LAIN URIEL OHLWEILER

PARTICULARIDADES DO ESPERMATOZÓIDE E DA CÉLULA SOMÁTICA NA INTERAÇÃO COM O OOPLASMA: O BOVINO COMO MODELO NA FIV E O SUÍNO COMO MODELO NA CLONAGEM

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LAIN URIEL OHLWEILER

Particularidades do espermatozóide e da célula somática na interação com o ooplasma: o bovino como modelo na FIV e o suíno como modelo na clonagem

Dissertação apresentada ao Curso de Mestrado em Ciência Animal, Área de Concentração em Reprodução Animal, do Centro de Ciências Agroveterinárias da Universidade do Estado de Santa Catarina (CAV-UDESC), como requisito para obtenção de grau de Mestre em Ciência Animal.

Orientador: Alceu Mezzalira

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Banca Examinadora:

Professor Dr. Alceu Mezzalira, Orientador
Universidade do Estado de Santa Catarina

Professor Dr. Arnaldo Diniz Vieira
Universidade Federal de Pelotas

Professor Dr. Marcos Henrique Barreta
Universidade Federal de Santa Catarina

Professor Dr. Fabricio Desconsi Mozzaquatro
Universidade do Estado de Santa Catarina

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RESUMO

PARTICULARIDADES DO ESPERMATOZÓIDE E DA CÉLULA SOMÁTICA NA INTERAÇÃO COM O OOPLASMA: BOVINO COMO MODELO NA FIV E O SUÍNO COMO MODELO NA CLONAGEM

O desenvolvimento embrionário depende da adequada interação nucleo-citoplasmática, o que é influenciado pelo tipo de célula doadora e pela qualidade do oócito receptor na clonagem, assim como por características dos espermatozóides e oócitos na fecundação *in vitro* (FIV). O primeiro estudo foi constituído de dois experimentos. O primeiro experimento avaliou o tipo de célula doadora de núcleo (células fibroblásticas - FIB vs. células mesenquimais derivadas de adipócitos - ADMSC), com diferentes citoplastos receptores (suíno – reconstruído com dois hemi-citoplasto suínos; mosaico – reconstruído com um citoplasto suíno e um citoplasto bovino; bovino – reconstruído com dois hemi-citoplastos bovinos), no desenvolvimento de embriões suínos, clonados por transferência nuclear de células somáticas (TNCS). Os cultivos celulares foram estabelecidos a partir de dois suínos de raças ameaçadas de extinção (casco de mula e moura), sendo os embriões reconstruídos por clonagem manual e cultivados *in vitro* por 7 dias, em meio PZM-3. Os grupos mosaico e bovino apresentaram produção embrionária menor que o grupo suíno (5,5; 1,9 e 18,0%, respectivamente). O grupo ADMSC-mosaico do animal moura apresentou produção embrionária intermediaria em relação ao controle e ao bovino, e superior ao grupo FIB-mosaico do mesmo animal. A porcentagem de blastômeros fragmentados em embriões clivados e mórulas foi superior nos grupos mosaico e bovino, em relação ao grupo suíno. A dinâmica de fusão, observada conforme a migração mitocondrial entre os citoplastos, foi diferente em função do citoplasto empregado. No segundo experimento foi investigado o efeito do inibidor de desacetilases “Scriptaid” no desenvolvimento embrionário *in vitro* dos grupos suíno, mosaico e bovino, utilizando-se células fibroblastos do animal moura. Os embriões reconstruídos foram expostos a 500 nM de Scriptaid por 12 h, iniciando a partir da ativação, sendo então cultivados em PZM-3 por 7 dias. A taxa de produção de blastocistos do grupo controle (9,2 vs. 17,3%) e mosaico (1,0 vs. 9,2%) aumentou com o uso de Scriptaid ($p < 0,05$), enquanto a proporção de fragmentos em mórulas reduziu no grupo mosaico (9,8 vs. 2,8%) ($p < 0,05$). No entanto, o uso de Scriptaid não aumentou a produção embrionária no grupo bovino. No segundo estudo, constituído de três experimentos, avaliou-se a influência de distintas qualidades de gametas na produção embrionária por FIV, em bovinos. Nos experimentos 1 e 3, foram avaliadas as taxas de produção embrionária no sétimo dia de cultivo. No experimento 2, dois touros de comprovada eficiência na produção embrionária *in vivtro,* foram utilizados na FIV de oócitos de qualidade boa e ruim. O touro 1 não mostrou diferença na produção embrionária com oócitos de qualidade boa (19,8%) ou ruim (12,7%). O touro 2 apresentou maior produção embrionária com oócitos bons (25,7%) do que com oócitos ruins (9,2%). No experimento 2, a capacidade penetrante dos dois touros foi avaliada em oócitos de qualidade ruim através da técnica de injeção espermática sub-zonal. A taxa de penetração, observada 3 h apos a injeção, foi menor no touro 1 (34,0%) em comparação ao touro 2 (44,3%) ($p < 0,05$). No experimento 3, o sêmen de ambos touros foi usado para injeção intra-citplasmática de espermatozóides com oócitos de

ambas qualidades. Não foi observado nenhum efeito de touro ou oócito na produção embrionária. Os resultados permitem concluir que o uso de moduladores da reprogramação como Scriptaid e tecnologias como a ICSI são alternativas adequadas para incrementar, ao menos em condições particulares, o desenvolvimento embrionário.

Palavras-chave: *Clonagem manual, clonagem inter-espécie, ativação embrionária, qualidade dos gametas.*

ABSTRACT

PARTICULARITIES OF SPERM AND SOMATIC CELLS IN THEIR INTERACTIONS WITH THE OOPLASM: THE IVF AS A BOVINE MODEL AND THE INTER-SPECIES CLONING AS A PORCINE MODEL

The adequate embryo development depends on proper nuclear-cytoplasmic interactions in the embryo. Such interactions are influenced by the donor cell type and quality of recipient oocyte on cloning, as well as by the characteristics of the sperm and the oocyte on in vitro fertilization (IVF). The first study (two experiments) investigated the effect of donor cell type (fibroblastic-like cells - FIB vs. adipocyte-derived mesenchymal stem cells - ADMSC), and host cytoplast (porcine – reconstructed with 2 hemi-porcine cytoplasts; mosaic – reconstructed with one-hemi-porcine and one hemi-bovine cytoplast; bovine – reconstructed using 2 hemi-bovine cytoplasts), on development of porcine somatic cell nuclear transfer (SCNT) embryos. Somatic cell cultures were established from two animals of endangered pig breeds (Mule-foot and Moura) and embryos were reconstructed by hand-made cloning and cultured for 7 days in PZM-3 medium. Mosaic and bovine groups produced lower blastocyst rates than porcine (5.5, 1.9 and 18.0%, respectively). The group ADMSC-mosaic from Moura animal showed an intermediate embryo development on porcine and bovine groups, which is higher than the FIB-mosaic group of the same animal. The percentage of fragmented blastomeres in cleaved embryos and morulas from the mosaic and bovine groups were higher than the porcine. The dynamic of fusion was different according the group of cytoplasm, as observed through mitochondria staining. In the second study (three experiments), we investigated the effect of the histones de-acethylase inhibitor "Scriptaid" on the in vitro development of the three groups of SCNT reconstructed embryos. Reconstructed embryos were exposed to 500 nM of Scriptaid for 12 h starting just after activation, being then cultured in PZM-3 for 7 days. The blastocyst rates in the porcine (9.2 vs. 17.3%) and mosaic (1.0 vs. 9.2%) groups were increased by Scriptaid treatment ($p < 0.05$), and the proportion of fragmented morulas was reduced in mosaic ($p < 0.05$). However, Scriptaid treatment did not increase embryo development in the bovine group. In the second study (three experiments) the influence of distinct oocyte and spermatozoa qualities in their interaction in embryo IVP by IVF was evaluate. On experiment 1 and 3 the blastocyst rates were evaluated (day 7). On experiment 2, two bulls were used for IVF with good or poor oocytes. Bull A did not show difference according the oocyte quality: good (19.8%) and poor (12.7%). Bull B showed higher blastocyst rates in good quality (25.7%) than poor quality oocytes (9.2%). On experiment 2, sperm penetrating capacity was evaluated for both bulls in oocytes of low quality, by sub-zonal sperm injection. The penetration rate observed 3h after the injection from bull A (34.0%) was lower than for bull B (44.3%) ($p < 0.05$). On experiment 3, both bulls were used for ICSI of good or low quality oocytes and no bull or oocyte quality effect has affected blastocyst rates. In conclusion, the use of reprogramming modulators such as Scriptaid, and alternative technologies such as ICSI are adequate to provide, at least under particular conditions, an increase in embryo development.

Key-words: Hand-made cloning, inter-species cloning, embryo activation, gametes quality.

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LIST OF ABREVIATURES

AC	Corrente Alternada (*Alternate Current*)
ANOVA	Análise de Variância (*Analysis of Variance*)
ART	Assisted Reproductive Technologies
Bl	Blastocisto (*Blastocyst*)
BSA	Albumina sérica bovina (*Bovine Serum Albumin)*
CIV (IVC)	Cultivo *in vitro* (*in vitro culture*)
COC	Complexos *cumulus*-oócito (*cumulus-oocyte complexes*)
DC	Corrente Contínua (*Direct Current*)
FCS	Soro fetal bovino (*fetal calf serum*)
FIV (IVF)	Fecundação *in vitro* (*in vitro fertilization*)
HMC	Clonagem manual (*Handmade Cloning)*
HM	Meio de manipulação (Hepes-buffered medium - M-199)
ICSI	Injeção intra-citoplasmática de espermatozóides *(Intracytoplasmic sperm injection)*
ICM	Massa celular interna (*inner cell mass*)
MII	Metáfase II (*Metaphase II*)
MIV (IVM)	Maturação *in vitro* (*in vitro maturation*)
mRNA	RNA mensageiro (*Messenger RNA*)
mSOFaa	*modified Synthetic Oviductal Fluid medium supplemented with amino acids*
PIV (IVP)	Produção *in vitro* (*in vitro production*)
6-DMAP	6-Dimetil aminopurina (*6-DymethylAminoPurine*)
SUZI	Injeção sub-zona de espermatozoide *(sub-zona sperm injection)*
TCN	*Total cell number*
TN (NT)	Transferência Nuclear (*Nuclear Transfer*)

TNCS (SCNT)	Transferência Nuclear de Células Somáticas (*somatic cell nuclear transfer*)
TNCSi/iSCNT	Transferência nuclear de célula somática inter-espécie *(inter-specie somatic cell nuclear transfer)*
OT	Transferência de ooplasma *(ooplasm transfer)*
WOW	(*Well-of-the-Well)*

SUMMARY

1. INTRODUCTION

Epigenetic alterations involving changes in global DNA methylation and histone modifications are necessary to give to the embryo the ability to initiate the transcription of the genome followed by the development of two different cell lineages present in the blastocyst (FULKA et al., 2008). These events occur in all embryos derived from any methodology. The main difference is that the fertilized embryo starts with two haploid nuclei while the nuclear transfer embryo start with a diploid nucleus.

The oocyte is the unique common factor that is involved in any embryo production modality. The previous statement is true for *in vivo* embryo production, *in vitro* fertilization (IVF), intra-cytoplasmic sperm injection (ICSI) or cloning by nuclear transfer (NT). There is emerging evidence, though, that inherent oocyte quality is the primary factor that determines whether an incipient embryo will develop appropriately or succumb to premature embryonic failure (COMBELLES and RACOWSKY, 2005). The oocyte quality, classified basically by the morphological aspects, indeed can be classified in three individual aspects, yet interconnected processes: nuclear maturation, cytoplasmic maturation, and molecular maturation (SIRARD et al., 2006). The nuclear and cytoplasmic maturation can be evaluated by trained technicians, however, the molecular maturation is consider as an ‘‘invisible’’ component of the oocyte. The molecular maturation involves the acquisition and proper processing of mRNAs, the proper ‘‘priming’’ of the biochemical cascades responsible for oocyte activation, and the initiation of embryo development after fertilization (SIRARD et al., 2006). The present embryo production rates for domestic animals oscillate from 20 to 50% for the well morphological selected oocytes. This strongly suggests that significant determinants of intrinsic oocyte quality hidden from view are the molecular mediators of fertilization, oocyte activation, and downstream development (ISOM et al., 2012).

Known molecular factors hidden from the classical gamete classification as well as the inadequate factors during IVF as particularities observed in morphology and behaviour of sperm (HANSEN et al., 2009) and oocytes (KHURANA and NIEMANN, 2000; HANSEN et al., 2009) generally are closely related with failures in embryo production. Some of the events that occur during the normal development of IVF embryos are the adequate interaction and synchrony between male and female pronucleus (LAURINČÍK et al., 1998) and the adequate embryo genome activation. The sperm ability to activate correctly the oocyte, inducing the oocyte cleavage, not necessarily is related with the capacity of sperm to de-methylate adequately and develop a good quality male pronucleus. The opposite are also true, the plenum oocyte activation is not guarantee of development of a good quality female pronucleus. Experimentally, to overpasses deficiencies of embryo production, researchers use pre-tested semen to assure the sires are proven for *in vitro* fertility, and select oocytes based on morphological good quality, for the IVF procedures (SIRARD et al., 2006). However, mainly in commercial IVF programs that are established and profitable for the bovine, the specific breeding wanted causes as these variables cannot be controlled by the simple selection of only the good gametes.

In somatic cell nuclear transfer (SCNT), the events that control the embryo development are more problematic. Cell reprogramming events consist of the morphological remodelling of the donor nucleus, including the breakdown of the nuclear membrane, initial chromatin condensation, spindle assembly, the release of proteins from the nucleus into the cytoplasm, the movement of proteins from the cytoplasm into the nucleus, and the formation of a pronucleus-like structure after activation (BUI et al., 2006). By this way, the cell type as well as the cytoplast quality and a good interaction between both are key points, even in the early stages of the embryo development. Besides several factors that affect the cloning

efficiency, one of the best established aspects is that after SCNT, the genome of the donor cell does not respond to the active de-methylation activity of the oocyte cytoplasm, and in most cases, the DNA methylation level remains higher than in normal embryos (HAN et al., 2003; BEAUJEAN et al., 2004). Jin et al., (2007) showed that the use of nucleus derived from mesenchymal stem cells can increase the development of porcine embryos. However, the strategy of using cells less differentiated as donor of nucleus is not capable alone of increase the efficiency of cloning (MARTINEZ-DIAZ et al., 2010). By the other hand, the use of nucleus with different competence of reprogramming is a good strategy to study the nucleus-cytoplasmic interaction and activity.

A proposed strategy to overpass this situation is the treatment with histone deacetylase inhibitors (HDACi) such as Trichostatin-A (TSA) and Scriptaid, which stimulate nascent mRNA synthesis, increases histone acetylation, and enhance the transcriptional activity and histone acetylation.

The TSA treatment has no increment on bovine fertilized embryos production (IKEDA et al., 2009; OLIVEIRA et al., 2010) and rat fertilized or ICSI embryo production (YOSHIZAWA et al., 2010). However, it increases the development until blastocyst stage in cloning in pigs (LI et al., 2008; YAMANAKA et al., 2009; BEEBE et al., 2009; MARTINEZ-DIAZ et al., 2010), cattle (ENRIGHT et al., 2003; DING et al., 2008; AKAGI et al., 2011; SRIRATTANA et al., 2012), rabbits (SHI et al., 2008), mouse (BUI et al., 2010), and increase production of offspring in mice (KISHIGAMI et al., 2006, 2007), the weight at birth pig cloning tended to be higher (MARTINEZ-DIAZ et al., 2010) and the pregnancy rate in bovines (SRIRATTANA et al., 2012). The *in vitro* and *in vivo* beneficial effects of TSA treatment also depend of the donor cell type, mainly in pigs (MARTINEZ-DIAZ et al., 2010).

However, TSA treatment has the adverse effect to be teratogen (SVENSSON et al., 1998) and more severe, might causes placenta megaly in mouse (KISHIGAMI et al., 2006).

Scriptaid, which is less toxic than TSA has also shown a significantly improvement in the production of cloned embryos in pigs (ZHAO et al., 2009, 2010), mice (VAN THUAN et al., 2009) and bovines (AKAGI et al., 2011, WANG et al., 2011). However, Scriptaid treatment also did not show beneficial effect in parthenogenetic and IVF embryo production of porcine (ZHAO et al., 2009) and bovine (BARRETA, 2012).

One of the species that show the better constancy about *in vitro* and *in vivo* embryo development in IVF and SCNT is the bovine. This is one of the reasons why the bovine cytoplasts are also one of the most used to study the inter-specie somatic cell nuclear transfer (iSCNT). One of the early attempts of iSCNT using the enucleated bovine oocyte as recipient cytoplast was reported by Dominko et al. (1999). The interest for study iSCNT increased in the last years because of the appeal of preserving endangered animals (that are threatened with extinction), and also by the excellent potential to study nucleus-cytoplasmic interactions, as well as the embryo development.

As the nucleus-cytoplasmic compatibility are problematic in iSCNT embryos, a high *in vitro* developmental block from 8- to 16-cell stage has already been observed in the pig (YOON, 2001), horse (LI et al., 2002; SANSINENA, 2002), llama (SANSINENA et al., 2003), Siberian tiger (HASHEM et al., 2007), monkey (LORTHONGPANICH et al., 2008; SONG et al., 2009), marbled cat (*Pardofelismarmorata*) and flat-headed cat (*Prionailurusplaniceps*) (THONGPHAKDEE et al., 2010), and Tibetan antelope (ZHAO et al., 2007). An explanation for this block is that the mitochondria are incapable of maintaining proper respiration levels when species are more taxonomically apart from each other (KENYON and MORAES, 1997). This fact was confirmed by (WANG et al., 2009) in iSCNT

embryos, where they found the stage of 8- to 16 cells to show a significantly low level of ATP production. The strategy of combining cytoplasts of one species with a high availability of oocytes with cytoplasts of a species with low availability (i.e in risk of extinction) appears as a good technique to try to supply nucleus-cytoplasmic deficiencies caused by the incompatibility. Moreover, it also offers more cytoplasm content to support the embryo development. This idea is more viable in the hand-made cloning (HMC) technique, proposed by PEURA et al. (1998) and modified by VAJTA et al. (2001), where the association of two cytoplasts is easily performed.

With the purpose to evaluate nucleus-cytoplasmic interactions, regarding the oocyte and embryo genome activation, two studies have been performed: 1- Using the porcine as model with the association of bovine cytoplasm, we evaluated the embryo development characteristics of intra and inter-species cloning. 2- By combining bovine gametes with distinct morphological and behavioural characteristics, we evaluated the characteristics of embryo development. In both works the embryonic development was evaluated, during the early stages of the cloning: reconstruction/fusion, oocyte activation/fertilization, pronuclear formation and blastocyst development. After the identification of some of the key points of the blocking of embryo development, strategies such as the use of HDACi in cloning, and ICSI in fertilized embryos have been as an attempt to increase the efficiency of both techniques.

2. LITERATURE REVIEW

2.1 GAMETES MANIPULATION: EVOLUTION OF EMBRYO PRODUCTION TECNIQUES AND THE UNDERSTANDING OF EMBRYO DEVELOPMENT BIOLOGY

On early 1900s, Hans Spemman performed experiments considered elegant and genial, using amphibians as a model (TAGARELLI et al., 2004). His pioneering studies involved embryo splitting for studying events of early embryo development. Those have been indeed the first reports of clone production, where genetically identical individuals have been first artificially through embryo splitting. The same researcher started nuclear transfer studies about the year of 1920, and had been awarded with a Nobel Prize of Physiology/Medicine later, on 1935.

Hans Spemman tried to answer, for the first time, the following question: “Do cell nuclei change during development?” Using increasing development stages nuclear transfer with amphibian eggs (TAGARELLI et al., 2004) he was aiming to determine when exactly the developmental potential of nuclei would become restrict. At that point, many years ago, he was disseminating the principles of animal cloning. Those principles happen to be the base for studies on cell differentiation and totipotency.

After Spemman death, in 1941, studies on animal cloning had only been continued as late as in the 1950s, by Robert Briggs and Thomas King (BRIGGS and KING, 1952), using frogs as a model.

With a few understanding of *in vivo* embryo development added by a slight success in bovine embryo production using artificial insemination, the studies were directed to reach the total control of embryo developmental biology. To this, *in vivo* ovulated oocytes Brackett et

al. (1982) reported the birth of the first calf derived from *in vitro* fertilization (IVF). Few years later, in 1986, the first offspring derived from ovine blastomers was reported (WILLADSEN, 1986), again using *in vivo* maturated oocytes. The successful results with the ovine have been soon reported for the bovine, when Prather et al. (1987) and Robl et al. (1987) reported the birth of the first calves derived from blastomeres nuclear transfer (NT). This reinforced the process was possible to repeat, at that time, as long as totipotent or multi-potent cells were used.

Even with reports of positive results on *in vitro* fertilization and cloning by nuclear transfer, the efficiency was still considerably low to start considering to offer the technique in large scale. By the way, Lu et al. (1987) reported the birth of twin calves after *in vitro* maturation, fertilization and culture. This has been certainly a huge step towards the ability to control, *in vitro*, almost all embryo developing stages.

At this point, the use of blastomeres was still an impairing factor for cloning, due to the reduced number of identical offspring that could be eventually produced, derived from a single embryo. The search for an executable technique, to be used with undifferentiated cells has led to the use of cells derived from day-9 embryonic disc, from passages 6 to 13. Again, the results reported the birth of viable offspring (CAMPBELL et al., 1996). This improvement raised the possibility of using a higher number of identical cells, and maintained for a longer period of time.

Despite the great importance of all successive studies, the international acknowledgment of cloning happened on 1997, after results of 1996 have been reported. This breakthrough for the scientific community was the birth of Dolly, the sheep, cloned from differentiated somatic cells obtained from an adult (6 years old) donor (WILMUT et al.,

1997). This has opposed the biological dogma that persisted from as early as the XX century, and said a differentiated cell, already specialized, could not go back to its totipotent status.

The achievements reported above were not devoid of criticism and skepticism, though reduced by the reporting of the first mouse offspring derived from differentiated (cumulus) cells. The female mouse was born on 1997, (WAKAYAMA et al., 1998). Few time later, the first calf derived from fetal somatic cells was born (CIBELLI et al., 1998), being this report increased in importance as the calves were transgenic. During the same year, the first calf derived from adult differentiated cells nuclear transfer was born (KATO et al., 1998), providing a more solid pathway for researchers to continue.

2.1.1 IVF dissemination and its obstacles

Based on the first promising results on bovine IVF, the technique has been extrapolated for other domestic species. However, even after obtaining IVF viable offspring derived from different species such as sheep, goats, porcine, equine, buffalo and others, the efficiency does not increase over 20 to 50% of embryo development. Several factors affecting embryo development efficiency. They can regard gametes manipulation (culture media, culture conditions and technician skills) or gametes donor intrinsic characteristics and particularities, as well as oscillations regarding gametes quality. Despite the initial factor responsible for reducing embryo production, failures on nuclear reprogramming and genome activation appear as the most observed consequences.

2.1.2 Cloning: current status and impairing factors

Even with a considerable low efficiency of cloning (WESTHUSIN et al., 2001; RENARD et al., 2002), few years after the first somatic cell cloning offspring were born, a number of companies started to offer cloning services. A considerable number of companies, not long time later, failed.

Currently, the main role played by cloning is to be used as a tool for producing transgenic animals, and also to supply the demand for cloning superior animals. Recently, the biotechnology promise is the regular delivery of transgenic animals for use in biomedical drugs production, and not only as models for research. The first encouraging results on transgenic animal production were reported by Hammer et al. (1985), with micro-injection of rabbits, sheep and pigs pronuclear stage embryos. The efficiency of this protocol ranges around 10 to 15% among the animals produced. In 1997, Schnieke et al. (1997) reported the birth of the first transgenic sheep produces by NT, with the great efficiency of 100% of transgenic among animals born. In this context, cloning of genetically modified animals has become extremely applicable, although not yet efficient. Animals with the ability to secrete specific molecules by their saliva, blood, urine and mainly, in milk, are highly needed by biomedicine and agriculture.

Since Dolly the sheep was born, dozens of species have already been cloned, derived from a large variety of donor cell types. Nonetheless, despite the large success among species currently cloned up to now, the early detection of pregnancy abnormalities of even before, during early embryogenesis and organogenesis, is not possible yet. The understanding of such biological events that play an important role on epigenetic reprogramming after somatic cell

NT is not only an important step to reduce economical and time-wasting losses, but will represent another important breakthrough toward elimination of such problems.

Two of the determining factors for cloned embryo development are the nuclear donor and also the recipient cytoplast (usually an oocyte or an early zygote). Their interactions are, in fact, the most important factor or success.

It is known that different cell lines derived from the same donor are responsible for producing distinct embryo developing rates. Moreover, the same cell type from distinct donors may generate distinct embryo production efficiencies. This information illustrates how important it is to understand how nucleus-cytoplasm interactions occur and what exactly is the role played by them during nuclear reprogramming and embryo genome activation.

The recipient cytoplasm plays a key role in reprogramming the genome donor, however the ooplasmic components that are responsible for post-fertilization reprogramming events is not likely to be enough to alter the key-points for nuclear differentiation after SCNT (BIRD, 2002). This process is also known as “erasure” of somatic differentiation status (CEZAR, 2003).

The level of synchrony between cytoplast and karyoplast (nucleus donor) as well as the embryo activation process have already showed to influence embryo reprogramming and development of clones (FULKA et al., 1996; BORDIGNON and SMITH, 2006). Furthermore, the cytoplasmic mosaicism and the level of heteroplasmy caused by the fusion of distinct cytoplasts during the HMC steps might either impair or increase the capacity of cloned embryo reprogramming and developing (LIU and KEEFE, 2000; ALBERIO et al., 2001).

2.2 Strategies to increase efficiency of assisted reproductive technologies

With the aim of increasing embryo production efficiency among the different species, different assisted reproductive technologies (ARTs) have emerged. Besides they can overpass specific particularities intrinsic to each species, they have also been responsible for raising new knowledge regarding the embryo development events.

2.2.1 Intra-cytoplasmic sperm injection (ICSI)

About a century ago, G. L. Kite was one of the pioneers in the field of microinjected spermatozoa and the complete result of his experiment was never published (HIRAMOTO, 1962). These studies re-started a half of a century later with Y. Hiram to using sea urchin eggs (HIRAMOTO, 1962). Some year after the ICSI technique has been proposed with the main objective in attempt to overpass the negative aspects of non-fertilization or polisher (HAFEZ, 2003). However, this technique also presents some disadvantages, such as the need for an additional chemical activation for species such as equine, porcine and ruminants. Still, the need for additional chemical activation may eventually reinforce the opportunity for an adequate oocyte activation.

2.2.2 Sub-zonal sperm injection (SUZI)

The difficulty on reaching high oocyte fertilization rates has initially been observed for human IVF. This motivated the search for alternatives to revert these negative results. One of the proposed alternatives for resolving this issue has been the SUZI (URNER et al., 1993). Anyhow, to accomplish the acceptable fertilization rates, the technique initially consisted of

the injection of 6 to 12 spermatic cells per oocyte, resulting in a high polispermy rate (LIPPI et al., 1993).

The main importance of SUZI has been the possibility of evaluating more narrowly the particularities of sperm-oocyte interactions and its further effects on embryo development (LI et al., 2003).

2.2.3 Ooplasm transfer (OT)

Other than ICSI and SUZI techniques that advocated mainly for reverting problems related to male gametes, the OT has been proposed in 1980 decade (MUGGLETON-HARRIS and WHITTINGHAM, 1982) aiming to supply oocyte deficiencies. The purpose of this technique is to transfer a small ooplasm amount (ranging from 10 to 30% of total oocyte volume), from an excellent/good quality, to a low quality oocyte, in an attempt to aid the embryo development with this good cytoplasm boost (HAWES et al., 2001). Although controversial, the OT technique (JOHN et al., 2002; DONG et al., 2006) has already been used for human ARTs where it solved early- and mid- pregnancy losses. It has also been used in inter-species cloning (SANSINENA et al., 2011).

One interesting fact about HMC is that this cloning technique actually uses ooplasm transfer, by the reconstruction of one single embryo using the cytoplasm derived from 2 distinct oocytes. As the HMC dispenses the use of micro-manipulation working station, the enucleating is based on the oocyte manual splitting, and to overcome this cytoplast volume loss (of approximately 50%), 2 hemi-oocytes are used to recover the original volume (to 100%).

2.2.4 Inter-species somatic cell nuclear transfer (iSCNT)

What impairs most the maintenance of a wide range of genetic variability is the momentary commercial interest for determined strains and/or species of animals. The maintenance of animals that carry a high genetic value, and that are for many times threatened with extinction is very expensive and currently almost non-viable, due to their current low applicability. Thus, genetic banks generated from their cells are of a huge importance for preservation of genetic variability, and for reducing costs that would be extremely high for keeping these herds away from inbreeding.

The maintenance and also recovery of animal species can also be questioned due to the fact that it goes against the natural selection evolutionary process. However, it is still anyway more interesting to keep a genetic bank with gametes and cells stored for long term, even if the utility of such genomes is not promptly visualized. This enhances the opportunity to insert important characteristics that would be otherwise simply lost to industrial breeding.

When a certain species in already threatened with extinction, the availability of cytoplasts (i.e. oocytes) is severely reduced. To this, the solution appears as the application of inter-species cloning by nuclear transfer, for recovering and preserving not only the genotypes, but also the epigenetic variability of these animals.

The iSCNT consists of the use of cytoplasts and karyoplasts from distinct species. The results obtained until now suggest that the cytoplasts obtained from a given species might acquire the competence needed to play a role as a nucleus recipient (LOI et al., 2007), promoting subsidies to additional studies on cell reprogramming. The efficiency of this technique is lower than the conventional cloning, due to a number of factors such as failure on nuclear donor reprogramming, mainly caused by a deficient action of histones soon after

oocyte activation, poor respiratory efficiency of embryos and also lack of the minimum ATP level availability, mainly from the stage when cells start to become compact. This corroborate to failure on genome activation that per se would already be a serious cause of embryo losses. Therefore, according to the state above, the SCNT between different species with the aim of understanding the distinct events that play a role on the technique efficiency is per se a tool to understand the events affected by its application. Due to establishing a relationship with early embryo developing events, there has been an increasing interest from the scientific community for the iSCNT.

Although wild animals cloning has already reported encouraging results using recipient cytoplasts from distinct species, not only for embryo production up to the blastocyst stage, but also with alive, although not viable offspring (VOGEL, 2001; FOLCH et al., 2009). Little is known regarding what total or partial mitochondrial heteroplasmy involves in these embryos, fetuses of born animals. Also little is known regarding their nuclear reprogramming process. Due to the fact that so many uncontrolled factors affect iSCNT success, we can infer that the results are positively surprising and that cloning provides great expectations on preservation of threatened, or even extinct, species.

The strategy of combining cytoplasts from a certain species from which oocytes are more available and the embryo developing efficiency is better to cytoplasts from another species, phylogenetically close, is perfectly viable using the technique described by Peura et al. (1998) and modified by Vajta et al. (2001). This has been an alternative to increase the inter-species cloning efficiency. This promising tool may promote the cytoplasmic hemi-complementation that is a feature of the handmade cloning. This might reduce the negative effects of inter-species heteroplasmy through the combination of cytoplasts from the threatened species to cytoplasts from a non-threatened one.

2.2.4.1 The reprogramming capacity from distinct cell types

Fibroblasts are the most common cell type used for cloning. They are relatively simple to obtain and their population is wide. As fibroblasts are differentiated cells they are very stable in culture, conversely they are known to be difficult to be reprogrammed exactly to its stability, what makes the role played by the oocyte, during reprogramming, way more difficult.

Recently, mesenchymal stem cells have showed to be an important alternative to replace fibroblasts on SCNT. Such cell type presents a high tissue repairing and healing capacity, as well as self-renewal ability (HUANG et al., 2011). As they provide high differentiation plasticity in different cell lines the interest on them not only for cloning, but also for stem cells therapy, has been significantly increased. The mesenchymal cells can be obtained from different tissues, being the most used sources the bone marrow and the fat tissue, where it is found a higher population of them.

All these advantages presented by mesenchymal cells are generating a great expectation when it comes to increase the cloning efficiency. The high differentiation capacity displayed by the mesenchymal cells, associated to the complementation of a homologous cytoplasm during embryo reconstruction may represent a huge step to be taken by iSCNT.

3. ARTICLE 1:

THE ROLE OF THE OOPLASM TYPE AND THE DEACETYLASE INHIBITOR SCRIPTAID IN PORCINE INTER-SPECIES SOMATIC CELL NUCLEAR TRANSFER

ABSTRACT

The adequate embryo development in cloning depends on proper nucleus-cytoplasmic interactions, what is influenced by the donor cell type and recipient oocyte quality. In this study the first experiment investigated the effect of the donor cell type (fibroblastic-like cells - FIB vs. adipocyte-derived mesenchymal stem cells - ADMSC), and host cytoplast (porcine – reconstructed with 2 hemi-porcine cytoplasts; mosaic – reconstructed with hemi-porcine and hemi-bovine cytoplast; bovine – reconstructed using 2 hemi-bovine cytoplasts), on development of porcine somatic cell nuclear transfer (SCNT) embryos. Somatic cell cultures were established from two different pigs, both of endangered breeds (Mule-foot and Moura). Embryos were reconstructed by hand-made cloning and cultured for 7 days in PZM-3. Mosaic and inter-species groups produced lower blastocyst rates than controls. The group ADMSC-mosaic from Moura animal showed embryo development rate intermediate between the control and inter-species groups, and higher than the FIB-mosaic group of the same animal. The percentage of fragmented blastomeres in cleaved embryos and morulas from the mosaic and bovine groups were higher than in the control. The dynamic of fusion was different according the cytoplasm reconstruction, observed by mitochondria staining. In the second experiment we investigated the effect of the histones de-acethylase inhibitors “Scriptaid” on the in vitro development of the porcine, mosaic and bovine SCNT reconstruction models. Activated embryos were exposed to 500 nM Scriptaid during 12 h, and then washed and cultured in PZM-3 for 7 days. The blastocyst rate in the control and mosaic groups was increased by Scriptaid treatment, and the proportion of fragmented morulae reduced in mosaic. However, Scriptaid treatment did not increase embryo development for the bovine group. The results evidenced that the use of the homologous cytoplasm complementation for a certain type of cells help the embryo development of the iSCNT embryos. In a general point of view, for the mosaic reconstruction the ADMSC cells have a better embryo development than the fibroblast cells. The use of reprogramming modulator as Scriptaid is an adequate alternative to provide at least a partial increase in embryo development.

Key-words: Hand-made cloning, ADMSC, embryo activation, gametes quality.

Introduction

The ooplasm constitutes an unique mixture of factors that is critical for a successful reprogramming of the haploid maternal and paternal genomes at fertilization, as well as of the diploid somatic cell genome after somatic cell nuclear transfer (SCNT). As oocytes are more readily available in some species, such as cattle and pigs, than in others, the possibility of producing inter-species or even inter-generic SCNT embryos is promising for basic and applied research. Few live inter-species clones have been produced (LANZA et al., 2000; LOI et al., 2001; GÓMEZ et al., 2004, 2008, 2009; KIM et al., 2007; FOLCH et al., 2009; YANG et al., 2010) and all of these had been produced from cells reprogrammed after transfer into enucleated oocytes, that is, ooplasm, of the same genus. Inter-generic SCNT has so far only resulted in pregnancies that did not go to term (DOMINKO et al., 1999; CHEN et al., 2002; YIN et al., 2006). Recent research on inter-species (between different species of the same genus) and inter-generic SCNT (between different genii) has focused on improving the developmental rates of the embryos by modifying single steps of the SCNT protocol and the subsequent culture conditions (HASHEM et al., 2007; ZHAO et al., 2007). Only few reports studied initial interaction between the recipient ooplasm and the donor genome required for adequate reprogramming and initiation of embryonic development (WANG et al., 2009).

When it comes to preservationism, when a given animal species is threatened with extinction, the availability of recipient cytoplasts for cloning becomes an issue. The inter-species cloning appears as an alternative to overcome this situation, as well as the inter-generic cloning. Results on inter-species and inter-generic cloning suggest that cytoplasts from a given species can acquire competence to work as nucleus recipients in the host species (LOI et al., 2007), giving rise to still more studies regarding cell reprogramming. However, inter-species and inter-generic cloning efficiency is still low in comparison to conventional

cloning, due to a number of factors affecting embryo reprogramming, and a deficient operation of histones soon after embryo genome activation (MARTINEZ-DIAZ et al., 2010) arises as one of the main reasons for failure. Not only the inter-species and inter-generics cloning is an important tool to provide alternatives for endangered species, it is also one of the most exciting tools for studding early development events and the roles played by the ooplasm during this complex circuitry that rules mechanical and molecular pathways of genome activation and embryo reprogramming. This study aimed to evaluate inter-species ooplasm interactions on early porcine clone embryos as well as to elucidate peri- fusion and activation events, in an attempt to increase the global efficiency of cloning.

2 Materials and Methods

2.1 Chemicals

Unless otherwise indicated, chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 Experimental design

Experiment 1 analyzed the embryo development of porcine cloning reconstructed with three different cytoplasts combinations: porcine (porcine + porcine), mosaic (porcine + bovine) and bovine (bovine + bovine). The porcine group was considered as intra-species cloning, and the mosaic and bovine groups were considered as inter-species cloning. As cell donors, two cell types (fibroblast and adipocyte derived mesenchymal stem cells – ADMSC) were derived, each one from two distinct pig donors. Embryo development evaluation criteria were cleavage rate (evaluated on day 2 after activation), morula and blastocyst formation (evaluated on day 7

after activation), the proportion of fragmented blastomeres present in cleaved embryos and morulas, as well as the total cell number in the blastocysts. Mitochondrial migration through the fused cytoplasts as well as pronuclei swelling in the three groups of cytoplasts, were additionally evaluated.

In an attempt to increase the inter-species cloning development, the experiment 2 used the deacethylases inhibitor, Scriptaid treatment for the three groups of cytoplasts (porcine, mosaic and bovine) using fibroblast cells of the Moura animal. The same evaluations of the experiment 1 were performed, as well as the estimation of the blastocysts inner cell mass proportion.

2.3 Animal welfare

All animal procedures were approved by the Animal Care and Use Committee of Santa Catarina State University, and were in compliance with the guidelines from the Brazilian Council of Animal Care.

2.4 Nuclear donor cells collection and handling

Fibroblast cell cultures were derived from an ear biopsy of one three months old female Moura gilt and one five months old, Mulefoot gilt. The primary cultures were derived by explantion. Adipocyte derived mesenchymal stem cells (ADMSC) where obtained by a surgical collection of adipose tissue from the neck region of the same pigs described above, being dissociated by collagenase digestion. Cell cultures were established, expanded and cultured at 38.5°C, with 5% CO_2 in air and 95% of humidity. Cells were cultured in DMEM culture medium, supplemented with 0.22 mM sodium pyruvate, 26.2 mM sodium bicarbonate,

10,000 IU/mL penicillin G, 10mg/mL streptomycin sulfate, and 10% fetal calf serum (FCS) (Nutricell, SP, Brazil). Cells were cultured up to 90% confluence, being either passage or cryopreserved for further utilization. Cryopreservation was performed in 0.25 mL plastic straws in culture medium with 10% of dimethyl sulphoxyde.

For cloning, cells were thawed at least 72 hours before the cloning, and only cells at a high confluence (> 85%) and up to the fourth passage were used.

2.5 Oocyte preparation

2.5.1 Porcine oocytes

Porcine cumulus–oocyte complexes (COCs) were aspirated from 3–6 mm of diameter follicles from ovaries of pre-pubertal gilts collected from a local abattoir and transported to the laboratory for up to 6 hours. Only COCs completely surrounded by cumulus cells, with either a homogeneous or an evenly granulated cytoplasm were selected. Groups of 50 COCs were cultured in 0.4 mL of maturation medium using four-well dishes (Nunclon, Roskilde, Denmark) in a humidified atmosphere of 5% CO_2 in air, at 38.5°C and 95% of humidity. Maturation medium consisted of TCM199 (TCM-199 with Earle's salt, 25mM HEPES), supplemented with 25% (v/v) porcine follicular fluid, 0.1% polyvinyl alcohol (PVA), 0.1 mg/mL of cysteine, 10 ng/mL of epidermal growth factor (EGF; Invitrogen), 0.91 mM sodium pyruvate, 3.05 mM D-glucose, 0.5 mg/mL luteinizing hormone (LH, Lutropin, Bioniche, Animal Health, Canada), 0.01 UI/mL of follicle-stimulating hormone (FSH, Folltropin, Bioniche, Animal Health, Belleville, Ontario, Canada), 1mM of dibutyryl cyclic adenosine monophosphate (dbcAMP), 100 IU/mL of penicillin G and of 0.1 mg/mL streptomycin sulfate. After 22 h of maturation, oocytes were transferred to the same

maturation medium without LH, FSH, and dbcAMP for an additional 17 h under the same conditions.

2.5.2 Bovine oocytes

Bovine COCs were aspirated from 2–8 mm of diameter follicles from ovaries collected from a local abattoir and transported up to 6 hours to the laboratory. Only COCs completely surrounded by cumulus cells, with homogeneous or with an evenly granulated cytoplasm were selected. Groups of 50 COCs were cultured in four-well dishes containing 0.4 mL of maturation medium using four-well dishes in a humidified atmosphere of 5% CO2 and 95% air at 38.5 °C for 18 hours. Maturation medium consisted of TCM199 (TCM-199 with Earle's salt, 25mM HEPES), 10% of estrous mare serum (EMS), supplemented with 26.2 mM of sodium bicarbonate, 0.2 mM of sodium pyruvate, 0.01 UI/mL of FSH, 0.5 mg/mL of LH, 10 ng/mL of EGF, 10,000 IU/mL of penicillin G and 10mg/mL of streptomycin sulfate.

2.6 Nuclear transfer, oocyte activation, and embryo culture

Matured porcine oocytes were submitted to successive pipetting in HEPES-buffered M-199 with 20% FCS (HM20) for cumulus cells removal, and the oocytes were cultured in HM20 supplemented with 0.4 µg/mL demecolcine and 0.05M sucrose for 40 to 60 min. After rinsed in HM20, the partial zonae digestion was performed by exposure to 0.19% pronase in HEPES-buffered M-199 with 0.01% PVA and 25% FCS during 30 sec. After a recovery interval of at least 30 minutes in HM20, the oocytes with a loose and thin zona pellucida were incubated in 2.5 µg/mL cytochalasin B in HM20, in groups of up to 3 oocytes in 5 µL drops under mineral oil and were hand-bisected. Bisected halves were than stained with 10 µg/ml of

bisbenzmide 33342 (Hoechst) in HM20, being each hemi-oocyte placed into 2 μL drops in the same medium into a 60 mm Falcon Petri dish under mineral oil. The selection of cytoplasts was performed under ultra violet (UV) light in an epifluorescence microscope. Selected porcine cytoplasts were removed from Hoechst screening dish and maintained in HM20 until the embryo reconstruction.

The bovine COCs were submitted to successive pipetting in HM with 10% FCS (HM10), for cumulus cells removal, followed by the selection of maturated oocytes by the presence of the first polar body. Zona pellucida removal was performed in 0.5% pronase in HEPES-buffered M-199 with 0.01% PVA during 30 sec. Zona-free oocytes were rinsed several times in HM10, incubated in 5 μg/mL cytochalasin B in HM10 in groups of up to 3 oocytes in 5 μL drops under oil and hand-bisected (Ultrasharp Splitting Blade, Bioniche, Athens, GA). The cytoplast selection was performed upon the same procedures described previously. After selected, the cytoplasts were removed from the Hoechst screening dish and maintained in HM20 until the embryo reconstruction.

Embryos reconstruction was performed by the brief exposure of two cytoplasts (porcine + porcine - porcine group; porcine + bovine – mosaic group; or bovine + bovine – bovine group) and a somatic cell to 500 μg/mL phytohaemoagglutinin (PHA) solution in HEPES-buffered M-199 with 0.01% PVA. The embryo reconstruction technique was based in de Ribeiro et al. (2009). Briefly, a somatic cell was attached to one cytoplast, followed by the adhesion of a second cytoplast to the first, creating a 180° arrangement and recovering the original cytoplasm volume. In the mosaic reconstruction, the cell was attached to the porcine cytoplast.

Reconstructed embryos were exposed to electrofusion medium (0.3M mannitol, 0.1mM $MgSO_4$ $7H_2O$, 0.05mM $CaCl_2$ $2H_2O$, 0.5mM HEPES, 0.01% PVA), and fused with a 15 V

pre-fusion AC pulse, followed by two 1.6 kV/cm DC pulses for 24 μsec, in a BTX 453 chamber (BTX Instruments, Genetronics, San Diego, CA) coupled to an electro-fusion apparatus (BTX Electro Cell Manipulator 2001, Biotechnologies & Experimental Research Inc., San Diego, CA). After electro-fusion, the oocytes were cultured in porcine zygote medium (PZM-3) for 1 h. Fusion was assessed and only completely fused embryos were activated.

The SCNT embryos were activated through a brief exposure to ionomycin (10 μM/5 min) in HM20 followed by exposure to 6-dimethyl aminopurine (2mM/3 h) in PZM-3. Zygotes were cultured using the well-of-the-well (WOW) system, described by Vajta et al., (2001), and modified by Feltrin et al., (2006). In vitro culture was under atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C. The cleavage and blastocyst rates of SCNT embryos were determined at 48 h and 7 days after activation, respectively.

2.7 Mitochondrial migration analysis during the fusion/activation process and pronuclear development evaluation

One cytoplast (porcine in porcine and mosaic reconstruction, bovine in bovine reconstruction) was stained with 200 nM of MitoTracker®Green FM (M-7514, Molecular Probes, Inc.) in HM20 for 45min at 38.5° C. Then cytoplasts were washed trice in HM20 and then maintained in HM20 until used on embryo reconstruction.

In all reconstructed groups, the cytoplast stained was the one attached to the somatic cell (Figure 2A).

All evaluations of mitochondrial migration were also performed with the staining of the cytoplast reconstructed in the opposite site of the cell.

The mitochondrial dynamics analysis was performed 0-60 min after oocytes fusion, with intervals of 5 min with concomitant analysis of the nuclear status and position by labeling with 10 µg/ml of Hoechst. After 60 min of fusion, the mitochondrial distribution was analyzed until 180 min, with intervals of 30 min. The structures used on this analysis were not activated.

After activation, the pronuclear growing was analyzed with 0, 5, 10 and 15h after fusion. Mitochondrial and nuclear fluorescence was assessed using an epifluorescent inverted microscope under UV light, using a x40 objective and filters at ex = 490 nm and em> 516 nm for Mito tracker, and ex = 355 nm and em = 465 nm for Hoechst.

2.8 Scriptaid treatment

After activation porcine, mosaic and bovine SCNT zygotes reconstructed with Moura breed fibroblasts were separated into two groups and cultured in PZM-3 supplemented or not with 500nM Scriptaid for a period of 12 h starting after ionomycin treatment. Oocytes were then washed three to five times in PZM-3 and cultured as described previously.

2.9 Embryo fragmentation analysis, cell density and inner cell mass proportion

In the experiment 1 and 2, cleaved structures from day 2 of culture that did not progress on development were stained with 15 µg/mL Hoecsht in HM10 at day seven of culture, being the number of blastomeres and nucleus counted under UV-epifluorescent inverted microscope. For this analysis the embryos were removed one by one from the culture micro-wells. For certifying the exact number of blastomeres in the morulas, a blastomeric disaggregation was

carried out, by gently pipetting. At least 30 cleaved embryos and 3 morulas were analyzed in each group.

The estimation of the total cell number (TCN) as well as the proportion of cells in the inner cell mass (ICM) per blastocyst were performed by differential staining, based on Mezzalira et al. (2011). Briefly, blastocysts from each group were incubated in Dulbecco's phosphate-buffered saline (DPBS) solution containing 10 µg/mL propidium iodide and 1 mg/mL Triton X-100 for 40 sec at room temperature. Then, embryos were fixed in absolute ethanol containing 15 µg/mL Hochest for additional 7 min. Fixed embryos were placed on in a 2-µL glycerol droplet between slide and coverslip, for immediate evaluation under the epifluorescent inverted UV-microscope.

Statistical analysis

All data were analyzed using the JMP software (SAS Institute Inc. Cary, NC, USA). Cleavage, blastocyst and ICM:TCN ratios were analyzed by the Chi-square test. All other data including morula and blastocyst TCN, fragmentation in cleaved embryos and morulas were analyzed by one-way analysis of variance (ANOVA). Fragmentation in cleaved embryos and morulas and the proportion of cells in ICM were normalized by the Arco sine of the square root. The means were compared by Tukey test for TCN in blastocysts. Percentages of development to the morula and blastocysts stages were based on total of cultured zygotes. A value of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1 Embryo development features from the two pig breeds and two cell types, post-fusion mitochondria migration dynamics and pronuclear swelling patterns

3.1.1 Embryo development

The detailed results of embryo development are illustrated in table 1. The cleavage rates for all groups were higher than 80%. Some particularities were found in the fibroblast groups, as well as between the different cell types within the same animal (table 1). For the Mule-foot, the porcine and bovine reconstructions showed lower cleavage than the mosaic. Still for the Mule-foot, the fibroblasts were responsible for higher cleavage rates than the ADMSC cells, the opposite was observed for the bovine reconstruction.

For the Moura breed, the cleavage of porcine and mosaic reconstructions were higher than the bovine, being the fibroblasts responsible for higher cleavage in the porcine reconstruction, whereas the ADMSC cells were for thebovine reconstruction.

For both animals, no differences were observed on cleavage among the three types of embryo reconstruction.

The percentage of fragmented blastomeres in the Mule-foot did not differ among the cytoplast reconstruction types, within each cell type. However, the ADMSC cells showed less fragmentation than the fibroblasts, for porcine and bovine reconstructions.

Moura fragmentation rate was increased according to the proportion of inter-species cytoplasts used for reconstruction, for both cell types. This animal has also showed a lower fragmentation when ADMSC cells were used for porcine and bovine reconstructions.

The reconstruction affected the percentage of morula production only for the Moura breed. When using fibroblasts as cell donors, the bovine reconstruction produced less morulas than the other two groups.

Also, for both Moura and Mule-foot, the use of ADMSC provided higher morula rates than the fibroblasts, when reconstruction was the bovine cytoplast. For Moura, this behaviour has also observed when the reconstruction was with the mosaic cytoplast. The proportion of enucleated blastomeres in morulas did not differ between the porcine reconstructions, despite of the cell line used (mean = 2.1%). Moreover, the porcine were lower than the mosaic (mean = 10.8%) and bovine (mean = 11.0%) reconstructions. The mosaic and bovine proportion of enucleated blastomeres in morulas also did not differ between the reconstruction and the cell types.

In the Mule-foot blastocyst production, the same pattern has been observed for both cell types, between the different reconstruction types. The higher blastocyst rate was observed in the porcine reconstruction, in comparison with the mosaic and bovine.

The Moura fibroblasts have also produced more blastocysts when porcine cytoplasts were used. It was only the association of ADMSC, Moura and mosaic reconstruction that showed an intermediated blastocyst rate, between the bovine and porcine cytoplasts reconstructions. This association has also provided a higher blastocyst rate than the association fibrolasts, Moura and mosaic cytoplast reconstruction.

In summary it was observed that only when fibroblasts were used, the cleavage of bovine reconstruction reduced, in comparison to the porcine and mosaic. Then it was analyzed based in the average of both cells, this difference disappeared (Figure 1).

The percentage of fragmented blastomeres present in embryos reconstructed with fibroblasts increased significantly, according to the higher proportion of inter-species cytoplast. For the embryos derived from ADMSC and also for the average of both cells, fragmentation rate was the lowest for the porcine reconstruction.

Table 1. *In vitro* development of porcine SCNT embryos reconstructed from distinct donor cells and recipient cytoplasts

Cell donor animal	Cell Type	Cytoplast	Fused n (replicates)	Cleavage		Morulas n (%)	Blastocysts	
				n (%)	Fragmented blastomeres (%)		n (%)	Total of nuclei (n)
Mulefoot	Fibroblast	Porcine	132 (5)	121 (91.7)^{b}	16.5 ± 3.7*	23 (17.4)	24 (18.2)^{a}	36.8 ± 3.9 (11)
		Mosaic	128 (5)	125 (97.7)^{a*}	22.8 ± 5.3	29 (22.7)	8 (6.3)^{b}	28.3 ± 7.5 (3)
		Bovine	122 (5)	107 (87.7)^{b*}	28.7 ± 3.9*	20 (16.4)*	4 (3.3)^{b}	24.0 ± 7.5 (3)
	ADMSC	Porcine	121 (5)	113 (93.4)	8.3 ± 4.5*	28 (23.1)	25 (20.7)^{A}	43.8 ± 3.3 (15)
		Mosaic	126 (5)	116 (92.1)*	18.9 ± 5.0	22 (17.5)	4 (3.2)^{B}	
		Bovine	123 (5)	117 (95.1)^{y*}	19.8 ± 4.4*	34 (27.6)*	3 (2.4)^{B}	30.0 ± 7.5 (3)
Moura	Fibroblast	Porcine	149 (6)	142 (95.3)^{x*}	17.1 ± 4.7^{x*}	26 (17.4)^{x}	20 (13.4)^{x}	34.8 ± 3.7 (12)
		Mosaic	151 (6)	137 (90.7)^{x}	20.9 ± 4.9^{x}	26 (17.2)^{x*}	3 (2.0)^{y*}	
		Bovine	139 (5)	115 (82.7)^{y*}	37.7 ±4.5^{y*}	13 (9.4)^{y*}	0 (0.0)^{y}	
	ADMSC	Porcine	119 (4)	105 (88.2)*	9.4 ± 5.4^{Y*}	24 (20.2)	25 (21.0)^{X}	39.5 ± 2.7 (23)^{XY}
		Mosaic	143 (4)	127 (88.8)	19.1 ± 4.7^{XY}	41 (28.7)*	15 (10.5)^{Y*}	31.8 ± 3.5 (14)^{Y}
		Bovine	138 (4)	128 (92.8)*	25.6 ± 5.0^{X*}	32 (23.2)*	3 (2.2)^{Z}	62.0 ± 7.5 (3)^{X}

a,b; A,B; x,y,z; X,Y,Z distinct letters indicate a difference between cytoplasts within the same animal and cell type.

* indicates a difference between cell type within the same cell donor animal and cytoplast.

The fibroblasts have also shown variations in morula production rates. The bovine reconstruction produced less morulae than the mosaic, whereas both groups were similar to

the porcine. When only ADMSC or the average of both cells was considered, no difference has been observed among the morula rates of the three cytoplasts reconstruction types.

For the fibroblasts, the blastocyst rate was lower for the mosaic and the bovine, in comparison to the porcine. However, the ADMSC as well as the average of both cell types showed a significant decrease in the blastocyst rate, as the proportion of inter-species cytoplasm increased (Figure 1).

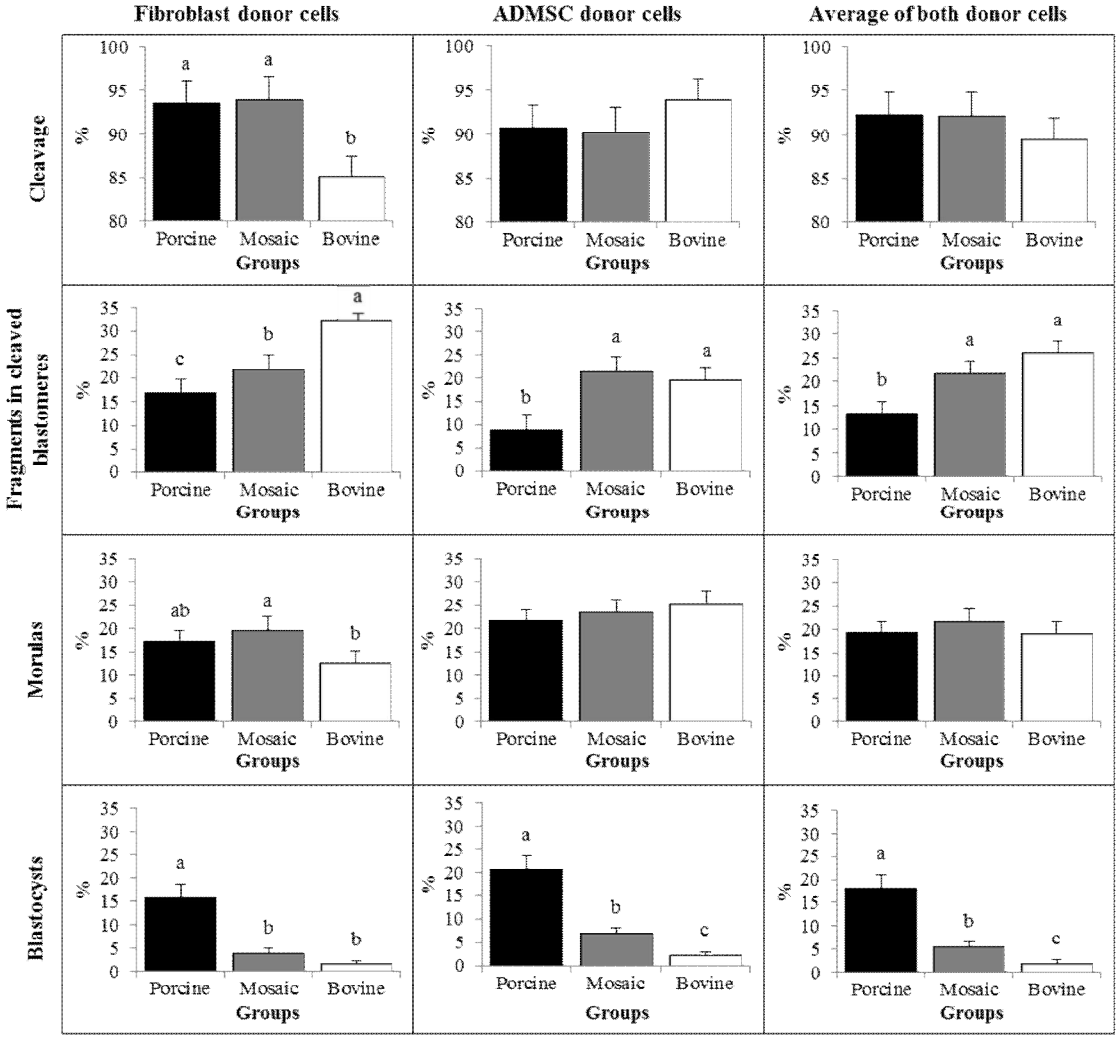

Figure 1. Cleavage, fragmented blastomeres, morulae and blastocysts rates in fibroblast cells, ADMSC and in the average of both cells reconstructed with porcine, mosaic and bovine cytoplasts, independent of the animal donor of the cells.

3.1.2 Mitochondrial dynamics after fusion

After fusion, all the cytoplasts reconstructions displayed the same phases of mitochondrial migration, however with different time-points, according to the cytoplasts combination. The only stage that was common for all the groups was observed at ten minutes after fusion, and ranged from the onset of mitochondrial migration until their placement in the opposite cytoplast (Figure 2, B). The following stage was the presence of stained mitochondria in both cytoplasts (from 15 – 30 minutes in porcine, up to 15 minutes in mosaic, and from 15 – 30 minutes in bovine; figures 3 A, B and C, respectively).

After the onset of migration to the opposite cytoplast, mitochondria polarized, totally in the opposite cytoplast (figure 2, C), for different time-points in the different groups (15 – 45 minutes in the porcine, 15 - 120 minutes in the mosaic, and 15 – 60 minutes in the bovine; figures 3A`, 3B` and 3C` respectively).

Mitochondria started to return to the original cytoplasm (Figure 2, D) approximately 45 minutes after fusion for the porcine and the bovine, and 120 minutes after fusion for the mosaic.

The onset of a homogeneous distribution of stained mitochondria in both cytoplasts (Figure 2, E) was observed approximately 60 minutes after fusion for the porcine and bovine, and 120 minutes after fusion for the mosaic cytoplasts reconstruction.

The mitochondrial migration to the opposite cytoplast, polarization on the opposite cytoplasm and return to the original cytoplasm occurred in the same time-points described above when the stained cytoplast was the one attached in the opposite site of the cell.

3.1.3 Pronuclear swelling patterns

The porcine cytoplast reconstruction allowed a pronuclear swelling with the highest diameter average after 10h of activation (24.1 μm) and nuclear re-condensation at 15h after activation (16.2 μm).

The mosaic and bovine embryos showed a significant pronuclear swelling from zero to 5h after activation, and no more significant increase in diameter average was observed until the 15h (Figure 4). However, at 15h post activation the average diameter of the mosaic (21.7 μm) was higher than the porcine and bovine (respectively 16.2 and 16.7 μm), and also close to the highest average acquired by the porcine, at 10h post activation.

The bovine cytoplasm reconstruction showed a similar distribution pattern (with the highest average diameter 10h post activation) to the porcine, however, with a significantly lower highest diameter average (figure 4).

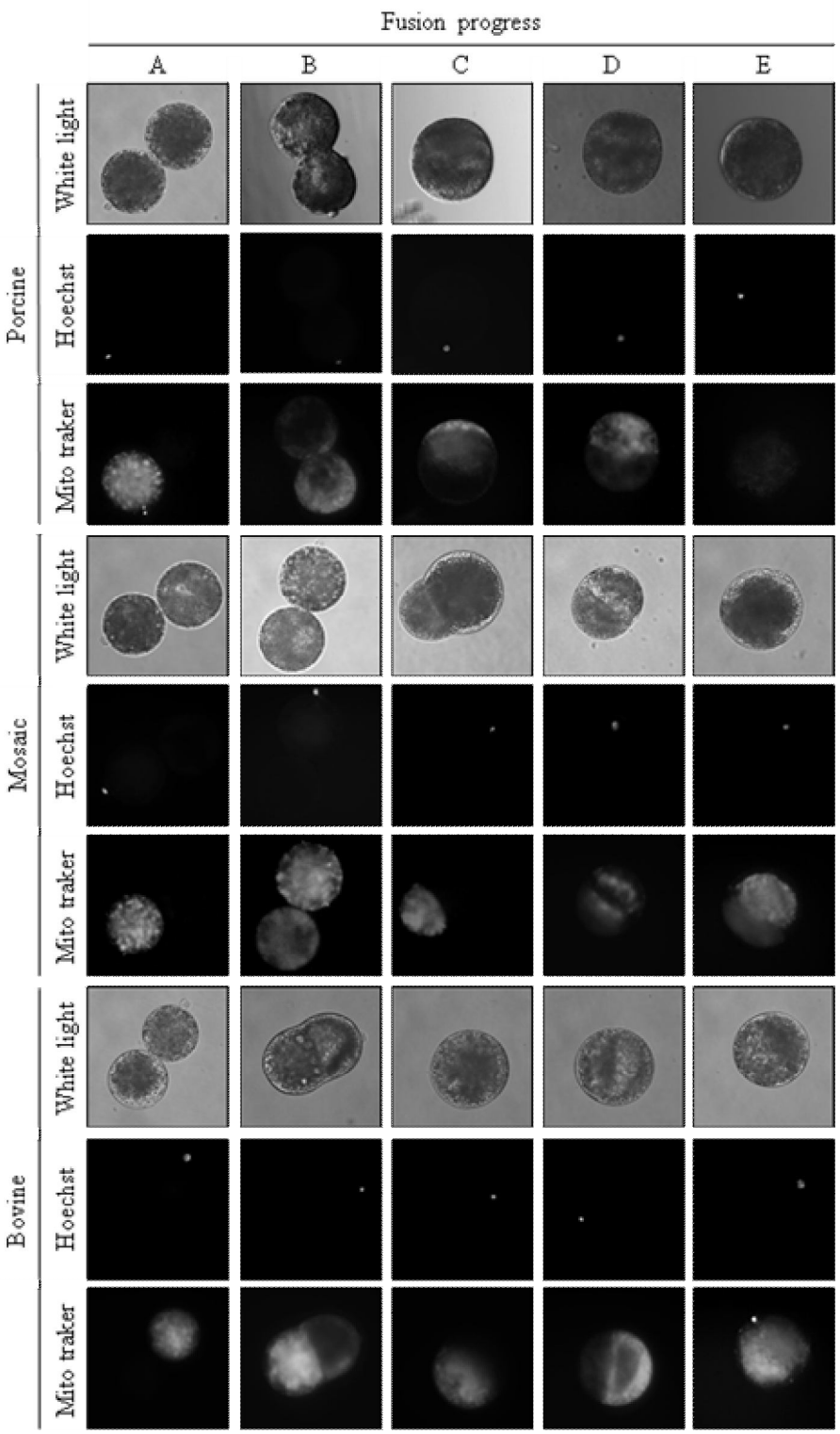

Figure 2. Representative pictures showing the progress of fusion and mitochondrial migration evaluated in porcine, mosaic and bovine cytoplasts. Hoechst staining represents the donor cell location, which stays close to the staining mitochondria original position. Mito-tracker staining represents the migration of mitochondria from one of the recipients cytoplasts, always reconstructed close to the donor cell. Reconstructed embryo immediately before fusion (A), with starting the mitochondria migration until 10 minutes after fusion (B), with mitochondria located in the opposite cytoplast (C), mitochondria starting to return to the original cytoplast and re-homogenate in the cytoplasm (D), mitochondrial homogeneous distribution (E). Original Magnification x400.

3.2 Embryo development of porcine, mosaic and bovine cytoplasts reconstruction, either treated or not with Scriptaid

The Scriptaid treatment did not play a role in cleavage and morula rates in comparison with the non-treated embryos (table 2). Also, the proportion of fragments in cleaved structures did not differ to the Scriptaid treatment. Howsoever, the morulas produced in mosaic reconstruction and treated with Scriptaid showed a lower number of anucleated blastomeres, being this proportion similar to the fragments present in the porcine (table 3). The treatment with Scriptaid increased the blastocyst production from the porcine and mosaic cytoplasts reconstruction (table 2). The Scriptaid treatment provided also a similar blastocyst rate between the Scriptaid-mosaic and the non-Scriptaid porcine group (table 2).

Furthermore, the inner cell mass proportion in the porcine embryos increased when the embryos where treated with Scriptaid (table 3).

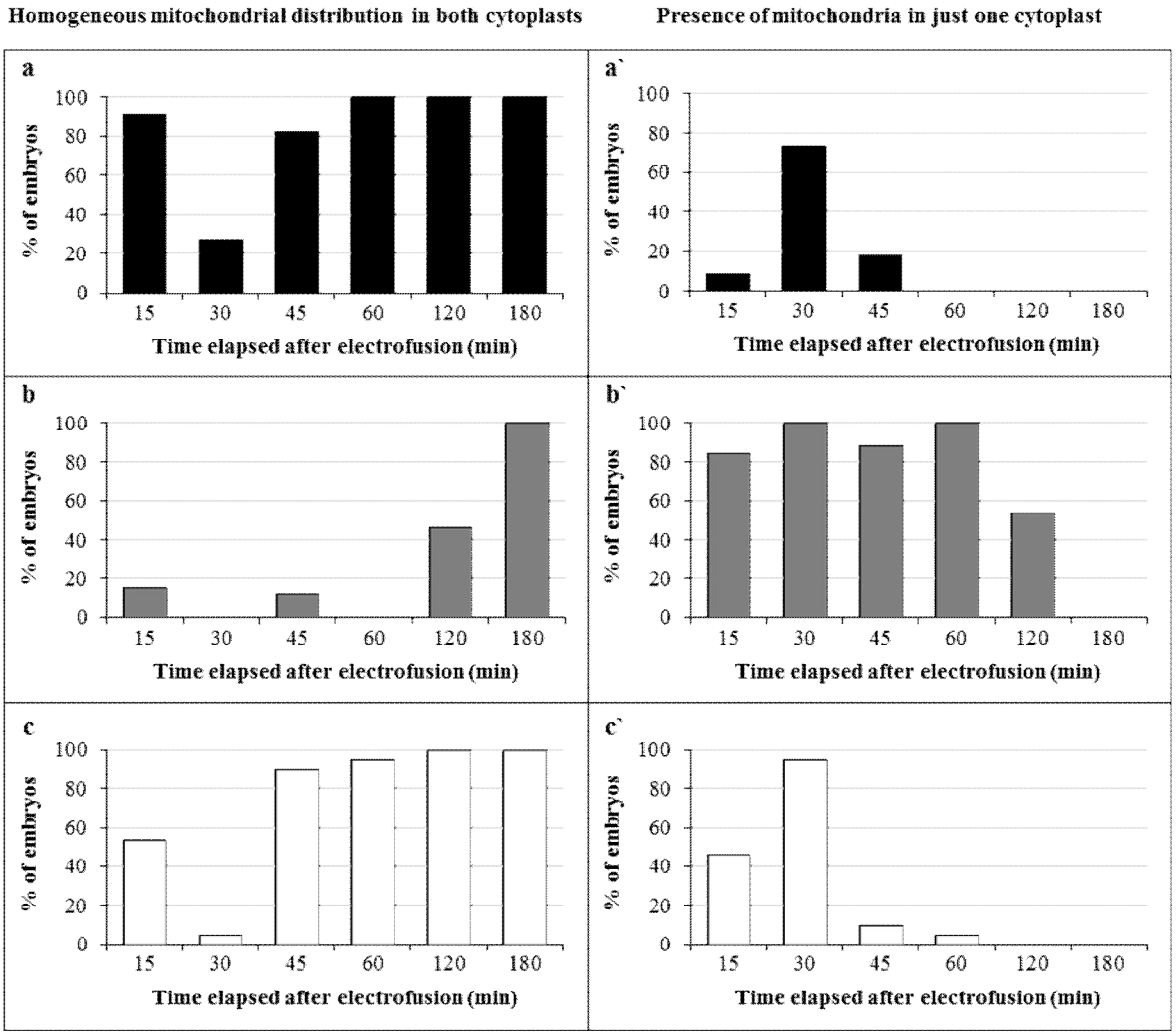

Figure 3. Mitochondria distribution after fusion of reconstructed embryos; Presence of stained mitochondria in both cytoplasts, for porcine (a), mosaic (b) and bovine (c) reconstructed embryos. Stained mitochondria in only one of the cytoplasts for the porcine (a`) the mosaic (b`), and the bovine (c`) cytoplasts reconstructions.

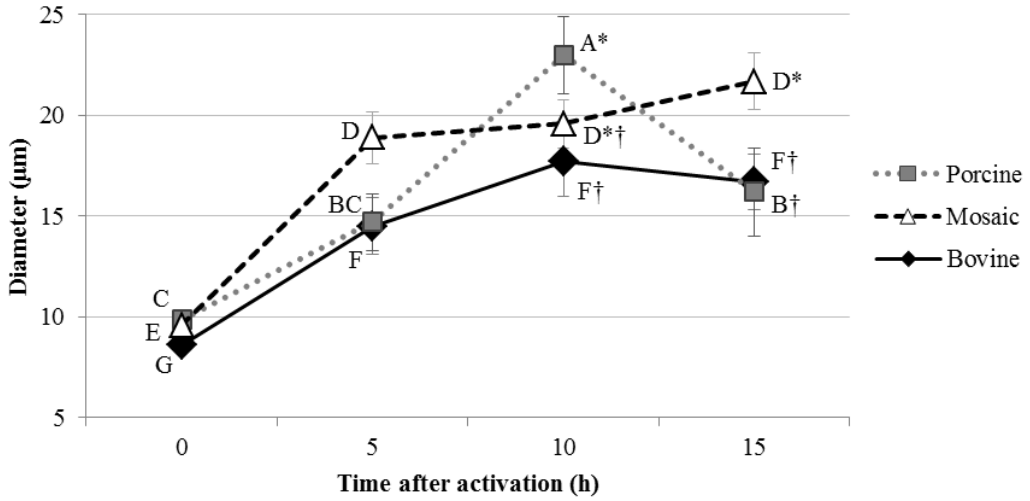

Figure 4. Pronuclear diameter (µm) 0, 5, 10 and 15 hours after activation of porcine clone embryos produced with porcine (2 x 50% of porcine), mosaic (50% of porcine + 50% of bovine) and bovine (2x50% of bovine) recipient cytoplasts. Different letters (A, B, C) denote a difference ($P < 0.05$) in porcine cytoplasts, (D, E) denote a difference ($P < 0.05$) in mosaic cytoplasts, (F, G) denote a difference ($P < 0.05$) in bovine cytoplasts. Different symbols (*, †) denote a difference ($P < 0.05$) between cytoplasts in the same time-point after activation.

Table 2. *In vitro* development of control, mosaic and inter-specie porcine SCNT embryos treated with Scriptaid

Specie of cytoplast	Scriptaid treatment	Fused n (replicates)	Cleavage n (%)	Morula n (%)	Blastocyst n (%)
Porcine	-	130 (5)	122 (93.8)^{ab}	35 (26.9)^{ab}	12 (9.2)^{b}
	+	156 (5)	149 (95.5)^{a}	46 (29.5)^{a}	27 (17.3)^{a}
Mosaic	-	97 (4)	92 (94.8)^{ab}	27 (27.8)^{ab}	1 (1.0)^{c}
	+	98 (4)	88 (89.8)^{abc}	25 (25.5)^{ab}	9 (9.2)^{b}
Bovine	-	86 (3)	75 (87.2)^{bc}	17 (19.8)^{ab}	1 (1.2)^{c}
	+	94 (3)	78 (83.0)^{c}	16 (17.0)^{b}	0 (0.0)^{c}

^{a,b,c} distinct letters in the same column differ ($p \leq 0.05$).

Table 3. Average nuclei number in morulas and blastocysts, and proportion of cells in the inner cell mass of clone blastocysts produced in control, mosaic and inter-generic porcine SCNT, treated with or not with Scriptaid

Specie of cytoplast	Scriptaid treatment	Morulas		Blastocysts	
		Average number of nuclei	% of anucleatedblastomeres	Average number of nuclei	Proportion (%) of cells in ICM:TCN
Porcine	-	17.6 ± 0.6 (10)[ab]	1.7 ± 1.4 [b]	61.6 ± 9.2 (7)[ab]	20.3 ± 4.1 (7)[b]
	+	18.2 ± 0.6 (11)[a]	0.5 ± 1.4 [b]	68.0 ± 9.2 (7)[a]	32.4 ± 4.1(7)[a]
Mosaic	-	15.5 ± 1.2 (3)[bc]	9.8 ± 2.6 [a]		
	+	17.1 ± 0.7 (8)[ab]	2.8 ± 1.6 [b]	33.7 ± 12.2 (4)[b]	
Bovine	-	14.8 ± 0.8 (6)[c]	11.0 ± 1.8 [a]		
	+	17.0 (2)	0.0		

[a,b,c] distinct letters in the same column differ ($p \leq 0.05$).

4. DISCUSSION

Dominko et al. (1999) showed that the bovine cytoplast supports development of embryos from various mammalian species. Sansinena et al. (2002) suggested that the bovine cytoplast seems to be capable of supporting a few mitotic divisions, but heteroplasmy or mitochondrial incompatibilities may affect nuclear-ooplasm events occurring at the time of genomic activation. As the nucleus-cytoplasmic compatibility are problematic in iSCNT embryos, a high *in vitro* developmental block from 8- to 16-cell stage has already been observed in the pig (YOON, 2001), horse (LI et al., 2002; SANSINENA, 2002), llama (SANSINENA et al., 2003), Siberian tiger (HASHEM et al., 2007), monkey (LORTHONGPANICH et al., 2008; SONG et al., 2009), marbled cat (*Pardofelismarmorata*), flat-headed cat (*Prionailurusplaniceps*) (THONGPHAKDEE et al., 2010), and Tibetan antelope (ZHAO et al., 2007). The mitochondria are largely responsible for the efficient production of the majority of cellular energy, in the form of ATP (PFEIFFER et al., 2001),

and most enzymes related to energy production come from the ooplasm in SCNT embryos (STOJKOVIC et al., 2001). According Kenyo and Moraes (1997), mitochondrial are incapable of maintaining proper respiration levels when the species providing the nuclei and mitochondria were evolutionarily more than 16 million years apart from each other, and Wang et al. (2009) confirmed these iSCNT embryos in the stage of 8- to 16 cells. In our study we confirm the fact of the developing block at morula stage (Table 1). Also, the lack of distinct nucleolar proteins related to transcription localization in intergeneric embryos indicates failures in sequence-specific interactions between the ooplasm and chromatin of another genus, what suggest a possible reason why the intergeneric SCNT embryos never reached the full term (ØSTRUP et al., 2011). Our elevated rate of morula production in the inter-species groups, independent of the cell used that did not developed to blastocyst (figure 1) confirm the molecular deficiency in such embryos, and also a high development blocking in the morula stage.

The early embryo development stages are regulated by maternally inherited genes that are derived from the oocyte cytoplasm, by a species-specific control of embryo genome activation (TELFORD et al., 1990). In cloning, the cytoplasm of the oocyte is capable of remodeling the transferred nucleus only to a certain degree (ZHAO et al., 2010). As observed, the disturbed relation between nucleus and cytoplasm (according mitochondrial migration, figures 2 and 3) is reflected in the pronucleus growing (Figure 4), and consequently in the embryo production. Moreover, we observed that the Scriptaid treatment enhanced the blastocyst formation of the porcine and mosaic groups (Table 2), probably by a better utilization of the cytoplasm beneficial factors as activate the cross-talk between nuclear encoded mtDNAtranscription by the nucleus better acetylated. Also, according nucleoli precursor bodies analisys, a superior role of the cytoplasm and its components in organizing

the initial steps of nucleologenesis was demonstrate by Maddox-Hyttel et al. (2007). What we observed related with this fact is that the growing of the pronucleus from the distinct groups of cytoplasts followed distinct pattern, probably caused by the incorrect crosstalk between nucleus and cytoplast. At least in part the Scriptaid treatment changed this scenario to an increment in the blastocyst production of mosaic embryos, showing the necessity of at least a little amount of specie-specific connection between nucleo-cytoplast for show an efficient result of and epigenetic mediator as Scriptaid.

It is possible that the mRNAs and organelles stored in the porcine cytoplast of the intra-specie group used for reconstruct the mosaic embryos only was effectively activated with Scriptaid treatment. In the porcine group, even the proportion of cells in ICM increased with the use of Scriptaid (table 3) with a close to the observed in *in vivo* blastocysts founded by Rath et al. (1995) (32%), and higher than the observed by Tao et al. (1995) and Fuente and Allan King, (1997) (22- to 24%).

Even with the known differences despite the differentiation and regeneration in the fibroblast cells and ADMSC, the embryo development did not differ significantly as showed previously by Jin et al. (2007) with the use of bonne marrow mesenchymal cells. In the three models of cytoplasts used for reconstruct the cloning embryos showed distinct patterns of cleavage, fragmentation, morulas and blastocyst, evidencing a probable major capacity of reprogramation of these cells. However, the potential of the ADMSC was better expressed just in isolated points. Development differences were observed depending of the animal donor, and no significant differences where confirmed when compared each type of cytoplast between both cellular types grouping both animals. One interesting case is that the ADMSC cells showed an intermediated result between porcine and bovine cytoplast, what was distinct from the fibroblasts. This fact evidence a possible major ability of those cells in using the

porcine homologous cytoplast factors. What evidence the major ability of reprogramming of the ADMSC is that when the fibroblasts where treated with Scriptaid, giving more competence to this nucleus, they also showed a higher embryo production then the bovine group (Table 2).

Not homogeneous at the time of activation – 1h after fusion (Figure 3), and sometimes far of the nucleus at this time (Figure 2C, mosaic), is probably that the non effect of the porcine mitochondria in the moment of the oocyte activation was responsible of the low embryo development in mosaic group in the first experiment.

In conclusion, the oscillations in embryo production observed in intra and inter-specie SCNT corresponds with to variations in mitochondrial migration during fusion and mainly to the pro-nucleus growing. The iSCNT embryo production has differences according the cell donor type and the level of homologous species cytoplast used as recipient. The efficiency of intra and inter-specie SCNT according embryo production and embryo quality can be increased with the use of Scriptaid.

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4. ARTICLE 2:

INTRACYT´PLASMIC SPERM INJECTION IMPROVES *IN VITRO* EMBRYO PRODUCTION FROM POOR QUALITY BOVINE OOCYTES

L.U. Ohlweiler[a], D.S. Brum[b], F.G. Leivas[b], A.B. Moyses[b], R.S. Ramos[b], N. Klein[a], J.C. Mezzalira[a], A. Mezzalira[a*].

[a]*Laboratório de Reprodução Animal prof. Assis Roberto de Bem, Universidade do Estado de Santa Catarina - UDESC, Lages, SC, Brazil.*

[b]*Laboratório de Biotecnologia da Reprodução - BIOTECH, Universidade Federal do Pampa - UNIPAMPA, Uruguaiana, RS, Brazil.*

Abstract

Poor quality oocytes and sperm reduce in vitro embryo development. Strategies such as ICSI and SUZI may be employed to assess oocyte/sperm interactions. This study aims to employ ICSI and SUZI as strategies to overpass low quality of bovine gametes. Good and poor quality oocytes IVF performance and sperm penetrating patterns were evaluated and compared between two distinct bulls, A and B (experiment 1). The blastocyst rate was higher for good versus poor quality oocytes (23.3 vs 11.1%, $P < 0.05$), regardless of the bull used. No difference in blastocyst rate was observed for bull A regardless of oocyte quality, whereas for bull B, blastocyst rate was higher ($P < 0.05$) for good quality (25.7%) than for poor quality oocytes (9.2%). The sperm penetration pattern was distinct between bulls, when oocytes of

*Corresponding author Tel/Fax +554921019122 /e-mail alceumezzalira@yahoo.com.br -Universidade do Estado de Santa Catarina. Av. Luiz de Camões, 2090.CEP 88520-000 Lages- SC / Brazil

poor quality were used. To understand such previously observed penetrating pattern, poor quality oocytes were submitted to SUZI (experiment 2). Oocyte penetration rate was lower for bull A than for bull B (29.6% vs. 53.8%, $P < 0.05$), when SUZI was performed within 1 h after sperm processing. When SUZI was performed up to 3 h after sperm processing, penetrating capacity was similar between bulls. However, bull B penetrating capacity significantly decreased after 3 h of sperm processing. In an attempt to overcome sperm penetrating disorders, poor and good quality oocytes were submitted to ICSI (experiment 3). Irrespective of the bull or of the oocyte quality grade, no differences have been observed in cleavage or blastocyst rates. Both of the bulls performed distinct embryo production rates, which we inquiry to be due to distinct sperm penetrating patterns. Ultimately, ICSI was an alternative to overpass gametes quality issues, on bovine embryo production.

Keywords: ICSI; SUZI; In vitro fertilization; Oocyte quality; Sperm penetration; Blastocyst.

1. Introduction

Protocols for *in vitro* production (IVP) of cattle embryos are relatively efficient and widely used. Factors such as oocyte and sperm quality may influence the success of IVP [1-2]. However, genetically important cows are frequently submitted to ovum pick up sessions without considering oocyte quality [3]. Consequently, poor quality oocytes are often retrieved, resulting in reduced IVP embryo yield. Variations in IVP results might also occur due to semen quality, what can be influenced by bull fertility, semen processing and storage conditions, or even to unknown factors [4-5]. Bulls with good pregnancy rates in AI programs (breeding after estrus detection or at a fixed time) are not necessarily effective for embryo

IVP [6-7]. Furthermore, there are differences among bulls in their contribution to embryonic development [8]; some are ineffective to produce embryos when used with oocytes from specific donors (due to apparent male-female interactions) [7]. To overcome such obstacles, especially in research, it is imperative to previously test semen used for IVF. However, in commercial IVP programs, results can be disappointing, especially because a particular sire is typically chosen for reasons other than fertility. Recently, progress has been made to improve *in vitro* embryo production for oocytes and/or semen of low quality [9]. Sub-zonal sperm injection (SUZI) has been used for *in vitro* evaluation of bull sperm [10], as it assesses sperm penetration competence. Another technique currently employed to circumvent fertilization problems is the intra-cytoplasmic sperm injection (ICSI), widely used in human reproduction to pass through poor quality of gametes. However, ICSI has not yet been widely used for cattle, as the technique is still under development [11-12]. The general objective of this work was to evaluate ICSI and SUZI strategies to improve the IVP efficiency of low quality bovine gametes. This study evaluated the fertilization interactions of semen from two bulls with either good or poor quality oocytes.

2. Materials and methods

2.1. Experimental design

2.1.1. Experiment 1: characteristics on sperm/oocyte interaction on embryo development by IVF

Oocytes (n = 1,566) classified as of good or poor quality were separately submitted to conventional IVF using semen of two bulls, here called bulls A and B, to evaluate the interaction of distinct semen characteristics and oocyte quality grades, on embryo development. A sample of zygotes of each treatment was pooled (approximately 44% of total IVF zygotes) for determination of nuclear status, based on morphological characteristics of the zygotes. Embryo development was compared to nuclear status of zygotes, among all experimental groups (five replications).

2.1.2. Experiment 2: determination of sperm penetration patterns

Based on the higher penetrating competence showed by Bull B in experiment 1, experiment 2 was carried out to further confirm the higher penetrating capacity of Bull B. Oocytes (n = 498) classified as of poor quality underwent SUZI using semen of both bulls A and B (three replications).

2.1.3. Experiment 3: comparison of ICSI and IVF on embryo production

With the aim of to overcome the limitations of sperm/oocyte interactions that impair IVF efficiency, experiment 3 aimed to compare ICSI and conventional IVF with oocytes of good or poor quality (n = 426), using semen from bulls A and B (three replications).

2.2. Media and reagents

Except where otherwise indicated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. Recovery of oocytes, classification and in vitro maturation (IVM)

Bovine ovaries from a local abattoir were transported to the laboratory at 25 to 30 °C in 0.9% sodium chloride solution, up to 6 h after slaughter. Follicles 2 to 8 mm in diameter were aspirated with the aid of a vacuum pump set at the pressure of 10-15 mL/minute. Cumulus-oocyte complexes (COCs) were classified based on morphological criteria [13] under stereomicroscope, in follicular fluid. Oocytes were classified as of good quality if they had homogeneous cytoplasm with none or few dark clusters, and also if they were completely surrounded with at least three compact layers cumulus cells. Poor quality oocytes had a heterogeneous cytoplasm with several dark clusters, completely or partially surrounded with non-compact and slightly dark cumulus cells. Degenerated oocytes were not used.

Oocytes were *in vitro* maturated in 400 μL of modified tissue culture medium (TCM-199, Cultilab, Campinas, São Paulo, Brazil), with 5.95 mg/mL HEPES, 2.62 mg/mL of NaHCO3, 0.025 mg/mL sodium pyruvate, 5 μg/mL of porcine follicle-stimulating hormone (NIH-FSH-P1; Folltropin-V, Bioniche Animal Health, Belleville, ON, Canada), 50 μg/mL of porcine pituitary luteinizing hormone (LH-P; Lutropin-V, Bioniche Animal Health) and 10% of estrous mare serum (EMS) [14], under mineral oil, for 18 to 24 h at 38.5 °C and 5% CO_2 in air, and saturated humidity.

2.4. Semen preparation

Frozen-thawed semen collected from two Bos taurus taurus sires (bulls A and B), previously proven to be efficient for IVF, were used [15]. The semen straw was thawed in a water-bath at 37 °C for 20 s and selected using mini-Percoll [16] gradients (90 and 45%), then re-suspended in Sperm-TALP medium.

2.5. IVF

Selected sperm and oocytes were incubated (for 20 h) in Fert-TALP medium with 6 mg/mL of BSA, 0.022 mg/mL of sodium pyruvate, 30 μg/mL of heparin and PHE (penicillamine, hypotaurine and epinephrine) at 38.5 °C with 5% CO_2 in air and saturated humidity. Semen final concentration used for IVF was 1.5×10^6 motile sperm / mL.

2.6. Evaluation of nuclear status of presumptive zygotes

After IVF, cumulus cells were removed from presumptive zygotes, by successive pipetting. Aproximately 44% of zygotes were pooled and used for evaluation of nuclear status, being the remainder submitted to conventional IVC, for embryo development evaluation. To nuclear status evaluation, presumptive zygotes were stained with 10 μg/mL of bisbenzmide (Hoechst 33342), and examined under ultra violet (UV) light. The nuclear developmental status was determined based on criteria as follows: matured oocytes (structures showing a metaphase plate or any structure ahead of this stage of development); or penetrated oocytes (structures showing a sperm head in the oolemma or any status ahead of this developmental stage). Penetrated oocytes were further classified, either in polyspermic zygotes (structures with more than one sperm head, or more than three pro-nucleus in the oolemma), or in one of the

six developmental stages of female and male pro-nucleus, as described previously [17]. Pro-nucleus one: female chromosomes condensed like a dot, as well as the acrosome, with no signs of de-condensation. Pro-nucleus two: chromosomes starting to de-condense and the acrosome enlarging in size. Pro-nucleus three: chromosomes further de-condensed, nuclear envelope starting to form. Pro-nucleus four: chromosome de-condensation complete, with a more or less spherical pro-nucleus surrounded by a complete envelope. Pro-nucleus five: pro-nucleus enlarging in size. Pro-nucleus six: pro-nucleus reaching the maximum size, closely located and spherical in shape. Zygotes were considered asynchronous when the male and female pro-nucleus had two or more stages of difference.

2.7. Sub-zonal sperm injection (SUZI) and sperm penetration assessment

After maturation, oocytes classified as of poor quality were successively pipetted for removal of cumulus cells. Only maturated oocytes were selected, based upon the presence of an extruded polar body. Groups of five oocytes were placed in 25 µL droplets of manipulation medium (Hepes-buffered TCM 199 with 6 mg/mL BSA) under mineral oil at 27 °C in air.
Sperm previously selected through the mini-Percoll gradients was maintained in 15 µL 10 % polyvinylpyrrolidone (PVP, 40 kDa, wt/vol) droplet, in HEPES-buffered TCM-199 at a final concentration of 1 x 10^5 sperm/mL. Semen was prepared at least 15 min prior to the onset of SUZI procedures. Micromanipulations were done under an inverted microscope (Olympus – IX51) and a Narishige micromanipulator (IM-9B; Narishige, Tokyo, Japan). The procedure has been adapted according to previous studies [10-21], with few modifications. Each oocyte was attached to a holding pipette (outer and inner diameter = 120 and 40 µm, respectively). A motile sperm was aspirated into an injection pipette (outer and inner diameter = 9 and 8 µm,

respectively) and injected into the oocyte perivitellinic space. For SUZI manipulations, semen was either prepared up to 1 h or 2-3 h in advance. After preparation, semen was maintained in the incubator at 38.5°C in Fert-TALP medium supplemented with 6 mg/mL of BSA.

Injected oocytes were maintained in manipulation medium at 38.5°C in air, for 3 h; then, sperm penetration rates were assessed using an inverted microscope. Oocytes were considered penetrated when they were negative for visualization of the sperm in the perivitelline space, confirmed with Hoechst 33342 (10 μg/mL) dye, under a UV light microscope, to confirm the presence of the sperm head in the ooplasm.

2.8. Intracytoplasmic sperm injection (ICSI)

Sperm and oocytes preparation was similar as previously described for SUZI procedures. The protocol used has previously described, and had few modifications [22]. Only maturated oocytes (with basis on the presence of an extruded polar body) were selected. Each oocyte was positioned with the polar body set at the 12 or 6 o'clock position, and the sperm was injected at the 3 o'clock position. Once sperm was aspirated to the pipette, the oocyte ooplasm was sucked until breaking the oolemma, and the sperm was placed into the oocyte cytoplasm. Following ICSI, oocytes were placed in manipulation medium for up to 1 h at room temperature, until they were chemically activated (5 min exposure to 15 μM ionomycin), followed by incubation in 2 mM 6-dimethylaminopurine (6-DMAP) for 2.5 to 3 h, in the incubator at 38.5 °C. Oocytes were then submitted to in vitro culture (IVC).

2.9. In vitro culture (IVC)

The onset of IVC was done in SOFaaci medium [16] with 5% EMS, under mineral oil in the same conditions used for IVM and IVF. After Day 2 (Day 0 was the day of IVF or ICSI), presumptive zygotes were loaded in a sealed foil bag filled with lung air, blown after 4 s of apnea, which was replaced on Day 5 after the onset of IVC. In experiment 1, IVC was performed into four-well dishes containing 400 µL of SOFaaci medium, whereas in experiment 3, IVC was performed into 100 µL droplets, both under mineral oil. Embryo development was assessed based on cleavage rates on day 2 and blastocyst rates (presence of early blastocysts, blastocysts, or expanded blastocysts) on day 7.

2.10. Statistical analysis

Data were analyzed using JMP software (SAS Institute Inc. Cary, NC, USA) with 5% of significance level.

Percentages of development to the blastocyst stage were based on the number of cultured oocytes. The percentage of sperm penetration in experiment 1 was based on total of matured oocytes. Percentages of polyspermy as well as developmental status of zygotes were based on total of fertilized oocytes.

Cleavage and blastocyst rates from experiments 1 and 3, polyspermy rates in experiment 1, and sperm penetration rates in Experiment 2 were analyzed by Chi-square test. All remaining data, including oocyte maturation, penetration and zygote status in Experiment 1 were analyzed through one-way ANOVA, on a completely randomized experimental design, being means compared using Tukey test.

3. Results

3.1. Experiment 1: characteristics on sperm/oocyte interaction on embryo development by IVF

The nuclear maturation rate (Table 2) was higher ($P < 0.05$) for good quality (88.6 and 86.8%) than for poor quality oocytes (70.4 and 74.8%). Likewise, the blastocyst rate was higher for good quality than for poor quality oocytes of (23.3 vs 11.1%, $P < 0.05$, Table 1). Also, distinct bull patterns were observed. Blastocyst rates obtained with semen of bull A were not significantly different between oocytes classified as good or poor quality. However, the blastocyst rate obtained with semen of bull B was higher when good quality oocytes were used (Table 1). Whereas, when oocyte quality was not considered, there was no significant effect of bulls.

Regarding sperm penetration rates, bulls A and B did not differ significantly when good quality oocytes were used. Conversely, when only poor quality oocytes were used, sperm penetration rate was significantly higher for bull B.

Polyspermy occurred when poor quality oocytes were fertilized with bull B sperm (Table 2), but was absent when good quality oocytes were fertilized with sperm from the same bull. In zygotes fertilized with bull A, pro-nucleus formation was more synchronous in poor quality than in good quality oocytes. Nonetheless, in zygotes fertilized with bull B, pro-nucleus formation was more synchronous in good quality oocytes. Moreover, the percentage of recently fertilized zygotes (with no sperm head de-condensation) was higher in good quality, than in poor quality oocytes (13.8% vs 8.3%, $P < 0.05$) with bull A semen (Table 2).

Table 1. Cleavage and blastocyst rates obtained after IVF and IVC using sperm from two bulls and oocytes of good* or poor** quality

Embryo stage	Bull A				Bull B				Average bulls			
	Good		Poor		Good		Poor		Good		Poor	
	%	(n)	%	(n)	%	(n)	%	(n)	%	(n)	%	(n)
Cleavage	58.3	(56/96)^{b}	57.2	(99/173)^{b}	67.1	(94/140)^{ab}	71.7	(109/152)^{a}	63.6	(150/236)	64.0	(208/325)
Blastocyst	19.8	(19/96)^{ab}	12.7	(22/173)^{b}	25.7	(36/140)^{a}	9.2	(14/152)^{b}	23.3	(55/236)^{A}	11.1	(36/325)^{B}

$^{ab; AB}$ Within a row, proportions without a common superscript differed ($P < 0.05$).

* Oocytes with homogeneous cytoplasm with no or few dark clusters and completely surrounded with more than three layers of compact cumulus cells.

** Oocytes with heterogeneous cytoplasm with a several dark clusters, completely or partially surrounded with non-compact and slightly dark cumulus cells.

Table 2. Maturation rates of good* or poor** quality oocytes fertilized with semen from either bull A or bull B. Penetration rates and developmental status of zygotes immediately after *in-vitro* fertilization

Bull / oocyte quality	No. oocytes evaluated	Matured oocytes		Penetrated oocytes [1]		Polyspermic zygotes [2]		Synchronous pro-nucleus[2]		Zygotes with no descondensed sperm [2]	
		No.	%	No.	%	No.	%	No.	%	No.	%
A/good	105	93	88.6^{a}	87	93.6^{ab}	0	0.0^{ab}	69	79.3^{b}	12	13.8^{a}
A/poor	115	81	70.4^{b}	72	89.0^{b}	0	0.0^{ab}	63	87.5^{a}	6	8.3^{b}
B/good	121	105	86.8^{a}	100	95.2^{ab}	0	0.0^{b}	87	87.0^{a}	12	12.0^{ab}
B/poor	103	77	74.8^{b}	76	98.7^{a}	3	3.9^{a}	58	76.3^{b}	7	9.2^{ab}

^{ab}Within a colunm, percentages without a common superscript differed ($P < 0.05$)

[1] Percentage based on total of matured oocytes

[2] Percentage based on total of penetrated oocytes

* Oocytes with homogeneous cytoplasm with no or few dark clusters and completely surrounded with more than three layers of compact cumulus cells.

** Oocytes with heterogeneous cytoplasm with a several dark clusters, completely or partially surrounded with non-compact and slightly dark cumulus cells.

3.2. Experiment 2: determination of sperm penetration patterns

The sperm penetration rate was lower for bull A than for bull B when the injection was done within 1 h after semen processing. Conversely, for bull B, sperm penetration rate was reduced

when injection was done within 2 to 3 h. Likewise, for bull A, sperm penetration rate was not influenced by the timing of injection (Table 3). However, when the time elapsed between sperm preparation and injection was not considered, sperm penetration rates differed between bulls ($P < 0.05$). As observed in table 3, bull A showed lower average penetration rates (34.0%) than bull B (44.3%, $P < 0.05$).

Despite the bull used, the timing of SUZI did not affect overall sperm penetration rates, which were 41.9% (98/234) for injection within 1 h after semen processing and 36.2% (71/196) for injection within 2-3 h after semen processing (Table 3).

Table 3. Sperm penetration rates after SUZI procedure within 1 h or 2-3 h after sperm processing.

	Injection within 1 h	Injection within 2-3 h	Average
Bull A	29.6% (34/115)^{aA}	39.4% (37/94)^{aA}	34.0% (71/209)^{a}
Bull B	53.8 % (64/119)^{bA}	33.3% (34/102)^{aB}	44.3% (98/221)^{b}
Average	41.9% (98/234)^{A}	36.2% (71/196)^{A}	39.3% (169/430)

^{ab}Within a column, values without a common superscript differed ($P < 0.05$)
^{AB} Within a row, values without a common superscript differed ($P < 0.05$)

3.3. Experiment 3: comparison of ICSI and IVF on embryo production

As showed on Table 4, cleavage rates after ICSI with bull A showed no differences between oocytes of good (76.7%, 23/30) or poor (71.4%, 25/35) quality ($P > 0.05$). Similarly, bull B ICSI showed no differences in cleavage rates for oocytes of good (73.3%, 22/30) and poor (65.6%, 21/32) quality ($P > 0.05$). The same pattern observed for cleavage rates between bulls has also been observed for the blastocyst rates, with no significant differences between bulls, despite of the oocyte quality. Blastocyst rates yielded by bull A were not statistically

different for oocytes of good and poor quality (26.7 and 22.9%), respectively. Likewise, bull B yielded higher blastocyst rates ($P < 0.05$) for good quality (30.0%), than for poor quality oocytes (21.9%).

Table 4. Cleavage and blastocyst rates obtained after IVF and ICSI using sperm from two bulls (A and B), and good* or poor** quality oocytes

Fertilization method	Bull/oocyte quality	Cleavage % (No.)	Blastocyst % (No.)	Average cleavage % (No.)	Average blastocyst % (No.)
IVF	A/good	60.0 (42/70)^{ab}	20.0 (14/70)^{ab}	60.0 (72/120)^{b}	22.5 (27/120)^{a}
	B/good	60.0 (30/50)^{ab}	26.0 (13/50)^{a}		
	A/poor	54.7 (52/95)^{b}	12.6 (12/95)^{b}	63.5 (106/167)^{ab}	11.4 (19/167)^{b}
	B/poor	75.0 (54/72)^{a}	9.7 (7/72)^{b}		
ICSI	A/good	76.7 (23/30)^{a}	26.7 (8/30)^{a}	75.0 (45/60)^{a}	28.3 (17/60)^{a}
	B/good	73.3 (22/30)^{ab}	30.0 (9/30)^{a}		
	A/poor	71.4 (25/35)^{ab}	22.9 (8/35)^{ab}	68.7 (46/67)^{ab}	22.4 (15/67)^{a}
	B/poor	65.6 (21/32)^{ab}	21.9 (7/32)^{ab}		

^{ab}Within a column, proportions without a common superscript differed ($P < 0.05$)
* Oocytes with homogeneous cytoplasm with no or few dark clusters and completely surrounded with more than three layers of compact cumulus cells.
** Oocytes with heterogeneous cytoplasm with a several dark clusters, completely or partially surrounded with non-compact and slightly dark cumulus cells.

4. Discussion

In most commercial IVF programs, oocytes are simply aspirated from small and medium antral follicles; this procedure is usually done with a pool of oocytes of unknown competence. When poor quality oocytes are used for IVF, they result in lower embryo development and pregnancy rates in comparison with good quality oocytes [1-2]. Our study yielded the same trend. Significantly higher blastocyst rates were obtained after IVF of

oocytes of good quality (Tables 1 and 4). Therefore, it is still necessary to enhance embryo IVP rates when poor quality oocytes must be used.

Despite the bull used, oocytes of poor quality submitted to IVF resulted in low overall blastocyst rates (Table 1). These findings corroborate with previous reports [1-2]. Overall blastocyst rates were similar when semen from bulls A and B were used to fertilize low quality oocytes. However, bull B showed higher cleavage rates with low quality oocytes, in comparison with bull A (Table 1). These differences (in cleavage rates) between bulls might have been due to previous observations of seminal patterns of these bulls (data not published). Immediately after thawing, bull B semen had higher vigor than bull A, corresponding to its higher IVF penetration rates for oocytes of poor quality. Bull B also had polyspermy, not detected for bull A, providing further evidence of their differences.

For further confirmation of the higher penetrating capacity of bull B, sub-zonal-sperm injection (SUZI) was used to inseminate poor quality oocytes. In that regard, SUZI is an important tool to study sperm attachment and penetration in oocytes [10], but its application for cattle is still limited. It was noteworthy that SUZI reinforced the distinct patterns presented by two bulls (bull B had a higher overall penetration rate than bull A). The distinct patterns between bulls were manifest by the higher cleavage rates presented by bull B, as well as by the greater incidence of polyspermy in poor quality oocytes. Presumably, good quality oocytes had more competence to block polyspermy.

In Experiment 2, Bull B had a lower sperm penetration rate when SUZI was done 2-3 h after sperm preparation (Table 3). Conversely, for bull A, sperm penetration rate was not significantly different for 1 h versus 2-3 h after sperm preparation. When the time interval between semen preparation and injection was not considered, bull A penetration rate was significantly lower than for bull B, supporting that sperm penetration is the main event

responsible for oocyte activation, and for further cleavage. All these findings emphasized the importance of choosing adequate bulls for IVF, regardless of oocyte quality.

After conventional IVF, bull A showed similar blastocyst rates with good or poor quality oocytes, whereas bull B had a significant decrease in the blastocyst rate with poor quality oocytes (Table 1). These results corroborated previous findings [2] regarding the influence of sire on embryo ability to develop and to establish pregnancy after IVF [17]. Those authors have additionally reported a presumable male-female interaction, similar to previously observed [7].

As all oocytes injected with a sperm cell in the perivitellic space (SUZI) were of poor quality, the distinct sperm penetration rates were attributed to the sire used. Such distinct patterns between the 2 bulls also explain the premature sperm penetration by bull B, where penetration in the oocytes has occurred in a manner not synchronous with oocyte maturation status. Furthermore, there was an asynchrony between male and female pro-nuclei formation.

It has been previously observed (data not shown) that Bull A semen presented lower spermatic vigor after thawing, despite the motility has been the same (40% of motile sperm) for both of the bulls. Thawing conditions were in a water-bath at 37 ºC, and evaluation was done under light microscopy on a slide warmed to 39 ºC, covered by warm coverslip. Bull A presented vigor of 2, whereas Bull B presented vigor of 4, on a 0-5 score [15]. Presumably, these features provided the significantly higher pro-nucleus synchrony for oocytes of low quality after IVF using semen of Bull A.

As time elapsed after semen preparation for SUZI, bull B reduced its penetrating capacity, with similar penetration rates observed between bulls A and B after three hours of semen processing. Bull B, presented higher spermatic vigor at thawing, i.e. opposite seminal characteristics in comparison to bull A. So far, bull B presented higher synchrony of pro-

nucleus formation when co-incubated with oocytes of good quality. This inquiry is strongly suggested by the higher presence of recently penetrated oocytes by semen of bull A, at the end of good quality oocytes IVF, based on number of zygotes with no de-condensed sperm (Table 2). This might have also resulted in higher asynchrony observed between good as compared to poor quality oocytes (Table 2).

Recently, the increasingly use of sex sorted frozen and re-frozen semen [20] and cryopreserved oocytes have been added to protocols for embryo IVP in cattle, especially for endangered species and preservation of rare breeds. This suggests the need to adjust *in vitro* procedures [7-8] to maximize embryo *in vitro* production. A promising way to overcome these limitations is the application of technologies such as ICSI. This procedure bypasses sperm-egg binding, sperm fusion as well as the events associated with conventional sperm-egg interactions, including plasma membrane and cortical region interactions [21]. Although ICSI is widely used in human reproduction in an attempt to circumvent poor gamete quality, in cattle there are few publications in the topic and the technique is still under development [11-12]. In Experiment 3, ICSI enabled both bulls to have similar cleavage and blastocyst rates; however, this was not possible after conventional IVF. This confirms ICSI as a tool for the use of genetically important male gametes for preserving both domestic and wild animals [10-21]. Moreover, these data taken together reinforced ICSI as a strategy to increase viability of poor quality oocytes. Nonetheless, despite the bull used, ICSI significantly increased cleavage rates for oocytes of good quality as well as the blastocyst rates for oocytes of poor quality.

In conclusion, good quality oocytes have higher competence to develop to embryos, than poor quality oocytes. Different bulls present distinct embryo production patterns, presumably due to particular sperm patterns and sperm-oocyte interactions, as evidenced by

SUZI. Furthermore, ICSI is a viable alternative to increase IVF embryo production for poor quality bovine oocytes.

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5. GENERAL CONCLUSIONS

The mitochondrial migration during fusion and pro-nuclear swelling in cloned embryos showed similar behaviour between intra- and inter-species SCNT. The iSCNT presented some differences, according to the percentage of homologous recipient cytoplast that was used for reconstruction. The cell lineage, and specially, the cell type, implies directly in the capacity of embryo development since the early events of mitochondrial migration or later, during pro-nucleus swelling. The efficiency of this technique, according to the iSCNT embryo production and blastocyst quality can be increased with the use of Scriptaid, showing that the better modulation of nuclear reprogramming can help in the cross-talk between nucleus and cytoplasm.

The characteristics of sperm vigor and penetration capacity are related with the embryo production, according to the oocyte quality. Techniques such as ICSI, that provide a most controlled activation, showed a beneficial effect in embryo development, despite of the gametes characteristics.

The ability of the zygote nucleus to better interact with the oocyte cytoplasm is significantly related with the efficiency in embryo production by IVF in the bovine, and by SCNT and iSCNT in porcine. The use of tools that have the ability to synchronize as a most physiological manner the occurrence embryo development events are widely efficient and applicable.

The use of assisted reproductive techniques such as SCNT, ICSI and SUZI as tools to understand and interfere on embryo IVP are convenient and help improve biomedical and economical applications of reproduction research.

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