]	6	453	73.73	32.04[a] (107)	23.62[a]	81.86[b] ±6.05 (92)

Data with different superscripts (a, b) indicate values with significant differences (P<0.05). *Assessed by metaphase II stage.

As a result of Experiment 1, we decided to increase the exposure time and the temperature of the HS treatment in the following experiment (see Experimental design). In Experiment 2 (Table 2), the HSO group (41.5ºC, during the entire IVM period) showed a lower maturation, cleavage, and blastocyst rates, when compared to oocytes/embryos from the control group (54.31 vs. 81.45%, 60.74 vs. 78.40%, 17.17 vs. 29.37%, respectively; P<0.05). Moreover, the average number of cells was lower in blastocysts derived from HSO group than those derived from non-HSO group (74.93±4.40 vs. 101.87±2.86 cells/blastocyst, respectively; P<0.05).

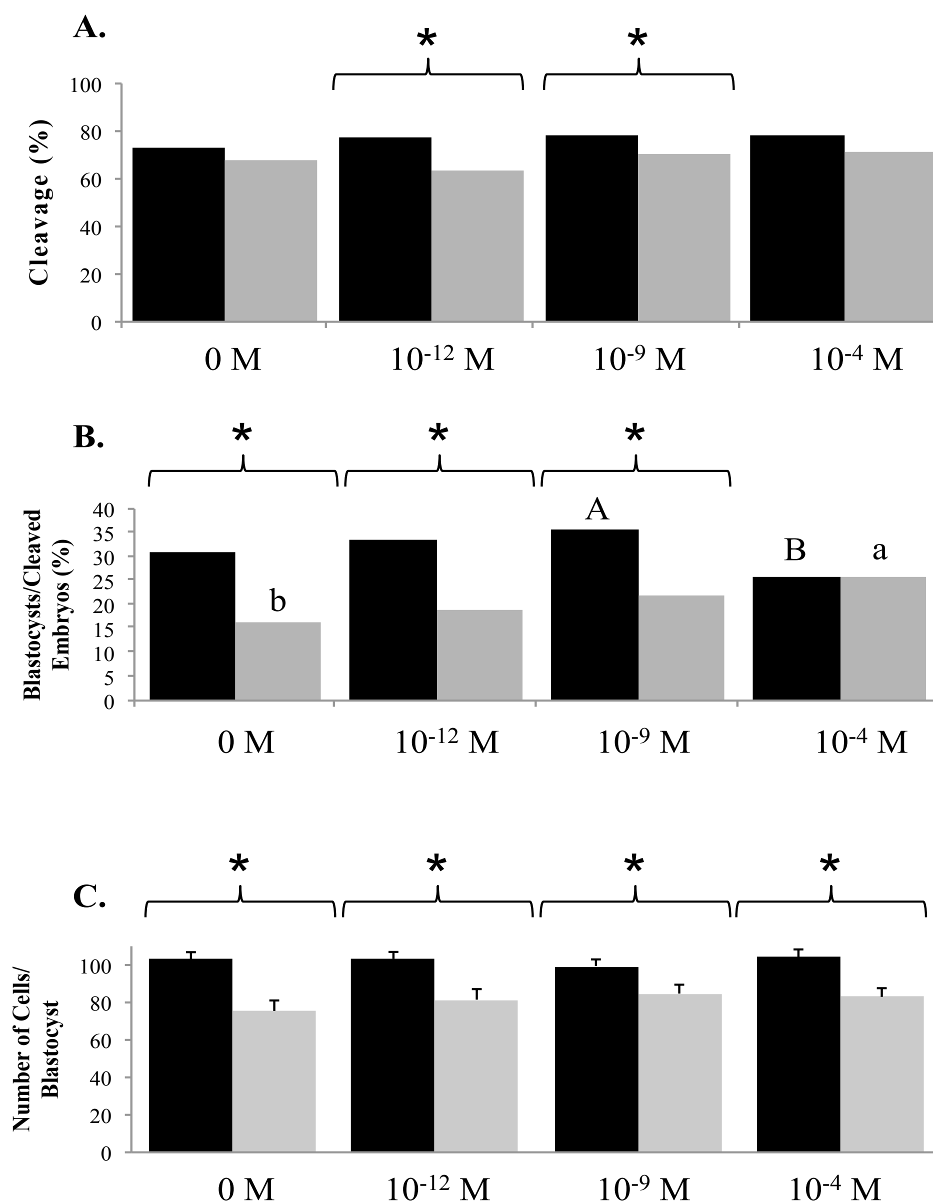
Table 2. Effect of heat stress (41.5ºC during the entire *in vitro* maturation period) on oocyte maturation, embryo development and average number of cells/blastocyst of bovine *in vitro* fertilized embryos.

Treatment	Oocyte maturation			Embryo development					
	Replicates	No. of oocytes examined	Rate of maturation*	Replicates	No. of oocytes examined	Cleavage rate	Blastocyst rate from cleaved embryos (n)	Blastocyst rate from oocytes	Average number of cells/blastocyst (LS Mean ± SEM)(n)
38.5ºC	6	275	81.45[a]	15	986	78.40[a]	29.37[a](227)	23.02[a]	101.87[a] ±2.86 (147)
41.5ºC	6	267	54.31[b]	15	978	60.74[b]	17.17[b](102)	10.43[b]	74.93[b] ±4.40 (61)

Data with different superscripts (a, b) indicate values with significant differences ($P<0.05$).*Assessed by metaphase II stage.

Results of Experiment 3 are shown in Figure 1. Similarly, as in Experiment 2, the HS treatment during oocyte maturation reduced the blastocyst rates in all the groups in comparison to embryos from non-HSO groups, except for the treatment group containing 10^{-4} M melatonin. Comparing blastocysts in the 10^{-4} M melatonin groups with its counterparts from the untreated melatonin groups it was noticeable that HS plus melatonin resulted in a higher rate of blastocysts than HS treatment without melatonin supplementation (25.81% vs. 16.20%, respectively; $P<0.05$). Whereas blastocyst development from non-HSO appeared to be the same irrespective of melatonin supplementation compared to control, the blastocyst rate of oocytes/embryos treated with 10^{-4} M melatonin was significantly lower than the blastocyst rate of oocytes/embryos treated with 10^{-9} M melatonin. The average number of cells/blastocyst was significantly lower in the HSO groups than in the non-HSO groups regardless of melatonin supplementation (75.45±5.62 vs. 103.17±3.67, 81.49±5.63 vs. 103.25±3.73, 84.73±4.75 vs. 99.44±3.54, and 82.98±4.64 vs. 104.01±4.30 cells/blastocyst, for 0, 10^{-12}, 10^{-9}, and 10^{-4} M melatonin, respectively; $P<0.05$).

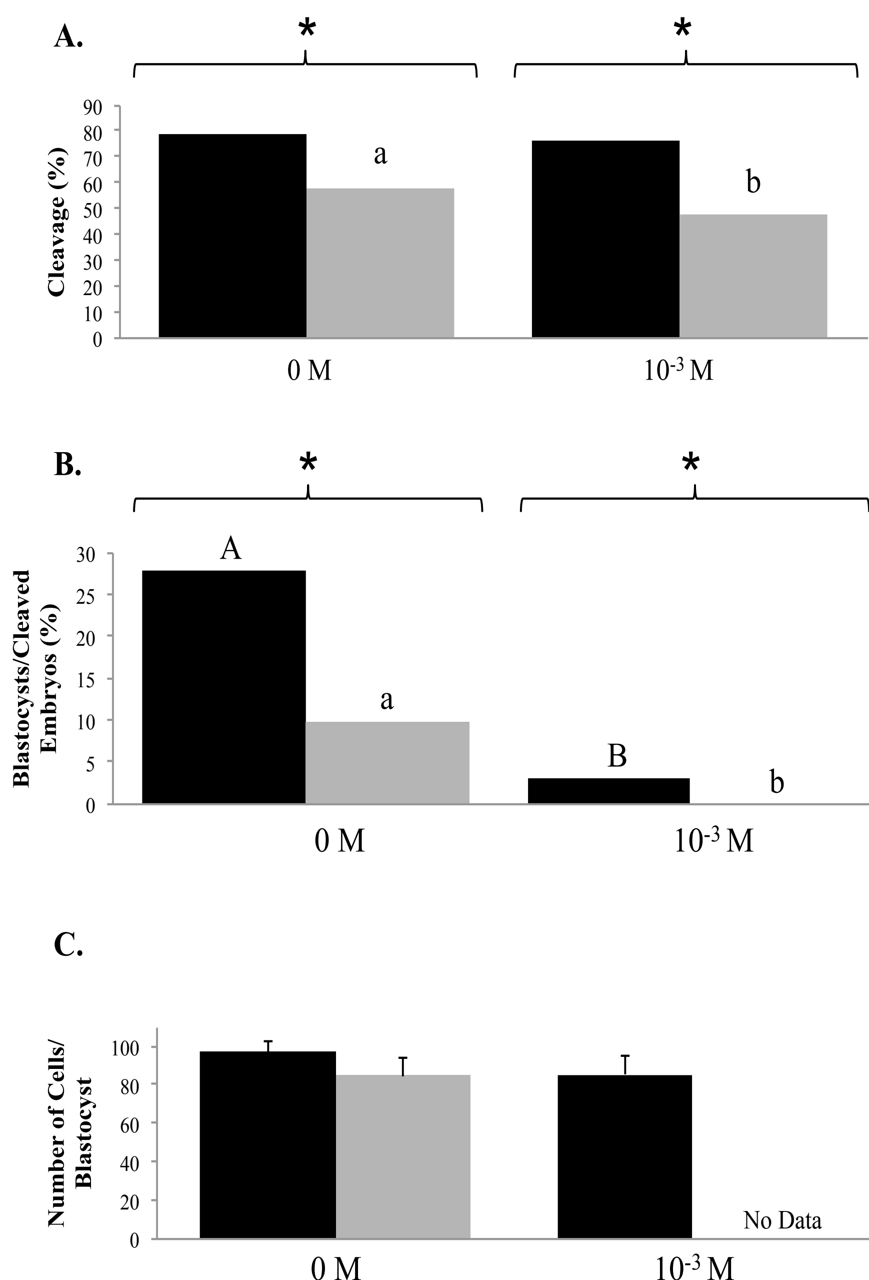
Figure 1. Effect of heat stress and melatonin on embryo development and average number of cells/blastocyst of bovine *in vitro* embryos (Experiment 3).



Horizontal axis, melatonin concentrations. A and B indicate significant differences in oocytes matured at 38.5ºC (black bars), while a and b indicate significant differences in oocytes matured at 41.5ºC (grey bars). An asterisk (*) indicates a significant difference between groups treated with the same melatonin concentration. A probability of $P<0.05$ was considered to be statistically significant.

In Experiment 4, a higher concentration of melatonin (10^{-3} M) was also tested using a similar experimental design to Experiment 3 (see Figure 2). The treatment with melatonin resulted in lower cleavage (47.83 vs. 58.52%; $P<0.05$) and blastocyst rates (0 vs. 9.70%; $P<0.05$) in embryos from HSO group and lower blastocyst rate (3.00 vs. 28.02%; $P<0.05$) in embryos from non-HSO group when compared to melatonin untreated embryos, respectively. In the absence of melatonin, differences between HSO and non-HSO groups were significant with regard to both cleavage and blastocyst rates (58.52 vs. 79.48, 9.70 vs. 28.02, respectively; $P<0.05$). On the other hand, the number of cells/blastocyst was not different between embryos from non-HSO group treated with 0 or 10^{-3} M melatonin when compared to the HSO group treated with 0 M melatonin.

Figure 2. Effects of heat stress and melatonin on embryo development and average number of cells/blastocyst of bovine *in vitro* embryos (Experiment 4).



Horizontal axis, melatonin concentrations. A and B indicate significant differences in oocytes matured at 38.5ºC (black bars), while a and b indicate significant differences in oocytes matured at 41.5ºC (grey bars). An asterisk (*) indicates a significant difference between groups treated with the same melatonin concentration. A probability of $P<0.05$ was considered to be statistically significant.

Discussion

Several studies have highlighted the potential harmful effects of HS on mammalian reproduction (Hansen, 2009). However, many questions remain without an answer about the HS effects on reproduction and embryology.

In Experiment 1, we observed that the maturation rate is decreased by the HS effect, but the difference between groups was offset by the time of cleavage. This fact could be interpreted as a maturation delay due to the HS treatment. A retarded development has been described in bovine oocytes following HS treatment (41ºC for 6 h) elsewhere (Payton *et al*., 2004). Moreover, Aroyo *et al.* (2007) in mice and Gendelman *et al.* (2010) in bovine observed a delay in the cleavage rate of embryos produced during the hot season in comparison with embryos produced during the cold season. On the other hand, some findings provided evidence for bovine HSO to mature faster than non-heat-stressed counterparts (Edwards *et al*., 2005; Schrock *et al*., 2007; Andreu-Vazquez *et al*., 2010). In agreement with our results, oocytes heat-stressed during the germinal vesicle stage (bovine: Payton *et al*., 2004; mouse: LaRosa and Downs, 2007) or during maturation (bovine: Edwards and Hansen, 1997; Ju *et al*., 2005; Schrock *et al*., 2007; Edwards *et al*., 2009; Zhandi *et al*., 2009; Rispoli *et al.,* 2011; Payton *et al*., 2011) have shown competence in overcoming the first cellular division similar to that found in non-HSO. In addition, we observed that HS treatment improved the ability of the oocytes to form blastocysts. In agreement with this paradoxical result, Isom *et al.* (2009) obtained an increase in the developmental rates of parthenogenetic pig oocytes submitted to HS (42ºC) during the first nine hours of embryo culture. In this regard, Pribenszky *et al.* (2010) highlighted a number of studies in which sub-lethal stress treatments (such as high hydrostatic pressure, osmotic stress, HS or OS) might increase the morphological survival, fertilization ability, and *in vitro* development, both in gametes and embryos. However, in contrast to these results, it has also been shown that

HS treatment during IVM had no effect (bovine: Ju et al. 1999; Roth and Hansen, 2004b; Schrock *et al*., 2007; Roth and Hansen, 2009), or may even decrease the blastocyst rate (bovine: Lawrence *et al*., 2004; Roth and Hansen, 2004a, b; Edwards *et al*., 2005; Ju *et al*., 2005; pig: Tseng *et al*., 2006; Sugiyama *et al*., 2007; Soto and Smith., 2009; Zhandi *et al*., 2009; Payton *et al*., 2011; Rispoli *et al*., 2011). This seeming controversy on the effects of HS could probably be explained by the different temperatures and timing schedule of the HS treatments and the methodology used in the cited reports. In addition, Rispoli *et al.* (2011) observed divergent responses on parthenogenetic and *in vitro*-produced bovine embryo development when oocytes were submitted to HS (41ºC) during the first 12 h of IVM.

To try to explain this phenomenon, it has been suggested that in response to HS, the stressed cells may trigger thermo-tolerance mechanisms (Davies, 1999), whereof the synthesis of heat shock proteins (HSPs) might be mainly responsible for the resistance (Hansen and Arechiga, 1999; Beere, 2005; Esfandiari *et al*., 2007). In fact, preimplantation bovine embryos can respond to HS treatment (42ºC for 80 min) by increasing synthesis of HSPs as early as the two-cell stage (Edwards and Hansen, 1996), as well as during subsequent stages of development (Edwards *et al*., 1997). Therefore, our results would reinforce the idea that the embryo could respond against a harmful thermal environment.

We have also observed that HS treatment resulted in a reduction in the cell number of blastocysts. In several studies, blastocyst quality has been shown to decrease after a HS treatment either during maturation (Lawrence *et al*., 2004; Roth and Hansen., 2004b; Ju *et al*., 2005; Soto and Smith, 2009; Zhandi *et al*., 2009) or during the first cleavage stages (Jousan and Hansen, 2004; Sakatani *et al*., 2004). It is likely that HS treatment during IVM could have a significant effect mainly on the trophoectoderm cells of the blastocyst (Ju *et al*., 2005; Zhandi *et al*., 2009).

In Experiment 2, an increase in exposure time and temperature in the HS treatment had a deleterious effect on all development parameters studied and on the average number of cells per blastocyst. In this line, Edwards *et al.* (2005) suggested that the consequences of HS during oocyte maturation may be time-temperature dependent. A delay in maturation may have occurred, but in this case, the treatment was probably too harmful. Taken together, the results of experiments 1 and 2 have shown that even HSO can form blastocysts, although the developmental competence of these blastocysts may likely be impaired in comparison to non-heat-stressed blastocysts.

The body temperature of heat-stressed cattle fluctuated during the day with a maximum peak of 40.5ºC (Rivera and Hansen, 2001). Nevertheless, in this study, we tested higher levels of temperature, as *in vitro* embryos can withstand higher temperatures than those *in vivo* (Ozawa *et al.*, 2002). Several authors have used the temperature of 41.0ºC with different exposure times to HSO during IVM (Edwards and Hansen, 1997; Lawrence *et al*, 2004; Roth and Hansen, 2004a, b; Schrock *et al*., 2007; Rispoli *et al*., 2011). In our study, and also in the case of other studies (already mentioned), a HS treatment of 41.0ºC might not be harmful enough to compromise embryo development, and subsequently may not be good to test whether melatonin could mitigate the harmful effects of HS on embryo development from HSO.

The different melatonin concentrations that we tested in Experiment 3 did not show any significant effect compared to the control group when oocytes were not heat-stressed. In agreement with our results, Papis *et al.* (2007) and Tsantarliotou *et al.* (2010) tested the melatonin effect with different treatment strategies and did not find any consequence on the development rates of the *in vitro*-produced bovine embryos. Recently, Takada *et al.* (2012) in cattle pointed out that melatonin (10^{-9} M) can decrease the DNA damage in cumulus cells during IVM, but without a significant effect on development rates. In contrast, other authors have

observed that melatonin improved the *in vitro* embryo development in buffalo (Manjunatha *et al*. 2009), pig (Choi *et al*., 2008; Kang *et al*., 2009; Shi *et al*., 2009) and mice (Ishizuka *et al*., 2000).

When HS was applied to the oocytes, 10^{-4} M melatonin significantly improved the blastocyst rate compared to the group untreated with melatonin and obtained similar development rates to those without HS and melatonin treatment. Therefore, melatonin improved the competence of the HSO and may mitigate some of the negative effects of HS on embryo development. In heat-stressed pig oocytes (40ºC for 3 h prior to IVF) supplemented by melatonin at 10^{-9} M, Rodriguez-Osorio *et al.* (2007) obtained an improvement in cleavage, but not in blastocyst rates. In other stress contexts, melatonin has been shown to have a protective action from OS in *in vitro*-produced bovine embryos cultured under high oxygen concentration (Papis *et al*., 2007) and in mouse oocytes *in vitro*-matured with different amounts of oxygen peroxide (Tamura *et al*., 2008), but not in porcine embryos short exposed to oxygen peroxide (Rodriguez-Osorio *et al*., 2007). In a study performed recently by Gao *et al.* (2012), melatonin significantly suppressed ROS production and promoted embryonic development in vitrified embryos compared with untreated ones.

Interestingly, melatonin used *in vivo* in heat-stressed pregnant mice reduced early embryonic death (Matsuzuka *et al*., 2005), suggesting that the topical administration of melatonin could also enhance the fertility rates in heat-stressed animals. Moreover, it seems that under HS conditions, melatonin treatment (subcutaneous implants) at dry-off period improved reproductive performance postpartum in high-producing dairy cows (Garcia-Ispierto et al. 2012). In fact, it appears that *in vivo* melatonin synthesis is up-regulated by many physical, chemical, and environmental stressors related to OS (Tan *et al*., 2007). So, these results, together with the low toxicity of melatonin even in pregnant animals (Jahnke *et al*., 1999), reinforce the idea that

melatonin could be an interesting compound to increase the fertility in heat-stressed animals. In this sense, in women who failed to become pregnant in the previous IVF-embryo transfer cycle (with a high intra-follicular oxidative stress), oral administration of melatonin caused a decrease in the concentration of two intra-follicular sensitive indicators of OS and improved the fertilization rate (Tamura *et al*., 2008). Further *in vivo* studies need to be performed to gain a better understanding of the benefits of using melatonin in reproduction.

It is suggested that melatonin may have a higher antioxidant action mainly when the production of oxygen free radicals in medium is exaggerated (Reiter *et al*., 2009) and our results might be in agreement with this observation. Although we have not assessed the oxidative status of oocytes and embryos, the link between increased ROS production in response to elevated culture temperature has been yet reported in oocytes (Nabenishi *et al*., 2012a,b) and in embryos (Sakatani *et al*., 2004, 2008). On the other hand, it is likely that part of the protection that melatonin has shown in cumulus cells (Hemadi *et al*., 2009; Takada *et al*., 2010), may lead to improved development competence of HSO. The fact that cumulus cells could provide protection during oocyte HS treatment (Edwards and Hansen, 1996, 1997) and the existence of melatonin receptors in COCs (El-Raey *et al*., 2011) further supports this idea.

However, melatonin did not produce any observable effect on average number of cells of the blastocyst. This is in alignment with some studies that showed a lack in an improvement of the blastocyst cell number (McElhinny *et al*., 1996; Papis *et al*., 2007; Kang *et al*., 2009), but also contrasts with others (Rodriguez-Osorio *et al*., 2007; Choi *et al*., 2008; Shi *et al*., 2009; Tian *et al*., 2010; Gao *et al*., 2012). In relation to embryo quality parameters, an anti-apoptotic effect of melatonin has also been described by Choi *et al.* (2008) and by Gao *et al.* (2012) in porcine and vitrified mouse embryos, respectively.

The results of Experiment 4 showed that 10^{-3} M melatonin in maturation and culture medium could be embryo toxic in the bovine both in non-HSO and HSO. Using the same melatonin concentration, this effect has also been observed under conventional *in vitro* conditions in other species such as porcine (Rodriguez-Osorio *et al*., 2007; Shi *et al*., 2009) and mouse (Tian *et al*., 2010), and in vitrified mouse embryos (Gao *et al*., 2012). However, the toxicity of 10^{-3} M melatonin was evident in the blastocyst rate but not in the cleavage rate in non-HSO, which is in contrast with Rodriguez-Osorio *et al.* (2007) and with Shi *et al.* (2009) (when melatonin were added in maturation medium). On the other hand, 10^{-3} M melatonin enrichment of the IVM medium did not affect either cleavage or blastocyst rates in cattle (Tsantarliotou *et al*., 2010). In mouse oocytes, Adriaens *et al*. (2006) reported lower maturation in the presence of 10^{-3} M melatonin while Sainz *et al*. (2003) described a reduced proliferation in Chinese hamster ovarian cells with the same melatonin concentration. In fact, the results from Experiment 4 were not unusual, as the concentration of melatonin tested was extremely high compared to the physiological concentration found in plasma (above mentioned, Berthelot *et al*., 1990). No other results were found in the available literature on the effect of 10^{-3} M melatonin on HSO.

In conclusion, the exposure time and the temperature of the HS *in vitro* treatments during IVM are important factors in the subsequent embryo development. A sub-lethal maturation temperature (41.0ºC) during 20 h of maturation improved the blastocyst rate, although a lower average number of cells/blastocyst was also observed. In contrast, a higher temperature (41.5ºC) and longer exposure time (24h) caused a decrease in the development rates and the average number of cells/blastocyst. In addition, melatonin at 10^{-4} M was found to alleviate bovine oocytes from the harmful effects of HS, enhancing the further development to blastocyst. It would be interesting to test in future works whether the *in vivo* application of melatonin can avoid the reduction of reproductive efficiency of cattle exposed to HS conditions. None of the

melatonin concentration tested (10^{-12}, 10^{-9}, and 10^{-4} M) showed a significant effect compared to the control group when oocytes were cultured under conventional *in vitro* conditions. Finally, melatonin at 10^{-3} M in the maturation and culture medium showed a harmful effect on bovine embryo development both in non-HSO and HSO.

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Experiment 5:

Effect of Sex-sorted Sperm on Development and Quality of *In Vitro*-Produced Bovine Embryos Derived from Ovum Pick Up Oocytes*

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Abstract

A combination of sex-sorted sperm and ovum pick up (OPU)-*in vitro* fertilization (IVF) techniques could improve the overall efficiency of the process and develop the commercial opportunities for sex-sorted sperm. Despite intrinsic differences have been found between bovine oocytes collected post-mortem from slaughterhouse ovaries and those collected by OPU, only a few studies about IVF with sex-sorted sperm have used oocytes collected by OPU. In addition, as far as we know, using OPU-oocytes, the effect of sex-sorted sperm on embryo development and quality have been studied in buffalo and in *Bos indicus*, but not in *Bos taurus*, and this was the aim of this work. Oocytes were retrieved by OPU from mature dry cows and *in vitro* matured. *In vitro* fertilization was performed with sex-sorted or unsorted sperm, and after that, presumptive zygotes were *in vitro* cultured for 9 days. Oocytes fertilized with sex-sorted sperm showed a lower cleavage rate (52.43 vs. 69.09%, respectively; $P < 0.05$) but similar blastocyst rate compared to oocytes fertilized by unsorted sperm. Moreover, the percentages of excellent or good blastocyst as well as the timing of blastocyst formation at day 7, day 8 or day 9 post IVF was not different between sex-sorted and unsorted groups. In conclusion, sex-sorted sperm and OPU-IVF are efficient tools to produce bovine embryos of predetermined sex and from individual donors.

Introduction

The possibility of determining the sex in domestic animals is a scientific challenge that has great implications for the productivity of livestock systems. The advantages of controlling the sex ratio of progeny are numerous: higher productivity, faster genetic progress, improvement in animal welfare (by decreasing obstetric difficulties in cattle and avoiding castration in pigs) and reduction of environmental impact due to the elimination of the unwanted sex before they

grow to adulthood (Rath *et al*., 2009). For the dairy industry, females (together with a few breeding males) are the most profitable animals, but little benefit can be obtained from male calves. In contrast, male animals have better growth rates than females (Seidel, 2003), so they are usually the desired sex in the beef industry. Nowadays, sperm gender preselection is a reality, as technical prerequisites such as adaptation of flow cytometers (Kamentsky and Melamed, 1967), identification of the X- and Y- bearing sperm differences (Moruzzi, 1979) and non destructive DNA labelling (Johnson *et al*., 1989) have been developed. In fact, sex-sorted sperm is one of the most important advances in biotechnology applied to agriculture over the past twenty years and offspring of predetermined sex have been born in several domestic mammals and humans using sex-sorted sperm (Maxwell *et al*., 2004).

Bovine sex-sorted sperm are commercially available in numerous countries. The method is efficient as the probability of the right sex is higher than 90% (Garner, 2006). However, despite the numerous optimistic predictions, sex-sorted sperm low efficiency still hampers its broader application because fertilization rates are lower and prices per AI are higher than non-sorted sperm doses (Seidel, 2003; Cran, 2007). A combination with different biotechnologies has been suggested to improve the efficiency of the process and develop the commercial opportunities for sex-sorted sperm (Rath and Johnson, 2008). *In vitro* fertilization is a preferred technique to be associated with sex-sorted sperm, as twenty oocytes could be fertilized with approximately 20,000 to 30,000 spermatozoa (Puglisi *et al*., 2006). In fact, sex-sorted sperm has been used for IVF to produce *in vitro* sexed bovine embryos, either employing the steps of conventional IVF (Lu *et al*., 1999) or intracytoplasmic sperm injection (Hamano *et al*., 1999). As for AI, the efficiency of *in vitro* production of embryos with sex-sorted sperm is still lower than with unsorted sperm (Vazquez *et al*., 2008).

Ultrasound guided OPU (Pieterse *et al*., 1991) allows us to obtain oocytes from highly valuable live donors during their reproductive life (Galli *et al*., 2001), which substantially increases the value of the *in vitro*-produced embryos in breeding programmes. A combination of sex-sorted sperm and OPU-IVF techniques could optimise the use of genetic and economic resources to produce sexed embryos. In this regard, some trials have been reported for *Bos indicus* (Pontes *et al*., 2010; Underwood *et al*., 2010), *Bubalus bubalis* (Liang *et al*., 2008) and *Bos taurus* (Pontes *et al*., 2010; Presicce *et al*., 2010; Senatore *et al*., 2010). However, as far as we know, using oocytes collected by OPU, the effect of sex-sorted sperm on embryo development and quality have been studied in buffalo (Liang *et al*., 2008) and in *Bos indicus* (Underwood *et al*., 2010), but not in *Bos taurus*.

The majority of IVF papers using sorted-sperm have made use of bovine oocytes derived from slaughterhouse ovaries to investigate embryo development and quality (Xu *et al*., 2009). However, intrinsic differences were found between OPU oocytes and oocytes from slaughtered ovaries (Merton *et al*., 2003; Lopes *et al*., 2006; Presicce *et al*., 2010; Plourde *et al*., 2012). On the other hand, the quality of the *in vitro*-produced embryos from sex-sorted sperm may be affected due to the stress inherent to the sex-sorting process (Rath and Johnson, 2008); nevertheless, the results in this regard are not conclusive yet. For instance, the mRNA expression patterns of some genes in *in vitro*-produced embryos with sex-sorted sperm were found to be different compared to those produced with unsorted sperm (Morton *et al*., 2007), but in other studies expression of studied genes were similar (Bermejo-Alvarez *et al*., 2010; Stinshoff *et al*. 2012).

The purpose of the present work was to study the effect of sex-sorted sperm on embryo development and quality, assessed as timing of blastocyst formation and blastocyst morphology, from bovine oocytes collected by OPU.

Materials and Methods

Animals

The experiment was performed at the Institute of Farm Animal Genetics (Friedrich-Loeffler-Institut, Neustadt-Mariensee, Germany), in the period from July to October. All procedures involving animal experiments in this study were carried out in accordance with the German Animal Welfare Law. Fifteen healthy and sexually mature dry Holstein-Friesian and Deutsche Schwarzbunte cows were used as oocyte donors.

Follicle aspiration and oocyte retrieval

Chemicals used in this study were obtained from Sigma-Aldrich GmbH (Taufkirchen, Germany), unless otherwise stated. Ovum pick up sessions were performed according to procedures described by Zaraza *et al*. (2010) with some modifications at 3 to 4 day intervals. Before each procedure, faeces were removed from the rectum and perineal area was cleaned with water and Octenisept® (0.1% octenidine dihydrochloride, 2% phenoxyethanol, Sulke and Mayr). Epidural anaesthesia was applied to each donor cow prior to OPU (5 mL of 2% procaine hydrochloride solution, Procasel®, Selectavet). Follicles bigger than 3 mm in diameter were aspirated using an ultrasound system (CS 9000, Picker) with a 6.5 MHz ultrasound transducer (EUP-F331, Picker) placed in a PVC tube with a needle guide. A 20G x $2^{3/4}$" needle (Neolus®, Terumo) was inserted in a plastic rod for follicle aspiration and connected to a vacuum pump adjusted to 65 mm Hg pressure (Aspirator 3, Labotect). Dulbecco's PBS (DPBS) (D5773) medium containing 1 g/L BSA (11924, Serva GmbH), 50 UI/mL penicillin G (PENP), 50 µg/mL streptomycin sulphate (35500, Serva GmbH) and 2.2 IU/mL sodium heparin (24590, Serva GmbH) was used to retrieve the oocytes and flush the collection needle. A 50-mL conical tube was used to store the aspirated fluid from each animal; the contents were immediately passed through a 50 µm filter and collected in a Petri dish with DPBS. The cumulus-oocyte complexes

(COCs) were found using a stereomicroscope and placed in TCM-air medium, consisting of TCM 199 (M2520) supplemented with 22 µg/mL pyruvate (P3662), 350 µg/mL $NaHCO_3$ (31437, Riedel–de Haen AG), 50 µg/mL gentamycin (G3632) and 0.1% BSA-fatty acid free (A7030). Oocytes were graded morphologically based on the cumulus investment according to Chaubal *et al.* (2006) with some modifications, as follows: category I, more than four layers of cumulus cells; category II, three or four layers of cumulus cells; Category III, one or two layers of cumulus cells; category IV, denuded oocytes; category V, oocytes with expanded cumulus; category VI, degenerated or lysed oocytes. Oocytes from categories V and VI were not used for IVF.

In vitro maturation (IVM)

Groups of up to 20 COCs were washed and *in vitro* matured in 100 µL drop of maturation medium consisting of TCM 199 containing 22 µg/mL pyruvate (P3662), 2.2 mg/mL $NaHCO_3$ (31437, Riedel–de Haen AG), 50 µg/mL gentamycin (G3632), 10 IU/mL eCG and 5 UI/mL hCG (Suigonan-Intervet) and 0.1% bovine serum albumin (BSA) (A7030). COCs were *in vitro* matured for 22 to 24 h in humidified atmosphere at 38.5ºC and 5% CO_2 in air. All the culture media containing bicarbonate were covered with silicone oil (35135, Serva GmbH) and equilibrated for at least two hours in culture conditions before use (see below).

Collection, preparation and sex sorting of sperm

Briefly, sperm sorting was performed according to the Beltsville Sperm Sorting Technology (Johnson *et al*., 1999). Ejaculates were collected with an artificial vagina from one mature Holstein-Friesian bull of proven *in vitro* and *in vivo* fertility and only ejaculates with initial progressive motility of at least 75% were used. Semen was diluted to a concentration of 100×10^6 spermatozoa/mL with Sexcess® sample fluid (Masterrind, Verden, Germany) and then stained with 125 µg bisbenzimide H 33342 trihydrochloride (bisbenzimide) (B2261) and

incubated for 1.25 h at 37ºC. Stained samples were filtered through a 51 µm nylon mesh (Falcon 2235, Becton Dickinson, Franklin Lakes, NJ, USA) and 0.001% (w:v) food dye (Warner Jenkinson, Inc., St. Louis, MO, USA) was added.

Sperm were then separated into X- and Y-chromosome- bearing populations using a high-speed cell sorter (SX MoFlo®, Beckman-Coulter, FL, USA) modified for sperm sorting (Johnson and Pinkel, 1986; Rens *et al*., 1999), operating at 2.76 bar. Labelled spermatozoa were passed through an orienting nozzle (Johnson *et al*., 1999) and illuminated with a 200 mW solid state UV-laser (Coherent Palladin, Coherent, USA). During flow cytometric sorting, gates were placed around viable and correctly oriented sperm to achieve purities greater than 92% in each of the enriched X and Y- chromosome-bearing sperm populations. Sorted sperm were collected into 10 mL centrifuge tubes, containing 500 µL of TEST catch medium (Johnson and Pinkel, 1986) supplemented with 20% (v:v) egg yolk.

Cryopreservation of sperm

Semen was cryopreserved using the Sexcess® treatment for sexed sperm (Rath *et al*., 2009). Briefly, sorted sperm in catch medium were centrifuged for 20 min at 800 × *g*. The supernatant was discarded and pellets resuspended in Sexcess® cooling extender to 26.4×10^6 sperm/mL. Samples were then cooled in 2 steps to 5ºC over 2 h and diluted with Sexcess® freezing extender (Masterrind, Verden, Germany) to a concentration of 20.5×10^6 spermatozoa/mL (final glycerol concentration 6.4%). Resuspended sperm were then loaded into 0.25 mL straws (IMV, L'Aigle, France). Samples were then frozen in an automated freezer (IceCube, Minitub, Landshut, Germany). Sperm from the same ejaculate which had not been sorted were frozen as described above as controls. Reanalyses were performed by resorting aliquots of sorted samples. Briefly, sperm were restained with ten-fold less concentrated DNA dye (bisbenzimide) as for sperm sorting and then incubated at 37°C for 30 min. Sperm were then sorted again at a sort rate of 60

to 80 events per second. Histogram data at a resolution of 256 channels were tested in a curve fitting program (Gauss 7, anonymous) to obtain the best fitting probability. Only samples with a purity of more than 92% were used for further experiments.

In vitro fertilization

Two semen straws (Y sex-sorted and unsorted sperm) of 0.25 mL from a bull with proven fertility for IVF were thawed at 30ºC in a water bath for 30 s and centrifuged for 10 min at 300 × *g* through a gradient of 1 mL of BoviPure® Bottom Layer (Nidacon). The sperm pellet was isolated and washed twice through 750 µL Fert-TALP medium (Parrish *et al*., 1988) by centrifugation at 400 × *g* for 3 min. In the first washing, heparin-hypotaurine-epinephrine (HHE) was omitted, but in the second wash HHE was included. Next, the sperm concentration was determined and a final concentration of 1×10^6 sperm/mL was used for IVF. Following IVM, COCs were washed thrice and co-cultured wfith spermatozoa in IVF medium in groups of up to 20 COCs per 35 µL drops, for 18 to 20 h at 38.5ºC in an atmosphere of 5% CO_2 in humidified air.

In vitro culture (IVC)

Presumptive zygotes were denuded from surrounding cumulus cells in TCM-air medium, washed and transferred to 30 µL drop of culture medium in groups of 5 to 8 embryos. Modified synthetic oviductal fluid amino acids supplemented (mSOFaaci) following Holm's recommendations (Holm *et al*., 1999) and supplemented with 4 mg/mL BSA (A7030) was used as culture medium (Zaraza *et al*., 2010). Culture drops were placed in a modular incubator chamber (Billups-Rothenberg- USA) with a gas mixture of 5% O_2, 5% CO_2, and 90% N_2 at 39 °C for a total of 9 days. Cleavage rate was evaluated on day 2 (IVF = day 0). Blastocyst rate, morphological quality and timing formation of the blastocyst produced were assessed on day 7, day 8, and day 9. Blastocysts were classified according to their morphological quality as:

excellent or good blastocysts (code 1 in the International Embryo Transfer Society [IETS] morphological classification [Robertson and Nelson, 1998]) and fair blastocysts (code 2 in the IETS morphological classification [Robertson and Nelson, 1998]).

Experimental design

To evaluate the effect of sex-sorted sperm following IVF on embryo development and quality, two hundred and seventy-two oocytes were retrieved from fourty-one OPU sessions. Oocytes recovered in different OPU sessions on the same day were pooled and randomly allocated into two different groups. Following IVM, IVF with sex-sorted or unsorted sperm was performed as described above. For the experiment, ten IVF sessions were carried out.

Statistical analysis

Ovum pick up-derived oocyte results are expressed as means ± SEM (standard error of the mean). Results of cleavage, blastocyst, morphological blastocyst quality, and timing of blastocyst formation rates were analysed using the chi-square test. A probability of $P<0.05$ was considered statistically significant.

Results and discussion

OPU-derived oocyte

The average collection rate was 6.8 ± 0.56 oocytes per OPU session and cow, of which 6.1 ± 0.52 were selected for maturation (see section: Follicle aspiration and oocyte retrieval). The number of oocytes recovered and classified as category III was the largest (2.3 ± 0.32), followed by category II (1.3 ± 0.19), category I (1.3 ± 0.20), category IV (1.2 ± 0.25), category VI (0.5 ± 0.15) and category V (0.2 ± 0.07). The number of oocytes recovered per OPU session in our experiment was similar to the values reported by Bungartz *et al*. (1995) and higher than those reported by other authors previously (Hasler *et al*., 1995; Goodhand *et al*., 1999; Rizos *et al*.,

2005; Chaubal *et al*., 2006; Lopes *et al*., 2006). Regarding the oocyte morphology classification, the absence of standardized classification make difficult to compare results between different scientific publications.

Effect of sex-sorted sperm on embryo development

The development rates of the bovine blastocysts *in vitro* fertilized with unsorted and sex-sorted sperm are shown in Table 1. We observed a higher cleavage rate in the oocytes fertilized with unsorted sperm in comparison with those fertilized with sex-sorted sperm (69.09 vs. 52.43%, respectively; $P < 0.05$). Interestingly, Ruiz *et al*. (2009) found no differences in penetration rate and syngamy between sex-sorted or unsorted sperm using a similar OPU and IVF setup to ours. In other studies but with oocytes recovered post-mortem from slaughtered ovaries, zygotes from sex-sorted sperm showed a delayed first cell cycle (Beyhan *et al*., 1999; Bermejo-Alvarez *et al*., 2010). This might not only be due to the *in vitro* conditions, since McNutt and Johnson (1996) observed *in vivo* a delay in embryo development when rabbits were inseminated with sex-sorted sperm. The effect only appeared 42 h after insemination, and was not evident at days 7, 14 or 21 post-insemination. Therefore, it would be interesting to look more intensively into the first cleavage cycle on a molecular basis.

The blastocyst rate that we observed in our experiment was not significantly different when unsorted or sex-sorted sperm were used for the IVF. Using OPU-derived oocytes, this finding has already been reported in buffalo (Liang *et al*., 2008) and in *Bos indicus* (Underwood *et al*., 2010). Pontes *et al*. (2010), Senatore *et al*. (2010) and Presicce *et al*. (2010) showed the efficiency of *in vitro* embryo production in dairy *taurus* cattle utilizing sexed sperm and oocytes recovered by OPU, but the absence of control group (unsorted sperm) in all three experiments makes difficult to establish connections between the results of these studies and our data.

Table 1. Development rates of bovine blastocyst derived from ovum pick up oocytes and *in vitro* fertilized with unsorted or sex-sorted sperm.

Group	IVF Replicates	Total oocytes	Cleavage (%)	Blastocyst/Cleaved embryos (%)	Blastocyst/ Oocytes (%)
Unsorted	10	110	69.09[a]	31.58 (24)	21.82
Sex-Sorted	10	103	52.43[b]	29.63 (16)	15.53

Data with different superscripts (a, b) indicate values with significant differences ($P < 0.05$).

Comparing the IVF efficiencies of both sperm types, the use of sex-sorted sperm did not affect significantly the percentage of oocytes forming blastocysts compared to unsorted sperm. With the aim to simulated the conditions of commercial OPU-IVF programmes, in our experiment, denuded oocytes were used for IVF and embryo culture was performed with small numbers of embryos (five to eight), even the supposed poor quality of those oocytes and the reduced number of embryos in culture would have been a handicap in this study (Hazeleger *et al*., 1995; O'Doherty *et al*., 1997; Nagao *et al*., 2008; Salvador *et al*., 2011). Nevertheless, the efficiency of the IVF in our experiment was similar to other works (Chaubal *et al*., 2006; Zaraza *et al*., 2010).

In other studies where oocytes were derived postmortem from slaughterhouse ovaries, IVF data are variable. Zhang *et al*. (2003) and Puglisi *et al*. (2006) obtained analogous results to ours for cleavage and blastocyst rates, whereas Peippo *et al*. (2010) did not find differences between sex-sorted and unsorted sperm. This is in comparison to other studies reporting lower cleavage and blastocyst rates (Beyhan *et al*., 1999; Bermejo-Alvarez *et al*., 2010; Presicce *et al*., 2010) or lower blastocyst rate (Merton *et al*., 1997; Lu *et al*., 1999; Lu and Seidel, 2004; Wilson

et al., 2006 Morton *et al*., 2007). The reported differences may be due to varying methods of oocyte and bull selection, quality of sorted sperm or type of culture medium.

Effect of sex-sorted sperm on the timing of blastocyst formation and on the blastocyst morphology

Regarding the timing of blastocyst formation (Table 2), no significant difference was found between unsorted or sex-sorted groups at day 7, day 8, or day 9. In addition, the percentages of excellent or good blastocyst yielded were similar between unsorted or sex-sorted sperm (Table 2). The kinetic of blastocyst formation was not influenced by the type of spermatozoa (unsorted vs. sex-sorted) in the work of Carvalho *et al*. (2010). Morton *et al*. (2007) reported that bovine embryos derived from sex-sorted sperm had a similar timing of development, morphology, and cell number to those derived from unsorted sperm, but a lower mRNA abundance of some developmentally important genes. In contrast, two studies found similar mRNA abundance between embryos produced with sorted or unsorted spermatozoa (Bermejo-Alvarez *et al*., 2010; Stinshoff *et al*. 2012). Lu *et al*. (1999) also observed from one-half- to one-day delay of embryonic development when bovine oocytes were inseminated with sorted sperm. Hayakawa *et al*. (2009) on the other hand used sex-sorted bull sperm in a multiple ovulation and embryo transfer programme and the quality of the *in vivo* embryos produced was no different to that in unsorted groups.

Table 2. Effect of *in vitro* fertilization with unsorted or sex-sorted sperm on morphological embryo quality and timing of blastocyst formation on bovine pre-implantation embryos derived from ovum pick up oocytes.

Group	Total blastocyst	Percentage of excellent or good blastocyst*(n)	Timing of blastocyst formation (n)		
			Blastocyst rate at Day 7	Blastocyst rate at Day 8	Blastocyst rate at Day 9
Unsorted	24	87.50 (21)	10.53 (8)	30.26 (23)	31.58 (24)
Sex-Sorted	16	85.71 (12)	5.56 (3)	24.07 (13)	29.63 (16)

*International Embryo Transfer Society (IETS) morphological classification, Robertson and Nelson 1998. Blastocyst rate calculated from cleaved embryos.

In conclusion, OPU derived-oocytes fertilized with sex-sorted sperm cleaved less than those fertilized with unsorted sperm. The embryo development to blastocyst stage was not further disturbed and the morphological quality and timing of blastocyst formation were similar to normal IVF. Sex-sorted sperm and OPU-IVF are valuable tools to produce bovine embryos of predetermined sex and from individual donors.

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GENERAL DISCUSSION

Simulation of the oviductal microenvironmental conditions *in vitro* has been reported as beneficial for the embryo development. In our first experiment, although we tried to simulate oviductal environment, we observed that a short bovine oviductal fluid (bOF) oocyte treatment applied prior to *in vitro* fertilization (IVF) had no effect on fertilization parameters, cleavage, blastocyst rates both on parthenogenetic and *in vitro* fertilized bovine embryos and neither on morphological quality of *in vitro* fertilized blastocysts. Significant differences in the expression of only two genes (*G6PD* and *SOD2*) from *in vitro* fertilized blastocysts after a bOF treatment were observed. However, comparing the genetic expression between excellent or good blastocysts (Grade 1) and fair blastocysts (Grade 2) were found that bOF treatment reduce the differences in much more genes (four genes over the control). These data suggest that bOF treatment could be useful to mitigate some lacks on the lower quality (grade 2) blastocysts produced *in vitro*. Further studies are needed to determine the potential positive effect of bOF treament with a view to improve bovine embryo development.

One of the issues that ovum pick up (OPU)-IVF practitioners have to face is to culture small groups of oocytes/embryos from an individual donor. In our second and third experiment we have addressed this question. Firstly, in our second experiment, we observed that post-fertilization culture conditions, regarding number of embryos in culture, seem to be the most important step (in comparison to *in vitro* maturation and IVF), determining drastically impairment of the blastocyst yield when single and/or a low number of embryos (< 10 embryos) are in culture. On the other hand, it seems that the final part of the culture (from day 3 to day 8) could be crucial for the manifestation of this deleterious effect when a low number of embryos are in culture. In our third experiment, we tried to improve the culture conditions of small groups of embryos by the addition of a mixture of epidermal growth factor, insulin, transferrin, and

selenium (EGF-ITS) or by the WOW system. The addition of EGF plus ITS increased the quality of the embryos cultured in low number, whereas culture embryos in WOW system improved the development rates. The introduction of EGF and ITS in cultures as well as the use of WOW system could be a good strategies to increase embryo developmental competence when reduced number of embryos are in cultured.

Moreover, we have attempted to deal with the issue of heat stress on bovine oocytes in the fourth experiment of this thesis. So, we assessed the protective role of the melatonin during oocyte maturation and embryo development against the damage subsequent to oocyte heat stress, but also subsequent to the *in vitro* production of embryos (IVEP) conditions. The addition of melatonin at 10^{-4} M to heat-stressed bovine oocytes enhanced their blastocyst rate. However, when oocytes were matured under conventional *in vitro* conditions, none of the melatonin concentration tested (10^{-12}, 10^{-9} and 10^{-4} M) showed a significant effect compared with control group. Finally, melatonin at 10^{-3} M showed a harmful effect on bovine embryo development both in heat stressed oocytes and non-heat stressed oocytes. It would be interesting to confirm in future works whether the *in vivo* application of melatonin can avoid the reduction of reproductive efficiency of cattle exposed to heat stress conditions. Indeed, a recent study showed evidence that under heat stress conditions, melatonin treatment (subcutaneous implants) at dry-off period improved reproductive performance postpartum in high-producing dairy cows (Garcia-Ispierto et al. 2012, referenced in the Experiment 4).

Finally, in our fifth experiment, we used oocytes derived from OPU to test the effect of sex-sorted sperm in the IVF and their further embryo development and quality. The fact that the combination of different technologies may optimize the *in vitro*-produced sexed embryos and the scarce number of sex-sorted sperm studies based on OPU-derived oocyte motivated this study. Also, it should be mentioned that the fertility of sex-sorted sperm has been questioned in many studies and is still an issue. In our results we observed that oocytes fertilized with sex-sorted

sperm showed a lower cleavage rate but similar blastocyst rate compared to oocytes fertilized by unsorted sperm. Indeed, the blastocyst morphological quality as well as the timing of blastocyst formation was not different between sex-sorted and unsorted groups. We concluded that sex-sorted sperm and OPU-IVF are valuable genetic tools to produce bovine embryos of predetermined sex and from individual donors.

As we have showed previouly, the application of *in vitro* embryo technologies into the cattle industry, mainly in *Bos taurus*, is very limited. To change this state of the art, it is needed *in vitro*-produced embryos with at least similar pregnancy rate to those *in vivo* derived. A strong focus is still required to solve issues such as the lack of more suitable media for IVPE, the harmful effects of low embryo numbers on embryo quality, the excess of oxidative stress during IVPE and the impact of sorting on sperm and on further embryo development. In this regard, the information generated in this Thesis have provided a better understanding of IVPE, giving a chance for further studies.

CONCLUSIONS

Conclusion from Experiment 1:

1- A short bovine oviduct fluid oocyte treatment had no effect on fertilization parameters, cleavage, blastocyst rates both on parthenogenetic and *in vitro* fertilized bovine embryos and neither on morphological quality of *in vitro* fertilized blastocysts.

2- The gene expresion of *G6PD* and *SOD2* genes from *in vitro* fertilized blastocysts showed significant changes in their expression after a bOF treatment. Significant differences were also found for the expression of *SCL2A1, GPX1*, *BAX*, *AKR1B1* and *PLAC8* genes between excellent or good blastocysts and fair blastocysts.

Conclusion from Experiment 2:

3- Post-fertilization culture conditions, regarding number of embryos in culture, seem to be the most important step determining blastocyst yield. Culture of single and/or a low number of embryos (< 10 embryos) drastically impaired the efficiency of blastocyst yield.

4- It seems that the final part of the culture (day 3 to day 8) could be crucial for the manifestation of this deleterious effect with a low number of embryos.

Conclusion from Experiment 3:

5- The culture of embryos in the well of well system improved the development rates compared to the conventional culture drop with a small number of embryos.

6- The addition of a mixture of epidermal growth factor, insulin, transferrin, and selenium mitigated some of the harmful effects of low embryo numbers on embryo quality, increasing the average number of cells/blastocyst and decreasing the apoptosis rate.

Conclusions from Experiment 4:

7- The exposure time and the temperature of the heat stress *in vitro* treatments during the *in vitro* maturation are important factors in the subsequent embryo development. A sub-lethal maturation temperature (41.0ºC) during 20 h of maturation improved the blastocyst rate, however, a lower average number of cells/blastocyst was also observed. In contrast, a higher temperature (41.5ºC) and longer exposure time (24h) provoked a decrease on the development rates and the average number of cells/blastocyst.

8- In heat-stressed bovine oocytes, the addition of melatonin at 10^{-4} M to the maturation and culture medium enhanced the blastocyst rate. None of the melatonin concentration tested (10^{-12}, 10^{-9} and 10^{-4} M) showed a significant effect compared to the control group when oocytes were matured under conventional *in vitro* conditions.

9- Melatonin at 10^{-3} M in the maturation and culture medium showed a harmful effect on bovine embryo development both in non-heat stressed oocytes and heat stressed oocytes.

Conclusions from Experiment 5

10- Ovum pick up derived-oocytes fertilized with sex-sorted sperm cleaved less than those fertilized with unsorted sperm. However, the embryo development to blastocyst stage was not further disturbed and the morphological quality and timing of blastocyst formation were similar to normal IVF. Sex-sorted sperm and ovum pick up-*in vitro* fertilization are valuable tools to produce bovine embryos of predetermined sex and from individual donors.