JOANA CLAUDIA MEZZALIRA

EFEITO DA HETEROPLASMIA NA DENSIDADE CELULAR E DESENVOLVIMENTO EMBRIONÁRIO *IN VITRO* DE EMBRIÕES BOVINOS CLONADOS POR TRANSFERÊNCIA NUCLEAR DE CÉLULA SOMÁTICA

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Efeito da heteroplasmia na densidade celular e desenvolvimento embrionário *in vitro* de embriões bovinos clonados por transferência nuclear de célula somática

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: Marcelo Bertolini

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RESUMO

EFEITO DA HETEROPLASMIA NA DENSIDADE CELULAR E DESENVOLVIMENTO EMBRIONÁRIO *IN VITRO* DE EMBRIÕES BOVINOS CLONADOS POR TRANSFERÊNCIA NUCLEAR DE CÉLULA SOMÁTICA

Na clonagem por transferência nuclear com célula somática (TNCS), o tipo de citoplasto receptor desempenha papel chave na reprogramação nuclear. Distintos citoplastos e carioplastos e condições de ativação foram utilizadas na reconstrução de embriões bovinos com o objetivo de avaliar o efeito do tipo de citoplasto (oócito e/ou zigoto) e do método de ativação (química, AQ, ou espermática, AE) no desenvolvimento de blastocistos clonados produzidos pela técnica de clonagem manual (*Handmade Cloning*, HMC). Após 17 h de maturação *in vitro* (MIV), 2.946 oócitos foram enucleados por bissecção manual, resultando em hemi-oócitos enucleados (citoplastos MII) e não enucleados (carioplastos MII). Outros 2.368 oócitos submetidos a 6 h de fecundação *in vitro* (FIV) foram bisseccionados manualmente e segregados em hemi-zigotos enucleados (citoplastos FIV) e não enucleados (carioplastos FIV). Células de um cultivo celular estabelecido a partir da biópsia auricular de uma fêmea bovina adulta foram utilizadas como núcleos doadores (carioplasto CS). As estruturas foram dispostas em (a) grupos controle: FIV; partenogênese com oócitos com (PG c/) ou sem zona pelúcida (PG s/); e clone por TNCS; ou (b) grupos experimentais: G1, citoplasto FIV + citoplasto MII + carioplasto CS; G2, citoplasto MII + carioplasto FIV; G3, citoplasto FIV + carioplasto FIV; G4, citoplasto FIV + citoplasto FIV + carioplasto CS; e G5, citoplasto MII + carioplasto MII. Após a eletrofusão das estruturas, os grupos experimentais G1 a G45 foram divididos em subgrupos de AQ ou AE. O cultivo *in vitro* foi realizado pelo sistema WOW (*well-of-the-well*). Após 20 repetições, as taxas de clivagem (D2) e blastocisto (D7) foram comparadas pelos testes de χ^2 e os valores para o número total de células e a alocação das linhagens celulares nos blastocistos, determinados por coloração diferencial, foram avaliados por análise de variância, com pareamento comparativo pelo teste de Tukey, para $P<0,05$. O único grupo experimental que apresentou desenvolvimento embrionário no D7 semelhante aos controles FIV (27,0%) e TNCS (31,4%) foi o subgrupo G1 AE (28,2%). Isso pode ser atribuído a uma melhor sincronia do ciclo celular entre citoplastos e/ou carioplasto e um mais adequado processo de ativação. O desenvolvimento embrionário nos grupos G1 AQ (13,7%), G4 AQ (6,4%) e G4 AE (8,7%) foi menor do que o G1 AE, possivelmente devido à assincronia do processo de ativação ou ciclo celular. O desenvolvimento embrionário nulo dos grupos G2 e G3, independente da ativação, possivelmente foi decorrente da manipulação das estruturas em um momento biologicamente sensível. Da mesma forma, a baixa clivagem (57,0%) e o desenvolvimento nulo no grupo G5 de ativação espontânea demonstraram de fato que a manipulação estimulou o processo de ativação embrionária de forma sub-limiar. Em geral, não houve diferença no número de células e alocação celular nos grupos onde houve desenvolvimento até o estádio de blastocisto. Conclui-se que o processo de ativação foi tão significativo para o desenvolvimento embrionário que o tipo de citoplasto e carioplasto usados na reconstrução embrionária. A produção de embriões clones com um método mais fisiológico de ativaçao (AE) mostrou-se como um procedimento viável, obtendo-se no grupo G1 AE a mesma eficiência observada na FIV ou na TNCS.

Palavras-chave: SCNT, *handmade cloning*, partenogênese, bovinos, ciclo celular, ativação embrionária

ABSTRACT

EFFECT OF HETEROPLASMY ON CELL DENSITY AND IN VITRO DEVELOPMENT OF BOVINE EMBRYOS CLONED BY SOMATIC CELL NUCLEAR TRANSFER

In somatic cell nuclear transfer (SCNT), the type of the recipient cytoplast plays a key role on nuclear reprogramming. Distinct cytoplasts and karyoplasts and different activation protocols were used for bovine embryo cloning aiming to evaluate the effect of the type of cytoplast (oocyte and/or zygote) and the activation protocol (chemical, AQ, or spermatic, AE) on development of cloned blastocysts produced by handmade cloning (HMC). After 17 h of in vitro maturation (MIV), 2,946 oocytes were enucleated by manual bisection resulting in either MII cytoplasts (enucleated) or MII karyoplasts (non-enucleated). An additional group of 2,368 oocytes, in vitro-fertilized (FIV) for 6 h, were manually bisected and segregated in either FIV cytoplasts (enucleated) or FIV karyoplasts (non-enucleated). Cells from a primary culture previously established from a skin biopsy from an adult female bovine were used as nuclei donors (karyoplast CS). Structures were allocated to (a) control groups: FIV; parthenogenesis using zona-intact (PG c/) or zona-free oocytes (PG s/); and clones by SCNT; or (b) experimental groups: G1, FIV cytoplast + MII cytoplast + CS karyoplast; G2, MII cytoplast + FIV karyoplast; G3, FIV cytoplast + FIV karyoplast; G4, FIV cytoplast + FIV cytoplast + CS karyoplast; and G5, MII cytoplast + MII karyoplast. Following electrofusion, experimental groups G1 to G5 were allocated to sub-groups of either sperm-mediated (AE) or additional chemical (AQ) activation. The in vitro culture was carried out in the WOW (well-of-the-well) system. After 20 replications, cleavage (D2) and blastocyst (D7) rates were compared by the χ^2 test, with values for total cell number and cell allocation in the blastocyst, determined by differential staining, being evaluated by ANOVA, with pairwise comparisons by the Tukey test, for $P<0.05$ ($P<0.05$). The only experimental group that yielded a blastocyst development similar to the FIV (27.0%) and SCNT (31.4%) control groups was the subgroup G1 AE (28.2%). This fact may be attributed to a more proper synchrony between the karyoplast and cytoplasts and/or to a more suitable activation process. Embryo development in subgroups G1 AQ (13.7%), G4 AQ (6.4%) and G4 AE (8.7%) was lower than in G1 AE, possibly due to a higher degree of asynchrony in the activation process or cell cycle. The lack of development in groups G2 and G3, irrespective of the activation protocol, was possibly due to the manipulation process during a highly sensible biological period. Likewise, the low cleavage (57.0%) and the lack of development in group G5 (spontaneous activation) in fact showed that the manipulation induced weak spontaneous oocyte activation. In general, total cell number and cell allocation were similar between groups with development to the blastocyst stage. In conclusion, the activation process appeared to be as important to embryo development as the type of cytoplast or karyoplast used for embryo reconstruction. The production of cloned bovine embryos using a more physiological activation process (AE) was proven as a viable procedure, with efficiency rates observed in subgroup G1 AE being similar to groups FIV or TNCS.

Key-words: SCNT, handmade cloning, parthenogenesis, cattle, cell cycle, embryo activation

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AC	Corrente Alternada (*Alternate Current*)
ANOVA	Análise de Variância (*Analysis of Variance*)
BL	Blastocisto (*Blastocyst*)
BSA	Albumina sérica bovina (*Bovine Serum Albumin)*
CIV (IVC)	Cultivo *in vitro* (*in vitro culture*)
CCM	Cell Culture Medium
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)*
MM	Meio de manutenção (Hepes-buffered M-199 + 10% FCS)
ICM	Massa cellular interna (*inner cell mass*)
MII	Metáfase II (*Metaphase II*)
MIV (IVM)	Maturação *in vitro* (*in vitro maturation*)
MPF	*Maturation/Meiosis/Mitosis Promoting Factor*
mRNA	RNA mensageiro (*Messenger RNA*)
miRNA	micro RNA (*micro RNA*)
mSOFaa	*modified Synthetic Oviductal Fluid medium supplemented with amino acids*
mtDNA	DNA mitocondrial (*Mitochondrial DNA*)
i.e.	Por exemplo (*For example*)
PIV (IVP)	Produção *in vitro* (*in vitro production*)
6-DMAP	6-Dimetil aminopurina (*6-DymethylAminoPurine*)
TC	*Tissue Culture*
TCN	*Total cell number*
TN (NT)	Transferência Nuclear (*Nuclear Transfer*)
TNCS (SCNT)	Transferência Nuclear de Células Somáticas (*somatic cell nuclear transfer*)
WOW	micropoços (*Well-of-the-Well)*
ZP	Zona Pelúcida (*Zona Pellucida*)

SUMÁRIO

1 INTRODUCTION

1.1 A brief history on animal cloning

When Hans Spemman tried for the first time in 1938 to answer the question: "Do nuclei change during development?" by using the transfer of cell nuclei from increasingly advanced embryonic stages into amphibian eggs (Tagarelli *et al*. 2004), he was aiming to determine at which point the developmental potential of nuclei becomes restricted. He was, then, seeding the principles of animal cloning. This visionary scientist, who is considered by many as the father of animal cloning and a major exponent on experimental embryology, contributed with his studies to the understanding of events on cell differentiation and totipotency. By using amphibians as salamanders and frogs as models for pioneering, elegant and ingenious experiments on embryo splitting in early 1900's and nuclear transfer in mid 1920's, he was rightfully awarded the Nobel Prize in Physiology/Medicine in 1935. After Spemman's death in 1941, animal cloning studies were resumed only in early 1950's, by Robert Briggs and Thomas King (Briggs & King 1952). By cloning tadpoles from northern leopard frogs to study gene activation and deactivation during cell development, the investigators laid out an experiment that was similar to that envisioned by Hans Spemann, in his 1938 proposal for a "fantastical cloning experiment". Interestingly, Briggs and King were unfamiliar with Spemman's earlier work. As Briggs' and King's few tadpoles cloned from differentiated cells grew abnormally, the authors concluded that the genetic potential reduces as cell differentiation increases, making it impossible to clone an organism from adult differentiated cells, a dogma in biology that persisted for almost 50 years, until late 20th century.

Luckily, the advancement of science comes with persistence. In 1962, John Gurdon announced that he had used fully differentiated adult intestinal cells to clone adult South African frogs (*Xenopus laevis*), daring to break the dogma (Gurdon 1962). Even though his

work had demonstrated that cells do retain the ability to form different tissues even after differentiation, some other investigators argued that some undifferentiated sex cells exist in the intestines of South African frogs, with Gurdon's clone possibly being derived from such cells. Years later, Gurdon's studies were complemented by McAvoy *et al*. (1975) with their report regarding the cell cycle stage influence on frog cloning. Almost concomitant to Gurdon's first report, Tong Dizhou, a Chinese embryologist, cloned the carp in 1963 (Liao *et al*. 2007), publishing his findings in an obscure Chinese science journal which was never translated into English to be scientifically recognized or validated. Again and again, the dogma was sustained.

The nuclear transfer technology took longer to attain success in mammals. In 1981, Illmenesee & Hoppe reported the first mouse cloned from inner cell mass (ICM) cells. Their work was not repeated by other researchers and suffered severe skepticism, with the investigatory inquiry finding chaos, but no fraud in the experiments (MacKenzie 1984). In 1983, McGrath & Solter demonstrated the viability of pronuclear exchange between mouse zygotes, also showing that cytoplasts derived from early zygotes had limited capacity to support development, data confirmed later by Prather *et al*. (1987). Then, in 1986, an unequivocal breakthrough was conquered in animal cloning after the birth of viable offspring derived from nuclear transfer of blastomeres from early-stage preimplantation sheep embryos, using *in vivo*-matured metaphase stage oocytes as recipient cytoplasts (Willadsen 1986). The success in the sheep was later confirmed in cattle (Robl *et al*. 1987), showing the repeatability of the process, when using totipotent or multipotent donor cells. However, the use of blastomeres was still limited by the low number of identical animals that could be produced from an individual embryo.

The search for a suitable nuclear transfer protocol using more undifferentiated culminated with the use of cultured embryonic cells as nuclear donors, which again resulted

in live offspring (Campbell *et al*. 1996b). Then, one of the greatest dogmas in biology was finally broken by the same group of researchers, after the birth of Dolly, the sheep, cloned using somatic cells from the mammary gland of a 6-years old ewe as nuclear donors (Wilmut *et al*. 1997), a procedure that was later named somatic cell nuclear transfer (SCNT). However, such feat did not come without skepticism by many, which was put to rest, at last, after the birth of the mouse Cumulina by Ryuzo Yanagimachi's group in Hawaii in 1997 (Wakayama *et al*. 1998), followed by the production of live offspring in cattle originated from somatic fetal fibroblasts (Cibelli *et al*. 1998) and from somatic adult cells (Kato *et al*. 1998). Since then, more than 20 animal species have been cloned from a widespread variety of donor cells (Bertolini *et al*. 2007), with the most recent cloned mammal claimed to be a camel named Injaz, or "Achievement" in Arabic, born last April in Dubai (media news).

1.2 Steps towards the "universalization" of animal cloning: technical challenges

Despite the expansion in use of animal cloning, a rather small number of laboratories in the world are well suited and equipped to perform nuclear transfer experiments successfully. The high cost of the necessary pieces of equipment and the need for highly skilled operators are among some of the limiting factors that have hindered the widespread use of the procedure. Many technical challenges remain as major obstacles for advancements in knowledge regarding biological issues associated with cloning. However, development of simplified cloning procedures over the years has allowed a broader dissemination of animal cloning as a tool for research, which is contributing to a gain in knowledge, efficiency and perhaps, to a more common commercial use of cloning by the industry.

An important step towards the simplification of animal cloning was the development of procedures without the need of micromanipulators, also known as the Handmade Cloning (HMC). Such zona-free technique was first described by Peura *et al*. (1998) with blastomeres and later adapted by Vajta *et al*. (2003) for somatic cells. The absence of *zona pellucida*

associated to the well-of-the-well (WOW) system proportionate an excellent toll for the study of embryo aggregation (Boiani *et al*. 2003, Misica-Turner *et al*. 2007, Ribeiro *et al*. 2009), cytoplasmic volume (Peura *et al*. 1998, Ribeiro *et al*. 2009) and cell allocation (Misica-Turner *et al*. 2007), for instance.

1.3 Steps towards the "universalization" of animal cloning: biological challenges

Despite the great number of possible applications for agriculture (Faber *et al*. 2003, Lewis *et al*. 2004) or biomedicine (Paterson *et al*. 2003), mammal embryos cloned by SNCT present an abnormal epigenetic configuration that is associated with phenotypical and physiological alterations during development (Bourc'his *et al*. 2001, Dean *et al*. 2001, Kang *et al*. 2001, Wrenzycki *et al*. 2001, Xue *et al*. 2002, Beaujean *et al*. 2004). This supports the hypothesis that failures on gene expression or embryo development are caused by an inadequate genome donor reprogramming, that usually takes place during or after SCNT, mostly due to faulty chromatin remodeling and re-establishment of an embryonic DNA methylation from the somatic cells pattern (Giraldo *et al*. 2008).

Many abnormalities established during early pre-implantation stages after cloning by SCNT may be only manifested during the course of pregnancy or after birth, resulting in phenotypical alterations that are often incompatible to life, becoming an important animal welfare issue (Bertolini *et al*. 2002, 2004). Unfortunately, the early detection of the severity and the type of abnormalities present in pregnancies and in calves derived from IVF and SCNT is not yet possible. The understanding of the biological basis behind the problems caused by cloning, mainly those linked to faulty epigenetic nuclear reprogramming after cloning, is important for the early diagnosis, prediction and elimination of the abnormalities.

The recipient cytoplast plays a key role on nuclei donor reprogramming. Nevertheless, the ooplasmic components responsible for nuclear reprogramming after fertilization appear

not to be sufficient to modify the differentiation marks from the donor nucleus after SCNT (Bird 2002), a process also known in cloning as erasure of somatic cell differentiation status (Cezar 2003). The level of cell cycle synchrony between cytoplast (recipient cytoplasm) and karyoplast (nucleus donor) and the embryo activation process have also been shown to influence reprogramming and development (Fulka *et al*. 1996, Bordignon & Smith 2006). Furthermore, the cytoplasmic mosaicism and level of heteroplasmy caused by fusion of distinct cytoplasms during cloning, usually originated from enucleated MII oocytes (cytoplast) and somatic cells (karyoplast), may be either detrimental or may promote or increase the development capacity of reconstructed embryos (Liu & Keefe 2000, Alberio *et al*. 2001). Taking together, the phenomena described above are merely fragments of a wide spectrum of a multitude of biological events, which demonstrates the complexity of the cell and molecular processes that still need to be elucidated in biology, for which experiments in cloning are very useful, as well initiated and proposed by Hans Spemman over a century ago.

Recently, Schurmann *et al*. (2006) demonstrated that the use of 1-cell stage IVF-derived embryos as cytoplasts for SCNT cloning, enucleated 4 h after the onset of IVF, resulted in similar *in vitro* development as embryos produced with enucleated MII oocytes. However, *in vivo* development of such cloned blastocysts, reconstructed with IVF-derived cytoplasts, was significantly higher, manifested by higher pregnancy and calving rates. As the use of a more physiological strategy to activate oocytes has been reported in cattle (Schurmann *et al*. 2006) and horses (Hinrichs *et al*. 2006), with sperm-mediated activation considered beneficial for epigenetic reprogramming, perhaps a better cell cycle synchrony or a favorable cytoplasmic mosaicism between cytoplast and karyoplast may have also played important roles in improving development, clearly demonstrating the differences with respect to the epigenetic reprogramming capacity between karyoplasts and cytoplasts, in the process cloning by SCNT.

Studies on *in vitro* development comparing the effect of embryo reconstruction with somatic cells (usually at the G0/G1 phases of the cell cycle) fused to single or a combination of cytoplasts at distinct cell cycle phases and/or activation status (hemi-oocytes at metaphase, hemi-zygotes at early post-fertilization stages, previously activated by the sperm), still need a more systematically investigation for bovine cloning by SCNT, as we hypothesize that (a) the use of IVF cytoplasts (pre-activated by the sperm) enhances *in vitro* viability after SCNT; (b) the association of two pre-activated cytoplasts provides better blastocyst rates than the association of one IVF and one MII cytoplasts; and (c) the additional chemical activation results in better *in vitro* viability than only the activation provided by the sperm. Consequently, the aim of this study was to determine the effects of the type of cytoplast (non-activated enucleated MII hemi-oocytes, early pronuclear stage enucleated IVF-derived hemi-zygotes) and/or karyoplast (somatic cells, non-activated MII hemi-oocytes containing the metaphase plate, early pronuclear stage IVF-derived hemi-zygotes containing the sperm and egg chromatin), and the activation process (sperm-mediated of chemically induced) on *in vitro* development, cell density and cell allocation of bovine embryos cloned by HMC.

2 LITERATURE REVIEW

2.1 Early fertilization events

The major function of fertilization is the transmission of genes from parents to the offspring. Fertilization, by the sperm penetration, is also responsible for initiating reactions in the egg cytoplasm that allows development to initiate and to go on. Fertilization is an active process in which highly motile sperm interact with the oocyte in a species-specific manner to bind and traverse the *zona pellucida*, ultimately fusing with the oocyte membrane to form the zygote. In most mammals, including the bovine, the egg nucleus is still diploid at the time of fertilization, and it is only when the sperm cells binds and/or enters the egg that the meiotic division is resumed (Perreault 1992). This active process takes place between gametes that are genetically quiescent. The oocyte is arrested at MII of meiosis, a chromosomal state that precludes DNA synthesis or RNA transcription (Crisp 1992). At this point, the sperm chromatin is uniquely compacted, into a highly dense and genetically inert format (Ward & Coffey 1998). Then, both the gametes "reawaken" each other, as the sperm activates the oocyte, with the sperm-egg fusion triggering a membrane depolarization in the oocyte, with a transient cytosolic calcium influx, as a peak, that repeats itself in predictable intervals, with consequent oocyte responses. Then, cortical granules are released, blocking polyspermy, the second polar body is extruded, and the gametes' chromatin initiate a gradual remodeling process into male and female pronuclei, a permissive DNA synthesis state. As half of the female genetic material is removed by the extrusion of the second polar body, the female pronucleus is haploid, as the sperm (Campbell *et al*. 1996a). However, ploidy abnormalities, *in vivo,* might exist due to two distinct common segregation errors: triploidy, subsequent to either dispermy (polyspermic penetration) or digyny, when the second polar body is not extruded (Gilbert 1991). Morphological consequences of oocyte activation and the timing

where such events take place appear to be controlled by the oocyte rather than the sperm. If the sperm decondensation is experimentally advanced, the sperm prematurely decondensed chromatin will not form a male pronucleus ahead of its female counterpart. It awaits conditions around an appropriate template (Perreault *et al*. 1987). The most important step for allowing egg development is the activation of egg metabolism, to aid development to proceed.

2.2 Epigenetic reprogramming

For nuclear transfer technique to succeed, the donor nucleus must undergo a series of reprogramming events. In the context of SCNT, the term "reprogramming" is used to define a multifaceted process by which a somatic cell nucleus is rendered into a physiological state capable of supporting embryonic development (Beyhan *et al*. 2007). These processes include remodelling somatic donor nuclei, silencing differentiation-associated genes, activating the genes critical for embryo development, and reestablishing imprinting patterns (Latham 2005).

Epigenetics is defined as the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence (Cezar 2003). The term epigenetic reprogramming is employed for the erasure of differentiation memory from the somatic (or more differentiated) cells used as donor nucleus for cloning. This erasure is defined as the turn such cells have to accomplish back to an embryonic-like totipotent stage. This is one of the main requirements for an efficient cloning, as faulty epigenetic reprogramming is highlighted as the major cause of developmental failure and abnormal phenotypes in cloned animals (Alberio *et al*. 2006). The epigenetic (and not genetic) nature of alterations can be evidenced by the fact that cloned animals are capable of producing offspring and generating normal progeny (Tamashiro *et al*. 2002). Progression through the cell cycle requires continuous changes in gene activity (Kono *et al*. 1996) that does not entail changes in DNA sequence and can therefore be classified as epigenetic (Wells *et al*. 2003,

Cezar 2003). The process of differentiation involves the assembly of specialized forms of a repressive chromatin, which must compartmentalize into functional domains still maintaining the stability of the differentiated state through successive cell divisions. Such functional domains include linker histones, Polycomb group proteins and methyl-CpG-binding proteins (Kikyo & Wolffe 2000) and many others.

2.2.1 DNA methylation and imprinted genes

DNA methylation at cytosine-rich regions, generally close to gene promoters, suppressors or enhancers, is the major epigenetic system that is essential for animal development (Okano *et al*. 1999). It fulfills the criteria of heritability (Pfeifer *et al*. 1990) by inducing the assembly of protein-DNA complexes that also involve suppressors with chromatin remodeling activity (Lei *et al*. 1996). One second role of DNA methylation in mammals is to function as the imprinting mark that distinguishes between silenced and expressed parental alleles for imprinted genes. Such genes are overall crucial regulators of growth, development and differentiation of mammalian embryo, fetus and placenta. As they display parental allele-specific expression established during gametogenesis (Surani *et al*. 1984), they are unique.

Imprinting is restricted to mammals and its evolutionary role is not completely understood. Imprinted genes are often organized in clusters, and most have differentially methylated regions (DMRs) that are CpG rich and subjected to epigenetic modifications (Tilghman 1999, Reik *et al*. 2001). The DMRs are proposed to serve as inactivation center when methylated, and as a chromatin insulator or boundary element when unmethylated, thus providing primary imprinting signals (Takada *et al*. 2002). In advancement, although methylation of DMRs in most genes result in their repression, in some instances methylation is essential for gene activation (Surani 2001). The aberrant expression of imprinted genes is possibly the responsible for some of the abnormalities seen in cloned animals (Shi *et al*.

2003, Yang *et al*. 2005). To date, what has been elucidated is that paternally expressed genes stimulate fetal growth and placental differentiation (Wang *et al.* 1994), while maternally expressed imprinted genes display the opposite effect, restraining or modulating fetal growth. Different methylation patterns at target sequences can occur in cloned animals generated from the same single embryonic stem subclonal population (Humpherys *et al.* 2001).

2.2.2 Zygote DNA methylation patterns and nuclear reprogramming

The normal DNA methylation pattern, described for normally fertilized embryos, has maternal and paternal genomes virtually erased during the first few rounds of cell division. Based on the established pattern, it is suspected that the paternal (sperm-derived) genome is actively demethylated before the onset of the first DNA replication. The maternal (oocyte-derived) genome, on the other hand, is demethylated passively, in a progressive fashion with each cleavage division (Oswald 2000, Bourc'his *et al.* 2001). Such passive process is believed to occur due to the lack of access of the maintenance methylating enzyme (DNA methyltransferase-1, or Dnmt1) to the nucleus during early cleavage (Oswald 2000).

Fertilized eggs undergo a wave of demethylation that erases part of inherited parental methylation pattern. Then, mouse blastocysts and 8- to 16-cell stage bovine embryos are relatively hypomethylated. The Dnmt1 functions as a major maintenance methyltransferase, ensuring "replication" of DNA methylation patterns, after each round of cell division (Okano *et al*. 1999). The other Dnmts, the Dnmt3a and 3b are not essential for maintenance of imprinted methylation patterns; however, they are essential for *de novo* methylation, as well as for the maintenance of methylation marks of imprinted genes (Hirasawa *et al.* 2008).

In mammals, the chromatin condenses after fertilization not to form a unique zygote nucleus, but both the chromosomes orient themselves upon a common mitotic spindle, being a true diploid nucleus only formed at the 2-cell stage (Gilbert 1991). In bovine embryos, shortly after the demethylation process occurs, a wave of *de novo* methylation (Okano *et al*.

1999) is observed (Barnes & Eyestone 1990) from the 8-cell to the 16-cell stage (the developmental block stage), establishing a new embryonic methylation pattern (Kang *et al.* 2001, Dean *et al.* 2001, Rideout *et al*. 2001). This *de novo* methylation is catalyzed by DNA methyltransferases Dnmt3a and Dnmt3b. It has been proposed that failure of the DNA to demethylate and remethylate causes incomplete nuclear reprogramming in cloned embryos, with methylation moieties in between the levels seen in normal zygotes and donor cells, with the pattern resembling more the donor cells (Bourc'his *et al.* 2001; Kang *et al*. 2001, Beaujean *et al*. 2004). This is in accordance to the findings that zygotic Dnmt1 not only is expressed in the preimplantation embryo, but is alone sufficient to maintain the methylation marks of imprinted genes (Hirasawa *et al*. 2008). However, differentiation also involves the assembly of specialized forms of repressive chromatin (McGrath 1984), which includes linker histones, polycomb group proteins and methyl CpG-binding proteins that compartmentalize chromatin into functional domains, keeping differentiation stable through the successive cell divisions (Kikyo & Wolffe 2000, Wrenzycki *et al*. 2001, Wrenzycki & Niemann 2003). It is not a surprise, then, that *in vitro* manipulations at such a crucial developmental time alter mechanisms involved in nuclear reprogramming, for which DNA methylation seems to be key.

Efficient cloning requires the erasure of repressive structures claiming the importance of enzymatic activities present in the oocyte and zygote, reestablishing the pluripotency (Wuensch *et al*. 2007). For highly differentiated cells, the challenge is supposed to be greater, as the reactivation of silenced genes tend to be hindered. Problems prior to or during the embryonic genome activation (EGA) result in embryos not developing beyond the 8- to 16-cell stage in cattle (Meirelles *et al*. 2004), the biological timing for EGA (Robl & Stice 1989). Developmental block, in fact, is a consequence of aberrant methylation of genes, especially imprinted ones. This species-specific block moment is concurrent with the maternal-embryo

transition, the developmental stage when embryos conclude the major genome activation (De Sousa *et al*. 1998) and must rely on the mRNAs transcribed from its own genome to continue development. It appears that somatic cell cloning impairs EGA in some embryos, irrespective of the cell cycle phase of the donor cell, probably due to faulty activation/inactivation of genes that should be adequately reprogrammed (Kanka 2003). After the EGA in cloned embryos, the transferred nucleus should have a pattern of gene expression compatible to the early normal embryo for further successful development. This change, generally termed reprogramming (Kim *et al*. 2002), is preceded by altering the configuration of chromatin, i.e., chromatin remodeling (Kikyo & Wolffe 2000). In spite of its biological importance, little is known about the molecular nature of the reprogramming event.

2.2.3 Oocyte activation events and the role of sperm factors on activation and embryo development

Oocytes in many species undergo parthenogenetic activation after exposure to physical or chemical processes that artificially induce a calcium influx into the ooplasm, such as electric currents or exposure to certain chemical agents (e.g., calcium ionophore, ionomycin), even in the absence of external calcium. The role of intracellular calcium has been defined as key in the activation process, as parthenogenetic activation and subsequent normal embryo development can be induced in mouse oocytes by intracytoplasmic calcium injection (Fulton & Whittingham 1978). That implies that increasing intracellular ionized calcium in an oocyte by injection induces the normal sequence of events known to follow fertilization, releasing the oocyte from the meiotic block at metaphase II followed by subsequent cell division. Classic studies have revealed the basis of most of our current knowledge related to the role of calcium on embryo activation. Measurements of cytoplasmic free calcium during the artificial activation and fertilization of single mouse oocytes injected with a calcium-sensitive photo-protein detected that free calcium rises exponentially from a

resting level to a peak over a period of 10 to 30 min. A series of oscillatory calcium peaks precedes such calcium rise during fertilization, but not during artificial activation (Cuthbertson *et al*. 1981). The local calcium concentration may fall in calcium-free medium due to calcium influx. Conversely, calcium-free medium is also capable of inducing oocyte activation, what suggests that calcium arises both from the citosol and the external medium (Steinhardt 1974, Cuthbertson *et al*. 1981). At fertilization, the signal during the calcium peaks does not arise from the entire oocyte but from a localized region, which may be at the site of sperm-egg fusion or near the plasma membrane of the oocyte (Cuthbertson *et al*. 1981). In mice, calcium features are of sustained oscillations continued in a regular pattern during 4 h after fertilization (Cuthbertson & Cobbold 1985). This confirmed that artificial activation differs from the pattern produced by the sperm.

A sperm-specific phospholipase-C (PLC) has been described as a PLC capable of inducing a series of calcium oscillations, with the mRNA involved in this process not being evident in other tissues (Parrington *et al*. 2000). Further studies about the complex signaling phenomenon for oocyte activation has been at least partially elucidated by the finding of the sperm-specific PLC-zeta, ou PLCζ (Saunders *et al*. 2002). This PLC isoform triggered calcium oscillations in mouse eggs that were indistinguishable from the fertilization events. Besides, the removal of PLCζ from sperm extracts abolished calcium release in mice eggs; in addition, the PLCζ content of a single sperm was proven enough to produce calcium oscillations capable of developing embryo to the blastocyst stage, as expected.

The absence of fertilization certainly results in the absence of such recently described important sperm factors and several other ones that still remain to be identified. In another important study, calcium waves were carefully analyzed (Deguchi *et al*. 2000) with results confirming that it would be quite impossible to reproduce with fidelity the natural pattern of calcium cytoplasmic movement. The initial calcium release that is the first experience for the

egg may involve mobilization of calcium from internal reserve stores. Once explosive calcium release takes place, the reserve stores may be hardly refilled. The progressive shortening of the duration of calcium waves could be due to facilitated calcium uptake to the endoplasmic reticulum during oscillations or down-regulation of calcium releasing channels. Cuthbertson *et al*. (1981) observed that a slight deformation of the egg is produced in such a way that cytoplasm calcium movement is guided by the preceding calcium wave. This was the first report of such a feature on mammalian eggs and the cytoplasmic contractions are believed not only to sweep the incorporated sperm nucleus and cortical organelles toward the contraction pole, but also reorganizes cytoplasmic determinants required for cell fate determination (Deguchi *et al*. 2000).

Besides all particular characteristics of activation events to the side view of the oocyte, activation completion through fertilization still depends on the individual particularities of every single male that donates sperm, as it is known that sperm from different bulls, under the same experimental conditions, demonstrate different kinetics of fertilization (Barnes & Eyestone 1990).

In summary, all the activation events contribute to one specific aim that is to activate the genome to develop an embryo and so on. In clones, however, the DNA methylation patterns typical of biparental chromosomes are not well reproduced (Bourc'his *et al*. 2001), and by consequence, the passive DNA demethylation process is less efficient. The DNA methylation patterns typical of normal development precede creating a differentiated cell state where the erasure and imprinting methylation profiles have to be reproduced (Kikyo & Wolffe 2000).

Many transcripts carried by the sperm appear to have important roles on embryo activation and development. Despite the virtual absence of cytoplasm and the insufficiency of ribosomes that hinders the sperm to support normal translation, it is known that male gametes

pass over more to the oocyte than merely the haploid male genome. For instance, sperm cells contain a complex repertoire of mRNAs (Ostermeier *et al*. 2004, Miller *et al*. 2005). These mRNAs are thought to provide an insight into past events of spermatogenesis, and the complexity and function of such repertoire still remain to be described. However, after microarray analyses, spermatozoal mRNA supports a view that a fingerprint can be obtained from normal fertile man, and this profiling might be useful for monitoring past gene expression events (Ostermeier *et al*. 2002). As sperm provides the zygote with a unique suite of paternal mRNAs, this might be an interesting tool for further investigations regarding the individual *in vitro* behavior of particular bulls.

After sperm maturation, the histones are replaced by transition proteins and then by protamines (Steger 2001, Meistrich *et al*. 2003), to optimize protein compaction. Sperm DNA is the most highly compact chromatid, at least six times more condensed than DNA in mitotic chromosomes. Such chromatin condensation gives high stability to the sperm but also inhibits the transcription of new RNA.

Despite these molecular facts, exposure of the early embryonic genome mice to micro RNA (miRNA) is known to induce permanent and heritable epigenetic change in gene expression (Rassoulzadegan *et al*. 2006). Mouse sperm RNA contains at least 20% of miRNA, which could be released into the oocyte during fertilization (Amanai *et al*. 2006).

Paternal messenger RNAs are delivered to the egg at fertilization (Ostermeier *et al*. 2004), and such transcripts (believed to be leftovers from spermatogenesis) are known to be important in early development. This information may have implications for the success SCNT, as conventional protocols are performed in complete absence of the sperm and its factors. Although the role of unknown sperm RNAs remains to be defined, it is presumed that they play specific roles at fertilization and on early embryo development (Ostermeier *et al*. 2004, Miller *et al*. 2005). There are also other unidentified molecules, such as small

interfering RNAs (siRNAs), that may participate in processes such as pronuclear formation, the orchestration of events leading to oocyte activation, the transition from maternal to embryonic gene control, and the establishment of imprints in early embryos (Ostermeier *et al*. 2004). However, this venue is still widely unexplored.

Developmental programming and phenotypic differences among species and individuals have been suggested to be heavily influenced, if not fundamentally controlled by, the repertoire of regulatory non-coding RNAs (Mattick & Makunin 2006), derived from the further processing of exons and introns of protein-coding genes and from non-coding RNA genes. The difficulty to determine such roles is due to the fact that a single cDNA sequence may define a transcriptional unit (TU). It is hard to determine where RNA is originated because a single TU could potentially produce more than one mRNA species through alternative splicing and alternate transcriptional initiation and termination (Mattick & Makunin 2006). Still, miRNA and RNA interference (RNAi) molecules are used by control mechanisms to inhibit gene expression at the level of mRNA degradation, translational repression, or chromatin modification and silencing genes (Kotaja & Sassone-Corsi 2007). There is still a universe to be explored regarding the mechanisms where sperm trigger any contribution, other than the haploid genome, for embryo development. The use of previously fertilized cytoplasts may contribute as a tool to investigate any clue for better pre-implantation development (Schurmann et al. 2006).

2.2.4 Aberrant reprogramming patterns in SCNT

SCNT and IVF blastocysts displayed surprisingly similar gene expression profiles, suggesting that a major reprogramming activity had been exerted on the somatic nuclei. Despite this remarkable phenomenon, a small set of genes appears to be aberrantly expressed and may affect critical developmental processes responsible for the failures observed in

SCNT embryos (Beyhan *et al*. 2007). The histone content can be affected by the stage of cell cycle, cell type and number of passages for cells used for cloning (Bordignon *et al*. 1999), with histone acethylation status being apparently remodeled by *in vitro* cell culture (Bordignon *et al*. 2000).

Pieces of evidence that aberrant expression of imprinted genes is present in SCNT aborted and deceased newborns (Cezar 2003, Yang *et al*. 2005). Such observations indicate that incomplete epigenetic reprogramming may be responsible for the developmental failure and abnormal phenotypes reported in cloned animals.

During the first cleavage stages, *in vivo*-produced and cloned embryos undergo drastic changes in their chromatin configuration (Dean *et al*. 2001). This implies that important genes become up-regulated (such as interferon tau) or down-regulated (such as histocompatibility complex class 1) during this period (Giraldo *et al*. 2008), being the abnormal expression profiles caused by faulty DNA methylation or histone acetylation (Enright *et al*. 2003b, Kanka 2003).

Attempts to change the epigenetic status of cells have ended in contrasting results, not yielding efficient protocols for efficient *in vitro* pre-reprograming of donor cells. For instance, cells treated with Trichostatin A or 5-aza-2'deoxycytidine (an enhancer of histone acethylation and an inhibitor of DNA methylation, respectively) were morphologically affected even with low doses of both the drugs. Despite toxic effects, the Trichostatin A improved blastocyst rate significantly, an indicative of better nuclear reprogramming or gene activation (Enright *et al*. 2003a).

As the nuclear reprogramming is a process that takes a few cell cycles to be completed, this development period is crucial for the fate of the embryo. The importance of understanding such mechanisms is related to the vast number of changes in gene expression

level during this period, supporting the complex mechanism of embryogenesis and implantation (Niemann *et al*. 2008).

2.3 Nuclear remodeling and the role of MPF on remodeling and oocyte activation

Nuclear remodeling after cloning, or remodeling of chromosomal architecture, is the designation for a series of molecular events that involve nucleus swelling, or the enlargement of the nucleus after transplantation (Prather *et al*. 1990), modification and expression of certain nuclear lamins (Kubiak *et al*. 1991, Prather *et al*. 1991), "blebbing" of the nuclear envelope (Szollosi & Szollosi 1988), and alterations in proteins synthesis (Fulka *et al*. 1996). Nucleolognesis (Kubiak *et al*. 1991) also plays a role in nuclear remodeling. Nucleoli from embryos after genome activation in cloned embryos usually resembles the nucleoli found in differentiated somatic cells (Fulka *et al*. 1996).

Conventionally, morphological (Kanka *et al*. 1999) changes and frequency of development have been used to monitor chromatin remodeling. Molecular and biochemical events can be used as alternative criteria to efficiently evaluate the extent of chromatin remodeling (Kim *et al*. 2002). Many proteins are specifically lost from the nucleus after cloning, while others are taken up from the egg cytoplasm (Kikyo *et al*. 2000). Whether differentiated nuclei might require specific global chromatin-remodeling activities on transplantation into eggs or during the generation of stem cell lineages is currently attracting strong interest (Wilson & Jones 1983). Successful remodeling of somatic nuclei is brought about by a process similar to that of normal fertilized embryos (Kim *et al*. 2002). The failure of activated oocytes to accomplish this is due to molecular events, like transcription silencing, TBP (TATA box binding protein) accumulation, and loosening of chromatin structure (Kim *et al* 2002).

Of prime importance for nuclear remodeling events is a cytoplasmic kinase termed MPF, or Maturation Promoting Factor (Masui and Markert 1971). This protein has been

identified as a complex of two proteins, cyclin B regulatory component and $p34^{cdc2}$ catalytic subunit, a protein which the kinase activity is regulated by changes in the phosphorylation state and by its association with other cyclins (Campbell 1996a). MPF is preferentially bound to the spindle-chromosome complex (Kubiak *et al*. 1993). The activation of $p34^{cdc2}$ kinase triggers the entry of the cell into mitosis or meiosis, resulting in nuclear envelope breakdown (NEBD), chromosome condensation, reorganization of the cytoskeleton and changes in cell morphology (Nurse 1990, Maller 1991, Masui 1992).

It has not been determined yet whether nuclear remodeling is an absolute need for clone development. Nevertheless, MPF has been shown to facilitate nuclear remodeling after cloning (Fulka *et al*. 1996). MII oocytes contain high levels of active MPF kinase, with activity level persisting until the oocyte is fertilized or activated (Fulka *et al*. 1992, 1996).

In conventional fusion/activation steps in cloning procedures, the donor chromatin, usually at the G0/G1 stage, newly introduced into the MII oocyte at the M-phase, responds by prematurely decondensing, with the assembly of the nuclear envelope occurring afterwards (Szollosi *et al*. 1988). For the chemically activated embryos, MPF activity remains high for an extended period of time (Kubiak *et al*. 1993). Consequently, cloning by HMC could be advantageous for studies on the role of temporal or spatial variations in MPF activity, depending on the cyto/karyoplast origin and type.

The effects of MPF on the transferred nucleus involve nuclear envelope breakdown and chromosome condensation. On nuclear transfer procedure, as the chromosome condensation is prematurely induced by the recipient cytoplast, that event is referred to as premature chromosome condensation (PCC). The cell cycle stage of the transferred nucleus has pronounced effects on the degree of premature chromosome condensation (Johnson & Rao 1970, Collas & Robl 1991). The degree of PCC can be variable and depends on the MPF activity, and the duration of exposure to MPF. When chromatin of S-phase nuclei are exposed

to MPF, they undergo premature chromosome condensation showing a typical pulverized appearance. When nuclei at G1 and G2 phases undergo PCC, the chromatin condenses to form elongated chromosomes with single and double stranded chromatids, respectively (Collas *et al.* 1992). This is important for cloning, as the synchronization of cell cycle stage is a very variable factor that plays important roles in the viability and ploidy of cloned embryos.

2.4 Synchronization of the cell cycle of the nucleus donor cell and recipient cytoplast

Briggs & King (1952) demonstrated that certain nuclei really could develop to a sexually mature adult. That was the rise of the concept that equivalent, totipotent nuclei from a single individual could, when transferred to an enucleated egg, give rise to genetically identical individuals (Campbell *et al*. 1996ab). Many reports on both amphibians and mammals have shown that the cell cycle stage of both the donor nucleus and the recipient cytoplasm, at the time of transfer, can have substantial effects upon the development of the reconstituted embryo.

The nuclear division cycle involves two major events. DNA replication (S-phase) and segregation of duplicated genetic material (M-phase) which is simply called mitosis (Campbell *et al*. 1996a). During a single cell cycle all the genetic material must be replicated once and only once and segregated equally to the two daughter cells. Failure to replicate or even any re-replication of a portion of the genetic material can result in aneuploidy.

The importance of cell cycle is still a matter of debate (Wells *et al*. 2003). In fact, not only the cell cycle stage, but the production system, the donor age and strain (genotype) may influence the viability of cloned embryos at distinct levels (Wakayama & Yanagimachi 1999; 2001). Thus, irrespective of other variations, the heterogeneity of cell cycle length between different cell types and even individual cells within a given cell population is mostly attributed to the variation in the early G1 period (Wells *et al*. 2003).

For successful nuclear transfer, the donor nucleus must adopt the same cell cycle

parameters as the zygote: these include nuclear envelope breakdown (NEBD), chromosome condensation and chromosome segregation so that it subsequently presents embryonic patterns of DNA replication and transcription (Wrenzycki *et al*. 2005). The recipient cytoplast is the one to direct the nuclear reprogramming. Conventionally, donor nuclei should be either in G1-phase, which is the interval between mitosis and the initiation of DNA replication, or in G0-phase (Heyman *et al*. 2002, Kato *et al*. 1998, Wilmut *et al*. 1997), in which they remain metabolically active but have exited the cell cycle (Oback & Wells 2002). If nuclei in G2 or S-phase are used, the potential reduplication of genome directed by the recipient cytoplasm can result in aberrant development. If the cloning protocol does not allow the second polar body extrusion, then the genotype usually becomes tetraploid. A second major problem is the initiation of premature nuclear breakdown and chromosome condensation (if DNA synthesis is not complete), which also leads to chromosome loss and aneuploidy (Barnes *et al*. 1993). When in S-phase, this phenomenon was described earlier as chromosome pulverization (Schwartz et al. 1971).

In unfavorable culture conditions, such as after serum starvation of highly confluent cell cultures, the levels of cyclin decline rapidly and cells do not pass the G1 checkpoint (Oback & Wells 2002). Eventually, cells exit the cell cycle and enter a quiescent state, the G0. The initial reports on cloning by SCNT claimed that the use of cells arrested in G0 stage by serum starvation was a requirement for complete reprogramming (Wilmut *et al*. 1997, Kato *et al*. 1998, Wakayama *et al*. 1998). Nevertheless, viable offspring was later on obtained with G1 arrested cells (Cibelli *et al*. 1998, Wells *et al*. 2003). Our synchronization protocol prepared cells at presumptive G0/G1, by contact inhibition upon high confluence, as described by Boquest *et al*. (1999). Both these strategies are standard in the laboratories around the world, although nuclear damage due to serum starvation is considered as one reason for the low offspring production of embryos reconstructed with cells that were

synchronized at the G0 phase (Kues *et al*. 2002, Wilmut *et al*. 1997).

Currently, no specific strategy or system provides a 100% synchronization of somatic cells in a defined cell cycle stage. It has been reported that in a batch of somatic cells growing in culture, less than half of the population is in actively dividing state and 60-80% are in G1 (Heyman *et al*. 2002). Donor cells in phases other than the G0 phase, for example in G1, can also be used to produce cloned animals (Cibelli *et al*. 1998), indicating that synchronization in the G0 phase is not a prerequisite for somatic cloning. In fact, early G1 phase cells (Kasinathan *et al*. 2001) enhanced fetal and calf survival. Furthermore, embryos reconstructed with transgenic fibroblast cells in G1 were more likely to develop to calves at term and had higher post-natal survival to weaning than with the G0 phase (Wells *et al*. 2003). Nevertheless, the production efficiency of cloned offspring is still low using G1 phase cells, and a simple method for recovering a synchronized G1 population has not been reported. The cell cycle has checkpoints at which DNA and/or abnormalities in the mitotic apparatus are checked (Hartwell & Kastan 1994).

Several protocols have been proposed over the years for the cell cycle synchronization of donor cells at the G0/G1 phase. Some protocols require the manipulation of the culture conditions, such as serum starvation (Wilmut *et al*. 1997) or cell contact inhibition by high culture confluence (Boquest *et al*. 1999). However, wide variations in time for proper cell cycle synchronization or culture confluence, with more unpredictable results, make such procedures less practical for scheduled SCNT routines, requiring certain level of expertise and knowledge of the cell lineage or type in use (Choresca *et al*. 2009). Alternatively, certain protocols make use of drugs that directly or indirectly halts the cell cycle, such as treatments with roscovitine (Gibbons *et al*. 2002), dimethyl sulphoxide (Hashem *et al*. 2006) or cycloheximide (Goissis *et al.* 2007). The use of such cell cycle synchronizing agents may be effective, but the rather broad spectrum of interference with cellular processes, along with

potential toxic side effects, may induce cell death, which may be consistent with DNA damage (Koo *et al*. 2009), along with unintended consequences of concern for further development (Gibbons *et al*. 2002).

2.5 The recipient cytoplast

The classical SCNT procedure uses enucleated MII-arrested oocytes as recipient cytoplasts. The choice of such recipients has been influenced by one main argument (Willadsen 1986, Wakayama *et al*. 1998): genomic reprogramming. There are pieces of evidence that the onset of embryonic gene expression occurs early in development (Flach *et al*. 1982), leading to the assumption that a transferred nucleus requires time to adapt to a new cellular environment. Being the environment in a state of transcriptional flux, a recently inserted donor nucleus could possibly not to have time to get adapted, impairing nuclear reprogramming. Such incompatibilities do not themselves show that zygotes lack the activities required for reprogramming and it is possible that such activities persist after activation (Du *et al.* 2002). Being this the case, the referred evidence is presumably masked by distinct cytoplast activities that may be detrimental to development.

2.5.1 Embryo reconstruction using pre-activated cytoplasts

The recipient cytoplast cell cycle stage is also of major importance, as *in vitro* development is improved when MII cytoplasts were used in association with post-fusion activation (Heyman *et al*. 2002). In their experimental conditions, the use of previously activated cytoplasts provided lower developmental rates. Conversely, S-phase cytoplasts had been reported more competent for post-natal survival of sheep clones (Wilmut *et al*. 1997), despite the also reported epigenetic instability of S-phase cytoplasts (Humpherys *et al*. 2001). Although variations among experimental conditions blurry the determination of the most optimized combination of cell cycle stages, it is established that donor nucleus must adopt the

cell cycle parameters of the zygote (Kikyo & Wolffe 2000). That is the reason why cells in G1 are considered the karyoplasts of choice, as this is brought to the onset of mitosis. This is true for cells that still do not go through the G1/S-phase checkpoint (Oback & Wells 2002). If this is the case, the combination of a nucleus in G1/S-phase might result in aneuploidy, if the recipient cytoplast is on G2/M-phase (Holliday 1987).

The higher developmental potential of embryos reconstructed using oocytes at telophase stage obtained by Bordignon & Smith (1998) may be well attributed to the pre-activation process *per se*, but it can also be attributed to other factors, such as the selection of oocytes that activate and respond by extruding the second polar body, avoiding the use of DNA dyes and ultra-violet, and also to the removal of limited cytoplasm during enucleation. The inactivation of MPF in MII arrested oocytes occurs within a short period after sperm penetration of after parthenogenetic stimuli, such as chemical activation (Collas *et al.* 1993). However, when the recipient cytoplasm is pre-activated prior to nuclear transfer, the nuclear envelope breakdown and premature chromosome condensation of donor cells do not occur due to the low activity of MPF, but DNA synthesis occurs relative to the nucleus cell cycle stage at the time of nuclear transfer (Campbell *et al*. 1996a).

As the oocyte ages in the oviduct or in culture, the cytostatic factor (CSF) responsible for stabilization of MPF is degraded, leading to an increasingly propensity to activation by exposure to environmental stimuli, such as chemical or electrical activation procedures (Wakayama *et al*. 2003). However, although easier to activate, aged oocytes have significantly lower potential to support development after fertilization (Chian *et al*. 1992). In fact, it has been demonstrated that the activity of histone H1 kinase in aged oocytes is lower (Wakayama *et al*. 2003).

2.5.2 The influence of cell cycle stage on nuclear reprogramming

Since the landmark study by Wilmut *et al*. (1997), it has been speculated that some cell cycle stages may be more effective than others for nuclear cloning. Consequently, considerable effort and debate has focused on the relative importance of the donor cell cycle phase on cloning efficiency. Although progress has been achieved to alter the cell cycle stage of blastomeres used as nuclear donors, reports have shown limited ability to identify completely effective non-toxic protocols for bovine blastomere cell cycle synchronization (Samaké & Smith 1997).

For any given cell cycle stage, the differences between cell types were also reported to be significant (Wells *et al*. 2003). Campbell *et al*. (1993b) has shown that bovine embryos reconstructed by nuclear transfer using MII cytoplast, all nuclei that undergo nuclear envelope breakdown also undergo DNA synthesis after reformation of nuclear envelope (nuclear remodeling), regardless of their cell cycle stage. When nuclei are transferred after the decline of MPF (i.e., after activation/fertilization), when no nuclear envelope breakdown occurs, replication will depend on the cell cycle stage of the transferred nucleus. If nuclei are in G1 or S-phases, the replication initiates or continues, respectively. Then, the MII cytoplast is one good receptor only for G1 and S-phase donors. It has been observed that when using oocytes at MII as cytoplasts, only nuclei in the G1 phase of the cell cycle should be used as nuclear donors. In contrast, when nuclei are transferred after the decline of MPF activity, chromosomal damage induced by PCC is avoided and all nuclei, regardless of their cell cycle stage undergo coordinated DNA replication. This cytoplast, which was termed “the universal recipient” may provide an increase in blastocyst yield, as long as the donor nucleus donor is totipotent. In fact, Campbell *et al*. (1994) obtained the highest blastocyst development when sheep oocytes were reconstructed with blastomeres (from 16-cell stage embryos), for which universal recipient yielded 55.4% blastocysts *versus* 21.3% for the MII cytoplasts.

Nonetheless, the optimal donor cell cycle stage for cloning appears to depend on the choice of the donor cell type (Wells *et al*. 2003), with two main reasons for the difference in cell "clonability": the inherent genetic or epigenetic errors in the donor genome, and introduced errors caused by faulty or incomplete epigenetic reprogramming. While our effort should focus on both, the understanding of the second reason may shed light to events still unknow related to genomic reprogramming, totipotency and cell dedifferentiation.

2.5.3 The enucleation process

Production of a cytoplast requires removal of the genetic material, with the effects of chromosome absence being unknown, but hypothesized to be one of the potential negative factors impacting subsequent embryo development (Wakayama & Yanagimachi 1999, Wakayama *et al*. 2003). The enucleation of oocytes may remove proteins that are essential for development (Simerly *et al*. 2003) or even reduce the levels of cytoplasmic kinases (Fulka *et al*. 1986). Such proteins, not to mention others, might play a role on reprogramming of the donor nucleus. Moreover, it is known that chromosomal removal preserves at least part of the machinery used in metaphase arrest since enucleated oocytes evidently support nuclear envelope breakdown, as well as chromosome condensation (Czolowska 1984, Collas and Robl 1991). Although the aspiration of a significant portion of oocytes still permitted acceptable development rates after IVF (Wakayama & Yanagimachi 1998) or IVF and SCNT (Westhusin *et al*. 1996), it is still hypothesized that the process of enucleation removes important factors that might be critical for the reprogramming of an incoming donor nucleus. The potential negative impact of MII oocytes spindle removal on developmental potential of SCNT embryos is double: by removal of factors critical for chromatin remodeling and reprogramming, or by disruption of subcellular supra-molecular organization in such a way that subsequent development is impaired. However, the absence of DNA has been reported as exempt (Wakayama *et al*. 2003) of failures in nuclear reprogramming after cloning by SCNT.

2.6 The role of cytoplasmic mosaicism and heteroplasmy

In assisted reproductive technologies, the cytoplasm transfer and nuclear transfer lead to a mixture of cytoplasmic components and a partial or complete modification of the mitochondrial background in an embryo. The cytoplasmic mosaicism and heteroplasmy caused by the fusion of cytoplasts from distinct structures after cloning, especially when by HMC, may affect *in vitro* embryo developmental capacity either positively or negatively depending on the biological status (e.g., activation status, cell cycle stage, aging, etc.), viability and quality of the structures.

Nuclear mitochondrial interactions appear to play a role during early development in mammals (Smith *et al*. 2005). During pre-implantation development, it appears that bovine zygotes lose some mtDNA molecules during cleavage up to the compaction, being later increased exponentially during blastocyst expansion. There is strong evidence that the ratio of mtDNA molecules is conserved among mammals, supporting previous reports that mitochondrial copy numbers may play a role in developmental competence (Smith *et al*. 2005, Barrit *et al*. 2001). Despite some mutations are accumulated with age, most often only a single sequence variant of mtDNA is transmitted through the offspring, to keep the DNA homoplasmy, and this is maintained in the female germ line due to elimination of paternal mitochondria soon after fertilization (John 2002). Heteroplasmy of mtDNA can occur in some human disorders resulting from rearrangements/deletions or single nucleotide mutations. However, the tissue distribution of these mtDNA mutations is linked directly to the disorder phenotype. Heteroplasmy does not necessarily associate with disease, as it is quite often likely to occur in nature (Meirelles & Smith 1997, 1998).

It is an ethical issue for human artificial reproductive technologies, since arguments have been published (Hawes *et al*. 2002) concerning the safety of using cloning in humans, based on a possible effect of mitochondrial heteroplasmy and the possibility of a subsequent

imprinting modification of one or both parental alleles. One of the main reasons for this debate is that the normal pattern of inheritance of mtDNA is strictly maternal (John 2002). Although it has been argued that heteroplasmy from donor ooplasm had been displaced by homologous mitochondria (Cohen *et al*. 1997) in that particular case, cytoplasm injection does allow transmission of donor cytoplasm mtDNA to the offspring (Brenner *et al.* 2000, Barrit *et al*. 2001). Moreover, SCNT is a useful tool to investigate such issues, and mainly regarding the use of fertilized eggs as cytoplasts, since there is suggestion that any foreign DNA introduced post-fertilization can be transmitted to the offspring (John 2005). Despite ethical aspects regarding the application of artificial reproductive technologies in humans, results in mice (Liu *et al*. 2003) suggest that the transfer of cytoplasts can be used to prevent transmission of mitochondria-related diseases.

To date, it is not clear if the cytoplasmic mosaicism or heteroplasmy caused by nuclear transfer in farm animals is detrimental to development. Most SCNT clones appear to contain none or few of the mitochondrial halotypes derived from the donor cell (Steinborn *et al*. 2000), whereas for embryonic donor nuclei it has been already reported that blastomeres haplotypes can take entirely the cloned offspring (Smith *et al*. 2000). In summary, mitochondria inherited at fertilization appear to undergo major changes during early embryogenesis in mammals. During their transition to the mature form (compaction to blastocyst stage) they rely on *de novo* replication (Perreault *et al*. 1992).

2.7 Future perspectives for cloning applications

The initial commercialization of nuclear cloning technology in cattle will focus on producing small numbers of high valuable animals for breeding purposes (especially cloning progeny-tested sires) and transgenic animals producing valuable biomedical products (Paterson *et al*. 2003). Until the technology is improved, however, these surviving clones come at a considerable ethical cost. From an animal welfare perspective, in addition to farmer

and consumer acceptance of the technology, the high rates of mortality throughout gestation and the post-natal period must be minimized before any large-scale cloning opportunities become practicable or tolerated. Ideally, cloning must reach pregnancy rates comparable with that for artificial insemination, which is around 55 to 60%. It is important to remember, however, that SCNT can be effective in producing what appears to be physiologically normal: fertile animals. This provides encouragement for eventually resolving technical issues with the nuclear transfer procedure and elucidating the molecular mechanism responsible for complete epigenetic reprogramming, as well as the improvement of production systems to enhance flexibility of protocols, to work, for instance, with cryopreserved cytoplasts (Forell *et al*. 2009), and for the production of transgenic animals for biomedical (Paterson *et al*. 2003) or agricultural (Faber *et al*. 2003, Lewis *et al*. 2004) purposes.

3 MATERIALS AND METHODS

All chemicals were from Sigma Chemical Co. (St. Louis, MO, USA), unless stated otherwise.

Establishment of Primary Somatic Cell Cultures

The primary somatic cell culture was established from the ear biopsy of a Nellore female bovine, according to our established procedures (Ribeiro *et al*. 2009). The biopsy was segregated in skin and cartilage explants that were cultured in cell culture medium (CCM) composed of Dulbecco´s modified Eagle Medium (D-1152) supplemented with 0.22 mM sodium pyruvate (P-4562), 26.2 mM sodium bicarbonate (S-5761), 10,000 IU/mL penicillin G, 10 mg/mL streptomycin sulfate (S-1277) and 10% fetal calf serum (Nutricell, SP, Brazil). The primary culture was performed in 35-mm cell culture dishes (Corning Incorporated, NY, EUA), under controlled atmosphere of 5% of CO_2 in air, at 38.5ºC and saturated humidity. Cells were dispersed at high confluence (>90%) upon exposure to 0.25% of trypsin (Gifco, Becton Dickinson, MD, USA) and 5 mM EDTA solution (E-6511) for 5 to 7 min. After trypsin inactivation by the addition of CCM, cells were centrifuged at 300 g for 5 min and re-suspended in CCM. Cells were cultured until they achieved an 85-95% confluence, to be either re-plated for culture (passage) or cryopreserved in 0.25 mL straws (Ohlweiler *et al*. 2007) and stored in cryogenic tanks for further utilization.

The cell cryopreservation protocol was based on the addition of 10% DMSO (D-2650) in CCM at room temperature; then, straws were loaded with approximately 150 µL of the cell suspension (1,000 cells/µL), and equilibrated for 15 min at 4^{o}C, followed by exposure to liquid nitrogen (LN_2) vapor for 10 min (>-100^{o}C) before plunging into LN_2. Approximately three days prior to utilization, cells were thawed in a water-bath at 35-37ºC, seeded in 4-well plates containing up to 400 µL cell culture medium and placed to culture.

Cell cycle was synchronized for use as nucleus donors (karyoplasts) for cloning by cell contact inhibition under high confluence (>90%), according to Boquest *et al*. (1999). Only cells up to the fourth passage were used for cloning.

Recovery and Selection of Bovine Cumulus-Oocyte Complexes (COCs)

Bovine ovaries were collected at a local slaughterhouse and transported to the laboratory at 30ºC in saline solution. Follicles between 2 and 8 mm of diameter were aspirated (19G needles) into 15 mL centrifuge tubes (BD Falcon, 352096) connected to a vacuum pump using an aspiration flow of 15 mL/min. Following a 10-min precipitation time, pellets were collected for the search of cumulus-oocyte complexes (COCs) in 100-mm Petri dishes. Upon harvesting, COCs were placed in another Petri dish containing centrifuged follicular fluid. Classification and selection of oocytes were based on morphological criteria (Gonçalves *et al*. 2008), with only COCs of grades 1 and 2 being used for the experiment.

In vitro maturation (IVM)

In vitro maturation was performed as described previously (Bertolini *et al*. 2004, Vieira *et al*. 2002). Briefly, selected COCs were *in vitro*-matured in groups of approximately 50 per well in 4-well dishes (Nunclon, Roskilde, Denmark, 176742) containing 400 μL of IVM medium, composed of manipulation medium (MM; TCM-199 with Earle´s salt (M-2520), 25 mM Hepes (H6147), supplemented with 26.2 mM sodium bicarbonate, 0.2 mM sodium pyruvate, 0.01 UI/mL FSHp (Folltropin, Bioniche, Animal Health, Canada), 0.5 μg/mL LH (Lutropin, Bioniche, Animal Health, Canada) and 10% estrus mare serum (EMS) (Figueiró *et al*. 2004). Dishes were incubated at 38.5°C in 5% CO_2 in air and high humidity. Matured COCs were used either for cloning or for *in vitro* fertilization, as below.

In vitro fertilization (IVF)

Bovine *in vitro* capacitation and *in vitro* fertilization were carried out as previously described (Bertolini *et al*. 2004; Vieira *et al*. 2002) with a few modifications. Bovine frozen semen straws were thawed in a water bath at 37°C and selected by swim up, with semen samples (40 μL) being placed in 1 mL TALP-sperm medium in tubes and incubated at 39ºC for 60 min. Then, the supernatants were pooled and centrifuged for 5 min at 500 g, when sperm concentration and motility were determined. After 17-18 h of IVM, matured COCs were co-incubated with *in vitro*-capacitated sperm cells at an adjusted concentration of 1 x 10^6 motile sperm/mL, in 4-well dishes containing 400 μL TALP-fert medium per well, at 38.5ºC, with 5% CO_2 in air and 95% of humidity. Depending on the experimental group, IVF was done for 6 h (experimental groups for embryo reconstruction with zygotes) or for 18-22 h (IVF controls).

Removal of cumulus cells and zona pellucida, oocyte and zygote manual bisection, and selection of hemi-cytoplasts and hemi-karyoplasts

<u>*Selection for maturation or fertilization and cumulus cells removal*</u>. After 17 h of IVM, or 6 h after the onset of IVF, COCs or presumptive zygotes were submitted to successive pipetting for 5-8 min in MM for cumulus cells removal. Under stereomicroscopy, matured oocytes in metaphase II (MII) were selected by the presence of the first polar body, whereas zygotes were screened for the presence of the additional second polar body. Only matured oocytes were used in the experiment. For zygotes, both polar bodies were not always visibly close to one another, making necessary a careful examination before rejection as unfertilized.

<u>*Zona pellucida removal from oocytes or zygotes*</u>. Zona pellucida (ZP) digestion was performed in MII oocytes and zygotes by a brief exposure (around 30 s) to a 0.5% protease (P-8811) solution in serum-free MM, under visual control, with the exposure time being no

longer than 50 s for each pool of oocytes. Protease was inactivated by rinsing the structures several times in MM with fetal bovine serum. However, as zygotes were more sensitive to the protease, their exposure time was shorter (around 15 s), which was also followed by a rinse in pure fetal bovine serum to inactivate the enzyme more efficiently. Structures that remained with an intact ZP, even after 10 min of the first protease exposure, were briefly re-exposed for complete ZP removal.

In every replication, samples of zona-intact and zona-free oocytes were used as zona-intact and zona-free parthenogenetically activated control oocytes (PC and ZFPC). Such oocytes were kept in the incubator during all the cloning steps, up to the chemical activation time, as described below.

Oocyte bisection. Zona-free oocytes were allowed to recover for 10 to 15 min in MM, to be then exposed to a 5 mg/mL cytochalasin-B (CCB) solution (C-6762) in MM and distributed in dishes in groups of 2-3 oocytes per 5 µL microdrop under mineral oil (M-8410) for manual enucleation. Oocytes were manually bisected using splitting blades (Ultrasharp Splitting Blade, Bioniche, Pullman, WA, USA), dry-coated in Sigmacote (SL-2), under stereomicroscopic control. Resulting hemi-oocytes were exposed to a 10 µg/mL bisbenzimide solution (Hoechst 33342, B-2261) in MM for the screening of the nuclear material under UV light in an inverted epifluorescent microscope (XDY-1, China) and segregation of hemi-oocytes in enucleated (MII Cytoplasts) and non-enucleated (MII Karyoplasts). Both types of hemi-oocytes were used for embryo reconstruction according to the experimental groups.

Zygote bisection. After *zona pellucida* removal, zygotes were allowed to recover for at least 15 min in MM containing 30% FBS. Then, zona-free zygotes were incubated in a solution of 5 mg/mL CCB + 10 mg/mL bysbenzimide and individually placed in dishes containing 5-10 µL droplets to be enucleated by manual bisection using the ultra-sharp splitting blades dri-coated in Sigmacote. However, for the enucleation of zygotes, the manual bisection was

performed under UV light in an inverted epifluorescent microscope (XDY-1, China) to assure the correct enucleation (Ohlweiler *et al*. 2009), i.e., to obtain one enucleated half (enucleated hemi-zygote, IVF Cytoplasts) and another nucleated half (hemi-zygote with both the sperm and the haploid maternal chromosomes, IVF Karyoplasts).

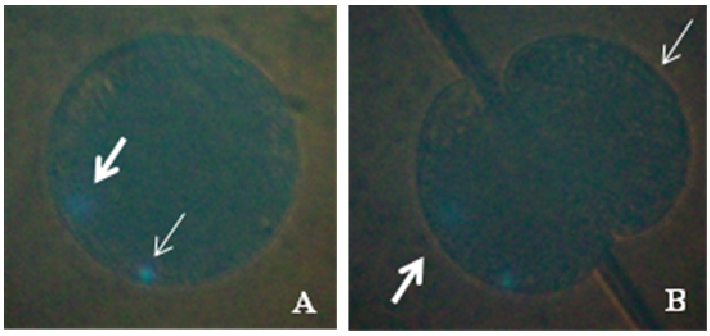

Figure 1. Zygotes after zona *pellucida* removal. A = Prior to manual bisection under UV-light monitoring, with sperm (narrow arrow) and MII-like (wide arrow) chromatin. B = Right after enucleation, with the obtained IVF-Karyoplast (wide arrow) and IVF-Cytoplast (narrow arrow).

The zygote exposure to UV light was not continuous, first being done quickly for the localization of the sperm and egg chromatin and polar bodies, if present. Then, the splitting blade was manually positioned close to the zygote (Ohlweiler *et al*. 2009), when the filter was re-opened, with the zygote maintained under UV exposure until the completion of the manual bisection (Figure 1). From the first UV light exposure to the completion of bisection, the average exposure time was no longer than 10 s.

Reconstruction of cloned embryos

Embryo reconstruction was accomplished by a quick exposure of MII or IVF hemi-cytoplasts and/or hemi-karyoplasts to a 500 µg/mL phytohaemoagglutinin (PHA, L-8754) solution in MM. To every 5 to 10 couplets that were reconstructed, a new pool of hemi-oocytes or hemi-zygotes and a new sample of somatic cells were exposed to PHA, so that the exposure was as brief as possible.

Figure 2 below illustrates the range of structures used for embryo reconstruction, according to each experimental group depicted in the design shown in Figure 3.

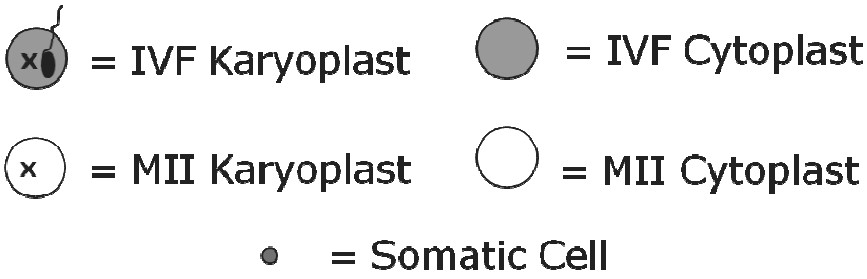

Figure 2. Cytoplasts and karyoplasts used for embryo reconstruction. IVF hemi-zygotes are represented as the dark-gray large circles, where IVF Cytoplasts (enucleated) and IVF Karyoplasts (oocyte and sperm DNA) were obtained after manual bisection performed under UV light exposure. MII-hemi-oocytes are represented as the light-colored large circles, where MII Karyoplasts (MII plate) and MII Cytoplasts (enucleated) were obtained after manual bisection and subsequent DNA screening. The small dark-gray circles are somatic cells, also used as nucleus donor (karyoplast) in some groups.

Reconstruction design performed for all fused groups using a somatic cell as a karyoplast (SCNT, G1, G4) provided better fusion rates than the reconstruction method proposed by Vajta *et al*. (2003), likely due to the linear arrangement of the structures (Figure 3) being more effective to the electric field into the fusion chamber (data not shown). Also, IVF Cytoplasts and IVF Karyoplasts were obtained from the same pool of IVF oocytes from the IVF control group, except that IVF lasted for only 6 h.

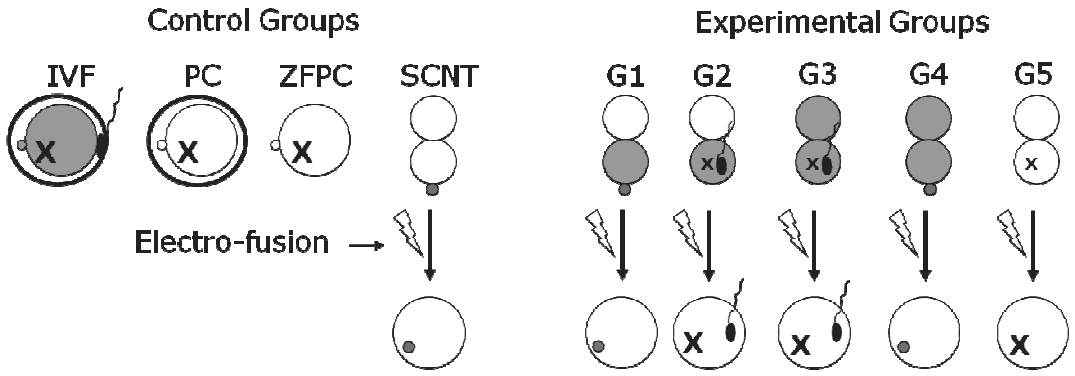

Figure 3. Experimental design for embryo reconstruction in the distinct control and experimental groups. Control Groups: The IVF Control Group was obtained after IVF for 18-22 h. The Parthenogenetic Control Groups, zona-intact (PC) oocytes, zona-free (ZFPC) oocytes, and Group G5 are control groups used for monitoring the gradual process of manipulation, culture conditions, and chemical and spontaneous activation. The SCNT Control Group is the conventional handmade cloning model, reconstructed by pairing two MII Cytoplasts and a somatic cell. Experimental Groups: Group G1 differed from SCNT control by the use of one cytoplast that had already been pre-activated by the sperm (IVF-Cytoplast) along with a MII cytoplast and a somatic cell. Both Groups G2 and G3 were reconstructed by pairing one IVF Karyoplast with either one MII Cytoplast (G2) or one IVF Cytoplast (G3). Group G4 used two IVF cytoplasts and a somatic cell for embryo reconstruction.

Electrofusion

Following reconstruction, according to the scheme in Figure 3, couplets from groups G2, G3, and G5, and triplets from groups SCNT, and G1 to G4 were kept separated by group in MM droplets until the electrical fusion. Pools of structures (up to 20 couplets/triplets) were equilibrated in electrofusion solution, containing 0.3 M mannitol (M-9647), 0.05 mM $CaCl_2$•$2H_2O$ (C-7902), 0.1 mM $MgSO_4$•$7H_2O$ (M-2643), 0.5 mM Hepes (H-7006) and 0.01% polyvinyl alcohol (P-8136). Structures were then placed in a 3.2-mm fusion chamber (BTX453) connected to an electrofusion device (BTX Electro Cell Manipulator 200, BTX Instruments Inc., San Diego, CA, USA). The alignment was performed using a pre-pulse of 15 V (AC) during 12 to 15 s, followed by a double pulse of 1.2 KV/cm (DC) for 20 μs each. Structures were rinsed in MM, placed individually in dishes containing microdrops of MM under oil, and incubated for at least 50 min until fusion assessment, with the mean interval between the onset of fusion and fusion assessment being of 2 h. Only structures with complete membrane fusion of all components were used on the following steps.

Embryo Activation Protocol

Parthenote PC and ZFPC and the SCNT control groups were always submitted to the chemical activation protocol. For parthenote group G5, approximately half of the fused structures were chemically activated (CA) and the other half was left without activation to assess spontaneous activation rates and developmental due to the manipulation procedures. Groups G1 to G4 were allocated to one of two sub-groups, being one of the subgroups subjected to chemical activation (CA), whereas the other was maintained in MM for the assessment of the effect of the sperm-mediated activation (SA), as depicted in Figure 4. The protocol used for chemical activation consisted of a 5 min exposure to 5 μM ionomycin (I-0634) in MM, followed by incubation in 2 mM 6-dimethyl aminopurine (6-DMAP D-2629) in MM for 6 h. The mean interval between fusion and activation was 2.0 ± 0.2 h.

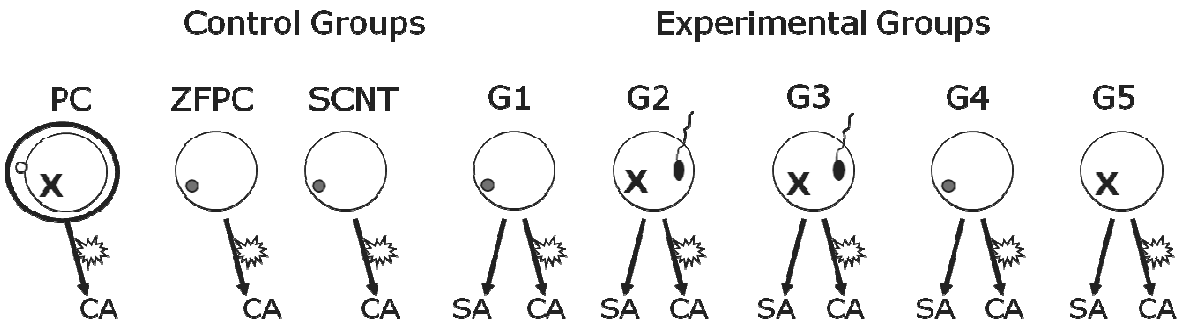

Figure 4. Embryo activation scheme for parthenote groups, (PC, ZFPC, G5) clone control (SCNT) group, and experimental groups (G1 to G4). Embryos in PC, ZFPC and SCNT groups were chemically activated (CA). Sub-groups of parthenote G5 were either chemically activated (CA) or non-activated (spontaneous activation). Experimental sub-groups from G1 to G4 were either spermatically (SA) or chemically activated (CA).

After the incubation, activated embryos were thoroughly washed in MM followed by a wash in *in vitro* culture (IVC) medium (modified SOFaaci medium, based on Vieira *et al*. (2002). Embryos that were not chemically activated were simply washed in modified SOFaaci medium. If the chemically activated embryos were placed in the culture dish before the non-chemically activated ones, the manipulation pipette was replaced to prevent a potential chemical cross contamination. The order used for placing the embryos in the culture dish alternated by replication.

The time elapsed at each significant *in vitro* manipulation step from the onset of IVM through IVC for zygotes used for cloning by HMC is shown in Table 1.

Table 1. Manipulation steps during early zygote development, from de onset of IVM to IVC, for zygotes used for cloning by HMC

***In vitro* manipulation process**	Time elapsed, h (mean ± s.d.)	
	After the onset of IVF	After the onset of IVM
IVF	0.0 ± 0.0	17.0 ± 0.1
Cumulus cells removal	6.0 ± 0.1	23.0 ± 0.1
Polar body selection	7.2 ± 0.4	24.2 ± 0.4
End of embryo splitting	9.8 ± 1.1	26.8 ± 1.1
Membrane electrofusion	11.1 ± 1.0	27.9 ± 1.2
Embryo chemical activation	13.0 ± 1.1	29.8 ± 1.1
In vitro culture	19.0 ± 0.4	35.8 ± 1.1

In vitro Culture

Embryos from all groups were cultured in the WOW system (well-of-the-well), in microwells, according to Vajta *et al*. (2000), and modified by Feltrin *et al*. (2006). Microwells were manually produced into 4-well dishes containing 400 μL SOF medium supplemented with 0.34 mM trisodium citrate (C-0909), 2.77 mM myo-inositol, 30 μL/mL essential amino acids (BME, B-6766), 10 μL/mL nonessential amino acids (MEM, M-7145), and 5% estrus mare serum (EMS), under 400 μL of mineral oil. The experimental subgroups for each group were always cultured in distinct wells to avoid cross contamination with the chemical activation reagents. The 4-well dishes were cultured at 39°C, in humidified gas mixture composed of 5% CO_2, 5% O_2 and 90% N_2, into laminated foil bags (Vajta *et al*. 2000).

As not all MII and IVF hemi-karyoplasts were used for embryo reconstruction, the remaining hemi-karyoplasts were also used as controls for development, with IVF hemi-karyoplasts being separated in two subgroups (CA or SA), as for groups G1 to G4 above. The MII hemi-karyoplasts were chemically-activated (CA). Hemi-embryos (50% of the embryo volume) from IVF hemi-karyoplasts and from MII hemi-karyoplasts were *in vitro*-cultured either individually (1 x 50%) or after aggregation of two hemi-embryos (2 x 50%) per WOW.

Assessment of in vitro viability, embryo quality and post-fertilization events

Cleavage rate was evaluated 48 h after the chemical activation, with the number of blastomeres in each microwell also recorded. Development to the blastocyst stage was evaluated on Day 7 of development, five days after cleavage assessment, with blastocysts classified according to their stage of development and morphological quality according to the IETS manual guidelines (Stringfellow & Seidel 1998). In the case of zona-free embryos, the stages of development were assessed by comparing embryo size and morphology with zona-

intact blastocysts.

The estimation of the total cell number (TCN) and the proportion of cells in the inner cell mass (ICM) and in the trophectodermal (TE) cell lineages per individual blastocyst was performed by differential staining, based on Cesari *et al*. (2006), with a few modifications. In brief, following the morphological evaluation, blastocysts from each group were incubated in a Dulbecco's phosphate-buffered saline (DPBS) solution containing 10 µg/mL propidium iodide (P-4170) and 1 mg/mL Triton-X100 (T-8532) for 40 s. Then, embryos were fixed in absolute ethanol containing 15 µg/mL bisbenzimide for additional 7 min. Fixed embryos were placed onto a slide in a 10-µL glycerol droplet and covered with a coverslip for immediate evaluation under an epifluorescent inverted microscope. Figure 5 demonstrates examples of blastocysts used for differential staining.

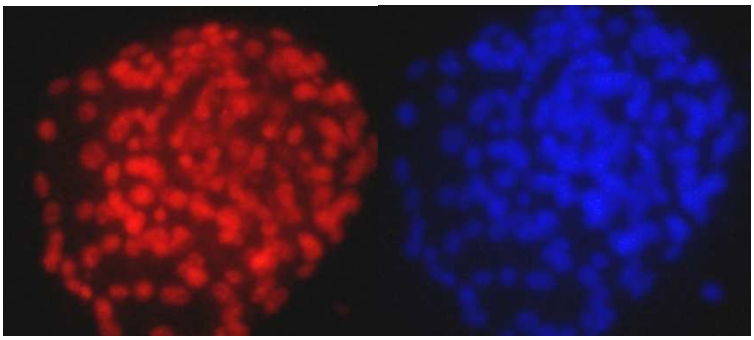

Figure 5. Day-7 bovine blastocysts stained for determination of total cell number, number of cells and proportion of cells in the embryonic lineages (trophectoderm, in red, and inner cell mass, in blue)

The proportion of the kinetics of post-fertilization and pronuclear formation events was evaluated by fluorescence microscopy after chromatin staining of groups of bovine IVF embryos with 10 µg/mL bisbenzimide from 6 to 19 h after the onset of IVF.

Statistical analysis

Data analyses were done using the Minitab software (State College, PA). Fusion, cleavage and blastocyst rates, and ICM:TCN, TE:TCN, ICN:TE ratios, were compared using the χ^2 test. Data regarding total cell number and cell number in the embryonic lineages (ICM, TE), based on morphological quality and stage of development, were analyzed by analysis of variance (ANOVA), for a level of significance of 5%, with embryo type (IVF controls, PC, ZFPC, and experimental groups G1, G2, G4 and G5), stage of development (early blastocyst, blastocyst, expanded blastocyst, or hatching/hatched blastocyst), and embryo quality (good, fair, or poor) as main effects. For zona-free blastocysts, the size of zona-intact embryos was used for comparison to assure a more accurate evaluation. However, the size of blastocysts can be highly variable, at least in our experimental conditions. For that reason, zona-free embryos potentially at stages 8 (hatching blastocyst) and 9 (hatched blastocyst) were pooled for the analysis. Pairwise comparisons between treatment groups were performed using the Tukey test. Simple Pearson's correlation and linear regression tests were used for the analyses of relationships and dependence between traits.

4 RESULTS

Oocyte and zygote manipulation, and embryo reconstruction

A total of 15,222 bovine COCs were *in vitro*-matured after 20 replications, with 4,638 COCs used for production of MII Cytoplasts and MII Karyoplasts, 9,845 for IVF Cytoplasts and IVF Karyoplasts, from which 9,280 (94.3%) were usable for the experiments, and 739 used for the IVF control group. The mean maturation rate for the pool of oocytes used for MII structures, based on polar body selection, was 63.5% (2,946/4,638), which was similar to the rate observed for the pool of oocytes 6 to 7 h after the onset of IVF (65.7%; 6,099/9,280), with 37.9% (3,731/9,280) and 25.5% (2,368/9,280) having one (MII unfertilized oocytes) and two (zygotes) polar bodies upon the screening, respectively. To note, 3.0% (71/2,368) of the zygotes were polyspermic, with the percentage of polyspermy being higher when oocyte quality was lower, based on their morphology and lower survival through the cloning steps.

The splitting of zygotes under controlled UV light exposure resulted in 79.7% (1,888/2,368) survival, whereas the screening of MII hemi-cytoplasts resulted in 54.5% MII cytoplasts (2425/4448) and 44.5% MII karyoplasts (1981/4448). A total of 1,756 IVF Cytoplasts, 787 IVF Karyoplasts, 1,748 MII Cytoplasts and 297 MII Karyoplasts were used for embryo reconstruction in the TNCS control group and in the experimental groups G1 to G5. The mean cell confluence in culture dishes used as somatic cell karyoplasts for cloning, assessed morphologically prior to cloning procedures, was 90.0 ± 6.4%.

Fusion rates for each group are shown and compared in Table 2. Fusion rates were higher in the G5 group and lower in the TNCS control group and in G3 and G4 groups ($P<0.05$).

Table 2. Fusion rates (%) between cloned control and experimental groups after embryo reconstruction

Embryo reconstruction groups	Reconstructed structures	Fusion rate	
	n	N	%
TNCS	365	274	75.1[cd]
G1	375	303	80.8[bc]
G2	346	291	84.1[b]
G3	441	332	75.3[cd]
G4	470	332	70.6[d]
G5	297	283	95.3[a]

[a-d:] Numbers in columns without common superscripts differ, P<0.05.

Cytoplast/karyoplast type, activation protocol and in vitro embryo development

Table 3 summarizes data from *in vitro* embryo development and cell density and allocation in control groups (IVF, PC, ZFPC, SCNT, G5) and experimental groups (G1 to G4, and subgroups within each group).

Control groups. Between the control groups (IVF, PC, ZFPC, SCNT) and group G5, cleavage was higher in chemically-activated structures, being lower in the IVF group, which did not undergo oocyte selection by polar body screening. Blastocyst rates also differed between control groups, being higher for PC and ZFPC parthenotes than IVF and SCNT controls; the lack of difference between both parthenote groups indicates the existence of no apparent effect of zona digestion on subsequent development. In addition, despite being lower than parthenotes, development in the IVF and SCNT groups was satisfactory and within the expected range, demonstrating a good overall developmental potential of the COCs used in the experiments. In G5, in spite of the high cleavage rate seen in the CA subgroup, development to the blastocyst stage was lower than anticipated. Also, a fair amount of structures cleaved in the non-activated G5 subgroup (by spontaneous activation), yielding no blastocyst development.

Table 3. Cleavage and blastocyst rates for embryos produced by IVF, parthenogenetic activation (PC, ZFPC, G5), and cloning (SCNT, G1 to G4) using distinct cytoplasts and karyoplasts and activation protocols

Treatment group	Activation protocol	IVC	Cleavage rate		Blastocyst rate*	
		N	n	%	n	%
IVF	SA	739	544	73.6^{d}	147	27.0^{b}
PC	CA	176	166	94.3^{ab}	74	44.6^{a}
ZFPC	CA	173	162	93.6^{ab}	78	48.1^{a}
SCNT	CA	260	239	91.9^{bc}	75	31.4^{b}
G1	SA/CA	143	139	97.2^{a}	19	13.7^{c}
	SA	143	117	81.8^{c}	33	28.2^{b}
G2	SA/CA	141	116	82.3^{cd}	2	1.7^{e}
	SA	141	102	72.3^{c}	0	0.0^{e}
G3	SA/CA	150	123	82.0^{c}	0	0.0^{e}
	SA	155	125	80.6^{c}	0	0.0^{e}
G4	SA/CA	162	140	86.4^{c}	9	6.4^{de}
	SA	163	150	92.0^{bc}	13	8.7^{cd}
G5	CA	141	133	94.3^{a}	16	12.0^{cd}
	NA	149	85	57.0^{e}	0	0.0^{e}

$^{a\text{-}e}$: Numbers in columns without common superscripts differ, $P<0.05$

*Based on cleavage

SA: sperm-mediated activation; CA: chemical activation; SA/CA: sperm-mediated followed by chemical activation; NA: non-activated (spontaneous activation)

Experimental groups. Data for experimental groups G1 to G4 in Table 3 must be interpreted according to the variables in study, taking into consideration data between treatments and controls, between experimental groups (differences in cytoplast/karyoplast composition), and between subgroups within each group (differences in activation protocols).

Cleavage rates in G1 to G4, irrespective of the activation protocol, fell well within the range observed in control groups. However, development to the blastocyst stage was significantly affected by the type of cytoplast/karyoplast and/or activation protocol. The use of IVF hemi-karyoplasts (G2 and G3) for embryo reconstruction was proven deleterious for

development to the blastocyst stage. Moreover, under most experimental conditions tested in this study, IVF hemi-cytoplasts were not as effective in supporting blastocyst development when used for embryo reconstruction with somatic cells as karyoplasts (G1 SA/CA and both G4 subgroups). Surprisingly, blastocyst rate in the G1 SA subgroup was similar to controls, being lower for G1 CA subgroup.

Results for *in vitro* development from a total of 574 and 364 IVF and MII remaining hemi-karyoplasts (structures not used for embryo reconstruction by cloning) employed as additional controls are presented in Table 4.

Table 4. Cleavage and blastocyst rates (%) for IVF and MII hemi-embryos from activated IVF or MII hemi-karyoplasts *in vitro*-cultured individually (1 x 50%) or as aggregates (2 x 50%) per microwell

Treatment group	Activation protocol	IVC scheme	IVC	Cleavage rate		Blastocyst rate*	
			N	n	%	n	%*
IVF hemi-embryos	SA/CA	1 x 50%	83	57	68.7[b]	0	0.0[a]
		2 x 50%	77	56	72.7[b]	1	1.8[a]
	SA	1 x 50%	77	41	53.2[a]	1	2.4[a]
		2 x 50%	130	102	78.5[b]	0	0.0[a]
MII hemi-embryos	CA	1 x 50%	189	169	89.4[c]	17	10.1[b]
		2 x 50%	175	165	94.3[c]	32	19.4[c]

[a-e:] Numbers in columns without common superscripts differ, $P<0.05$

*Based on Cleavage

SA: sperm-mediated activation; CA: chemical activation; SA/CA: sperm-mediated followed by chemical activation

Cleavage rates were lower in IVF hemi-embryos than for MII hemi-embryos, being even lower in sperm-activated halves (1 x 50%) than aggregates and/or chemically-activated counterparts. Nevertheless, embryo development was impaired, as seen for groups G2 and G3 in Table 3. Hemi-embryo aggregation exerted a positive effect on development for MII hemi-embryos, but blastocyst rates were lower than parthenote controls in Table 2, except for G5.

Total cell number and cell allocation in blastocysts from control and experimental groups

In general, the total cell number (TCN) in blastocysts and the cell density in the embryonic lineages (trophectoderm, ICM) varied more according to the stage of development and embryo quality than to embryo type or group. Embryos of better morphological quality and/or in more advanced stages of development contained more cells, and vice-versa. In turn, TCN correlated better with the stage of development ($r=0.696$, $P<0.0001$; regression equation $y = 25.9x - 73.9$, where y is TCN and x is stage of development) than with embryo quality ($r=0.459$, $P<0.0001$; $y = -34.5x + 171$, where y is TCN x is embryo quality).

Table 5 shows data regarding TCN in embryos from different controls and experimental groups, according to the stage of development. No differences existed between groups except for the group of zona-free parthenotes (ZFPC), which had fewer TCN than both the IVF and SCNT control groups. Also, no differences were seen between groups in terms of stage of development, with differences occurring within groups, by stage, mostly between early stages (5 and/or 6) and more advanced ones (8/9), but not in all groups. Such lack of difference was probably due to a large variation in TCN within each group. However, the combined overall TCN differed significantly between stages. Nevertheless, embryo quality ($y = -11.9x + 48.1$, where y is ICM number and x is embryo quality) and stage of development ($y = 6.60x – 20.4$, where y is ICM number and x is stage of development) were good predictors for ICM number in blastocysts.

Table 5. Total cell number (LSM ± SEM) in blastocysts produced by IVF, parthenogenetic activation (PC, ZFPC, G5), and cloning (SCNT, G1 to G4) using distinct cytoplasts and karyoplasts and activation protocols by stage of development

Treatment group	Activation protocol	Embryos n	Total cell number (TCN)	Stage of development			
				5	6	7	8/9
IVF	SA	94	112.2 ± 11.5^{a}	84.0 ± 20.6^{aA}	76.2 ± 20.6^{aA}	111.2 ± 13.3^{aA}	177.4 ± 11.9^{aB}
PC	CA	66	108.8 ± 7.4^{ab}	68.8 ± 18.8^{aA}	82.0 ± 15.4^{aA}	125.3 ± 10.1^{aAB}	158.9 ± 13.3^{aB}
ZFPC	CA	66	83.0 ± 8.4^{b}	62.5 ± 23.0^{aA}	59.4 ± 16.3^{aA}	82.4 ± 12.3^{aA}	127.8 ± 13.3^{aA}
SCNT	CA	69	112.3 ± 8.0^{a}	49.5 ± 23.0^{aA}	106.0 ± 13.3^{aAB}	130.4 ± 15.4^{aAB}	163.3 ± 9.0^{aB}
G1	SA/CA	19	102.5 ± 15.5^{ab}	47.7 ± 26.6^{aA}	87.3 ± 26.6^{aAB}	94.0 ± 46.1^{aAB}	181.0 ± 17.4^{aB}
	SA	31	106.3 ± 12.4^{ab}	33.3 ± 26.6^{aA}	73.5 ± 23.0^{aA}	146.0 ± 32.6^{aAB}	172.3 ± 12.8^{aB}
G2	SA/CA	2	173.0 ± 41.3^{ab}	-	-	83.0	263.0
	SA	-	-			-	-
G3	SA/CA	-	-	-	-	-	-
	SA	-	-	-	-	-	-
G4	SA/CA	13	153.8 ± 26.1^{ab}	74.0	-	83.0	150.3 ± 24.8
	SA	9	117.1 ± 17.3^{ab}	64.5 ± 32.6^{aA}	82.0 ± 32.6^{aA}	131.0 ± 46.1^{aA}	191.0 ± 23.0^{aA}
G5	CA	16	83.6 ± 15.0^{ab}	38.5 ± 32.6^{aA}	62.0 ± 32.6^{aA}	72.0 ± 32.6^{aA}	161.8 ± 20.6^{aA}
	NA	-	-	-	-	-	-
Overall mean		385	109.1 ± 3.0	60.2 ± 6.5^{A}	87.7 ± 6.7^{B}	112.8 ± 6.4^{C}	155.9 ± 5.5^{D}

$^{a,b:}$ Numbers in columns without common superscripts differ, P<0.05
$^{A-D:}$ Numbers in rows without common superscripts differ, P<0.05
*Based on Cleavage
SA: sperm-mediated activation; CA: chemical activation; SA/CA: sperm-mediated followed by chemical activation; NA: non-activated (spontaneous activation)

The TCN was strongly correlated with the number of cells in the trophectoderm ($r=0.945$, $P<0.0001$), with such association not being as strong when with the ICM ($r=0.757$, $P<0.0001$). In other words, TCN reflected slightly more an increase in cells in the trophectodermal lineage than in the ICM. When analyzed separately, differences seen in TCN were a perfect match to differences in the number of trophectodermal cells. Consequently, data presented in Table 5 can also be transposed to the trophectodermal cell number.

The association between both embryonic lineages also existed ($r=0.501$, $P<0.0001$). However, differences in ICM cell number increased as a function of development, with early blastocysts (stage 5, 14.1 ± 3.1) having similar number of cells in the ICM than blastocysts (stage 6, 19.6 ± 3.2) but fewer than expanded blastocysts (stage 7, 22.7 ± 3.0) or hatching/hatched blastocysts (stages 8/9, 35.7 ± 2.6), for $P<0.05$; blastocysts and expanded blastocysts had similar ICM cell number, but lower numbers than stages 8/9.

No differences in the proportion of cells within the ICM existed between any groups, regardless of the stage of development (Table 6), with embryo quality being a good predictor for the ICM proportion than any other factor ($y = -2.40x + 27.3$, where y is proportion of ICM cells and x is embryo quality), as seen in Table 7.

Due to the lack of colinearity in the data, an extensive analysis also considering embryo quality was not feasible. However, when analyzed separately, embryo quality did affect total cell number and ICM proportion, as shown in Table 7, with differences being pronounced between embryos of excellent morphological quality (Grade 1) and embryos of lower quality (Grades 2 and 3).

Table 6 Proportion of ICM (% of total cell number, TCN) in blastocysts produced by IVF, parthenogenetic activation (PC, ZFPC, G5), and cloning (SCNT, G1 to G4) using distinct cytoplasts and karyoplasts and activation protocols by stage of development

Treatment group	Activation protocol	Embryos n	ICM:TCN %	Stage of development			
				5	6	7	8/9
IVF	SA	94	23.4 ± 2.0	30.5 ± 4.8	22.6 ± 4.8	18.4 ± 3.1	22.1 ± 2.7
PC	CA	66	27.4 ± 1.7	26.0 ± 4.3	26.5 ± 3.5	23.4 ± 2.3	33.5 ± 3.1
ZFPC	CA	66	22.3 ± 1.9	22.8 ± 5.3	20.2 ± 3.8	24.1 ± 2.8	22.1 ± 3.1
SCNT	CA	69	25.5 ± 1.9	30.7 ± 5.3	23.5 ± 3.1	25.3 ± 3.5	22.3 ± 2.1
G1	SA/CA	19	22.2 ± 3.6	16.9 ± 6.1	20.8 ± 6.1	23.4 ± 10.6	27.5 ± 4.0
	SA	31	24.2 ± 2.9	21.2 ± 6.1	26.3 ± 5.3	24.7 ± 7.5	24.73 ± 2.9
G2	SA/CA	2	31.5 ± 15.8	-	-	12.0	20.2
	SA	-	-			-	-
G3	SA/CA	-	-	-	-	-	-
	SA	-	-	-	-	-	-
G4	SA/CA	13	35.2 ± 10.0	31.1	-	12.0	22.9 ± 3.2
	SA	9	22.3 ± 4.0	28.7 ± 7.5	20.1 ± 7.5	29.0 ± 10.6	23.6 ± 5.3
G5	CA	16	18.0 ± 3.5	20.8 ± 7.5	21.0 ± 7.5	14.0 ± 7.5	16.1 ± 4.8
	NA	-	-	-	-	-	-
Overall mean		385	23.0 ± 0.5	23.8 ± 1.6	22.0 ± 1.6	19.6 ± 1.5	21.2 ± 1.3

*Based on Cleavage

SA: sperm-mediated activation; CA: chemical activation; SA/CA: sperm-mediated followed by chemical activation; NA: non-activated (spontaneous activation)

Table 7 Relationship between embryo quality and total cell number (LSM ± SEM), proportion of cells in the ICM (% of total cell number, TCN) in blastocysts on Day 7 and number of blastomeres at the cleavage assessment on Day 2 of development

Embryo quality	Total cell number	ICM:TCN %	Blastomere number at cleavage
1	124.1 ± 5.5^{a}	24.9 ± 1.3^{a}	6.5 ± 0.2^{a}
2	94.4 ± 5.1^{b}	20.5 ± 1.2^{b}	5.9 ± 0.2^{b}
3	94.0 ± 6.7^{b}	19.5 ± 1.6^{b}	5.7 ± 0.3^{b}

[a,b]: Numbers in columns without common superscripts differ, $P<0.05$

The number of blastomeres per embryo, recorded in each microwell, assessed along with cleavage rate on Day 2 of development, was a good predictor for stage of development, and consequently, TCN, in blastocysts on Day 7 ($y = 6.40x + 68.6$, where *y* is TCN and *x* is number of blastomeres on Day 2). In general, embryos with more blastomeres on Day 2 reached more advanced stages of development on Day 7, whereas less developed blastocysts on Day 7 were usually originated from embryos with fewer blastomeres on Day 2. Interestingly, after pooling data by embryo type, reconstructed cloned embryos were kinetically more advanced on Day 2 than IVF and parthenote (PC, ZFPC) control groups (6.5 ± 0.5 *vs.* 5.6 ± 0.3 blastomeres; $P<0.05$). Also, embryos from the SCNT control group (7.0 ± 0.3) were more advanced on Day 2 than embryos from the IVF (5.6 ± 0.3), PC (5.4 ± 0.3) and ZFPC (5.7 ± 0.3) control counterparts. However, number of blastomeres per embryo on Day 2 did not have a significant effect on blastocyst number on Day 7 of development.

The proportion of the kinetics of post-fertilization and pronuclear formation events evaluated by fluorescence microscopy after chromatin staining of bovine IVF embryos from 6 to 19 h after the onset of IVF are illustrated in Figure 6, with corresponding comparisons with events described in the literature (Campbell *et al*. 1993a).

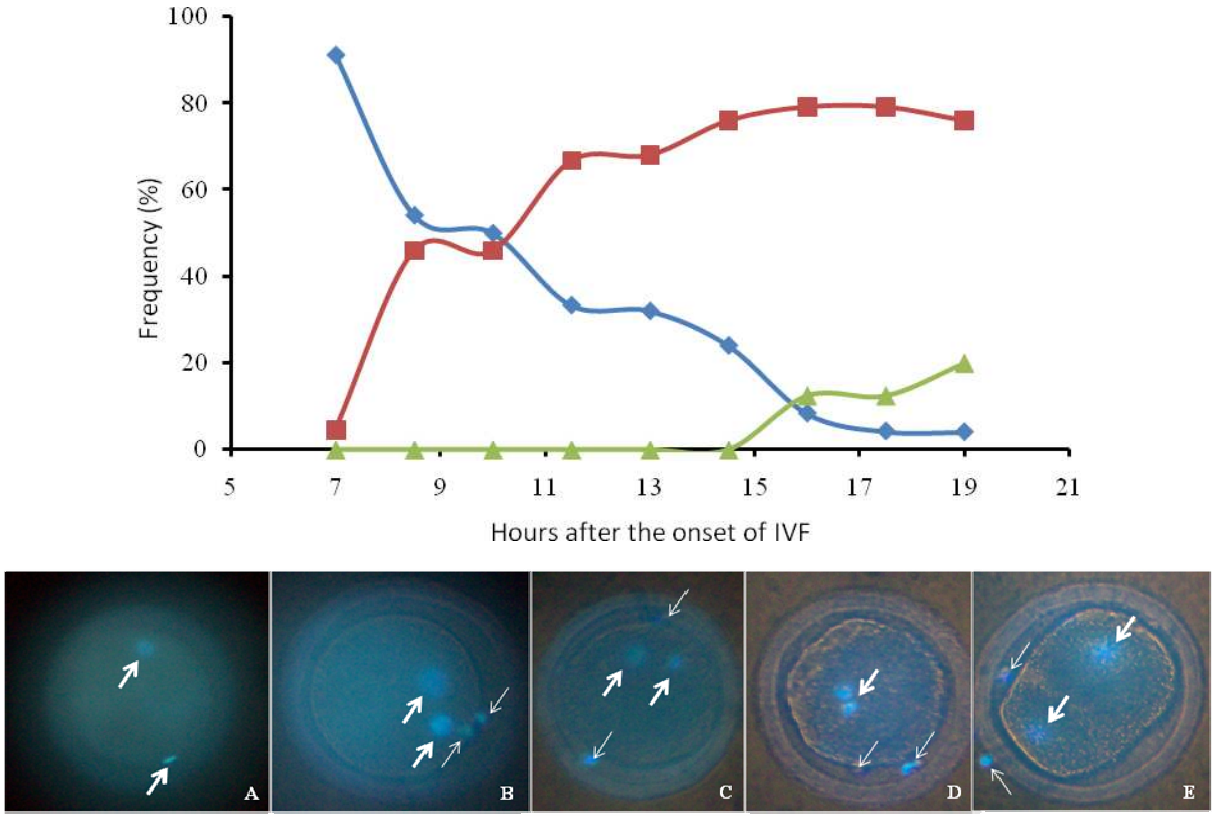

Figure 6. Proportion of main post-fertilization events after chromatin staining in *in vitro*-fertilized bovine zygotes 6 to 19 h after the onset of IVF.
Blue line (♦) in chart = zygotes with condensed sperm and MII-like oocyte chromatin (wide arrows in illustration A)
Red line (■) in chart = zygotes with uncondensed chromatin and polar bodies (wide and narrow arrows in illustration B, respectively) or showing visible pronuclei and polar bodies (wide and narrow arrows in illustration C, respectively)
Green line (▲) in chart = zygotes showing a mitotic spindle (wide and narrow arrows in illustration D, respectively) or cleaving/cleaved structures (illustration E, with wide arrows on the nuclei of blastomeres in a cleaved zygote, and narrow arrows on a polar body in the perivitelline space and on a cumulus cell attached to the *zona pellucida*)

As expected, the number of zygotes with condensed sperm and MII-like oocyte chromatin (blue, diamonds) decreased with time, reaching markedly lower levels after 10 to 12 h. Concomitantly, the proportion of zygotes with non-condensed chromatin or showing visible pronuclei (red, squares) increased significantly after 8 to 10 h, with zygotes showing a mitotic spindle or embryos that had undergone cleavage (2-cell stage embryos) starting to appear after 13 h following the onset of IVF. Most zygotes had pronuclei by 11 to 13 h after IVF, which is coincident to the mean fusion and/or activation procedures in groups G1 through G4.

5 DISCUSSION

Mammalian cloned embryos have been shown to develop faulty epigenetic configuration at a high and unpredictable level after cloning, usually associated with abnormal phenotypes in the course of development (Bourc'his *et al*. 2001, Dean *et al*. 2001, Kang *et al*. 2001, Xue *et al*. 2002, Reik *et al*. 1993, Beaujean *et al*. 2004, Bressan *et al*. 2009). This supports the hypothesis that temporal and spatial failures at gene expression level and embryonic development are caused by an inadequate reprogramming of the donor genome, during and after the SCNT procedures *per se*. It appears that the ooplasmic components responsible for genomic reprogramming that usually takes place soon after fertilization are not sufficient to modify the differentiation marks from most somatic cells (Giraldo *et al*. 2008, 2009). Many factors intrinsic to the cytoplast (e.g., activation status, stage of the cell cycle, cytoplasmic maturation, etc.) and to the karyoplast (cell type, origin and degree of differentiation, stage of the cell cycle, etc.) are known to play critical roles in the chromatin remodeling and nuclear reprogramming events after cloning. In fact, the use of MII cytoplasts are regarded as the most appropriate cytoplast type for cloning, despite the cell cycle asynchrony, as it is postulated to contain chromatin remodeling properties that are necessary for reprogramming somatic cells nuclei (Fulka *et al*. 1996), mainly when cells are arrested at G0/G1 (Bordignon & Smith 2006). The functional and molecular synchrony is yet another important factor to be considered on SCNT, since this synchrony between the donor nucleus and the ooplasmic components might lead to an inadequate or proper genomic reprogramming after cloning.

The use of pre-activated cytoplasts prior to cloning has been at least controversial, with reports demonstrating successful (Bordignon & Smith 1998) or faulty (Campbell *et al*. 1994) results after nuclear transfer, depending on the cell cycle of the donor cell (Oback & Wells 2002) and on the activation process (Yoshida & Plant 1992), among other factors. The

donor cell cycle was not determined in our study, but as our strategy to synchronize the cell cycle of cell populations in culture used the cell contact inhibition approach by high cell culture confluence (Boquest *et al*. 1999), in association with the selection of small cells for cloning (Kubota *et al*. 2000), it is likely that somatic cell karyoplasts were predominantly in G0/G1. Previous reports have demonstrated that cells in high confluence (putative G1/G0) are better reprogrammed into MII cytoplasts, whereas the use of late telophase cytoplasts does not require high cell confluence, i.e., the cytoplast is permissive to receive cells at any stage of the cell cycle (Bordignon & Smith 2006). The type of cytoplast and the activation process in our study had a significant role on the presented results. Zygotes used in this study as hemi-karyoplasts or hemi-cytoplasts were fused with the other related structures approximately 11 h after the onset of IVF. At this point, MPF activity was low or absent in the zygotes (Collas *et al*. 1993, Jones 2005). Then, we can consider that all manipulations were done when MPF was no longer active, with most cytoplasts or karyoplasts being presumably at the G1 stage of the cell cycle (pronuclear stage, Figure 6), which should be more synchronous to the donor cell, resulting in distinct results, depending on the association used for embryo reconstruction. In addition, and in general, chemical activation enhanced cleavage rates, but reduced blastocyst yield in the experimental groups. There was a biological tendency for cleavage to be higher in the chemically-activated subgroups. Nonetheless, such advantage was not reflected in further development.

The handmade cloning (HMC) procedure is an interesting tool for studies in cell and developmental biology. The cytoplasmic mosaicism and heteroplasmy caused by the fusion of cytoplasts from distinct oocytes after cloning by HMC might affect *in vitro* embryo developmental capacity either positively or negatively (Vajta *et al*. 2005), with an increase in total embryo cell number (Tecirlioglu *et al*. 2005, Giraldo *et al*. 2008) or having a healthy cytoplasm partly rescuing pronuclei from oxidative stress (Liu & Keefe 2000) when proven

to be positive, but reducing cloning efficiency when domonstrated to be negative (Vajta *et al*. 2005). Interestingly, the subgroup G1 SA in this study was the only experimental group to attain developmental rates to the blastocyst stage similar to the IVF and SCNT control groups (27.0%, 31.4% and 28.2%, respectively), with chemically-activated counterpart (G1 SA/CA) halving the blastocyst yield (13.7%). The possible biochemical and molecular pathways associated with differences in development between both G1 subgroups, and even the G4 subgroups, need some pondering. The fusion of one hemi-cytoplast with high MPF activity (non-activated) with a sperm-mediated activated hemi-cytoplast in G1 (and low MPF activity) and a somatic cell in presumptive G0/G1, determined a high rate of development. Data from the G1 SA subgroup indicated that the IVF hemi-cytoplast was not only able to entrain the non-activated MII hemi-cytoplast to its ongoing sperm-activated cytoplasm, providing sufficient support for embryo development and genome reprogramming as much as controls, but also that the additional chemical activation protocol imposed on the subgroup of reconstructed embryos (G1 SA/CA) compromised blastocyst yield. This agrees with a previous report by Campbell *et al*. (1993ab), in which it was postulated that a cytoplast with low MPF activity should maintain a proper ploidy, as a non-synchronized karyoplast could fail to develop due to re-replication. It is possible that the association of both cell cycle phases for the cytoplasts and the absence of chemical activation promoted a better synchronicity of the physiological events that were more favourable to development.

The lower blastocyst rate in that chemically-activated group might be explained by the interference with the activation processes set in motion by the sperm. Physiologically, the egg penetration by the sperm causes an increase in calcium concentrations (Cuthbertson *et al*. 1981), sustained in peaks for a while only in fertilized, but not in parthenogenetically activated eggs (Cuthbertson & Cobbold 1985, Yoshida & Plant, 1992, Crozet 1993). The same mechanism by which cortical granule migration is prevented by suppressing calcium

peaks (Kline & Kline, 1992) can be used to evidence that the absence or disruption of the events that play a role in fertilization does not prevent the SCNT embryo development, but certainly impairs the viability of animals originated from such embryos. In the G1 SA/CA subgroup, the additional chemical activation process, either by the sole peak of calcium influx induced by ionomycin (Yoshida & Plant, 1992) caused at an inappropriate time in development (approximately 13 h after IVF, for the fused sperm-mediated activated cytoplast), or by the kinase inhibition effect of the 6-DMAP incubation that followed (Susko-Parrish *et al*. 1994), or both, did not seem to affect the activation events that lead to cleavage, but did compromise further development, with non-developed embryos halted at or prior to the stage of embryo blockage (8- to 16-cell stages) (Ma *et al*. 2001, Meirelles *et al*. 2004), about the time of the embryo genome activation (EGA). The embryo block may somehow give the idea that an embryo that follows the block and death pathway stops development and dies because of lack of survival messages (Meirelles *et al*. 2004).

It is important to mention that the somatic cell in this study was always fused to the IVF hemi-cytoplast during reconstruction of the G1 group, being on the opposite side of the paired MII hemi-cytoplast. It is possible that such configuration might have promoted chromatin remodeling and genomic reprogramming more effectively in this subgroup in comparison with its chemically-activated counterpart or the G4 group. The MII cytoplast is known to cause premature chromatin condensation (PCC) and all events related to the M phase of the cell cycle to the donor nucleus (Campbell *et al*. 1993ab, Wakayama *et al*. 2000, Wakayama & Yanagimachi 2001, Heyman *et al*. 2002). Such events are believed to be important for genome reprogramming after cloning, providing the basis for the use o MII cytoplasts with delayed artificial activation (2 to 4 h) after membrane fusion (Alberio *et al*. 2001). Perhaps the introduction of the cell genome into the IVF cytoplast, presumably more synchronous to the donor cell, delayed PCC and M-phase-related events for some short

period of time, or even prevented it, before the cytoplasmic components between cytoplasts merged and homogenized at the molecular level, which may be beneficial to reprogramming. As our data shows, in such phenomenon, the presence of a non-activated MII cytoplast was key for development when compared with the use of two IVF cytoplasts (G4 group), even if the non-activated state was eventually overridden by the activated IVF cytoplast. Nevertheless, as we have not tested a configuration opposite to the G1 and G4 groups, having the somatic cell fused to the MII instead of the IVF hemi-cytoplast, and as the nuclear and activation events were not closely examined in G1 and G4 groups, such speculations require further testing.

The cytoplast association in G1 may exert a complementation effect instead of asynchrony, discrepancy or divergence effects, as initially anticipated. Interestingly, the cytoplasmic complementation by the microinjection of cytoplasmic fractions from viable oocytes into incompetent oocytes has been shown to restore subsequent embryo viability in mice and humans (Tesarik & Mendoza 1996, Barrit *et al*. 2001, Smith *et al*. 2005, Cohen *et al*. 1997). In fact, despite the possible assynchrony (Smith & Wilmut 1989) among cytoplasts and karyoplast, and considering the still present shortage in scientific knowledge regarding genomic reprogramming by cloning, it seems logical that a potential strategy to promote development in cloned embryos is to give as many options as the cell cycle and cytoplasmic components could offer, with an adjustment of physiological events still occurring by chance, but perhaps with better odds for normalcy. Intriguingly, lower development to the blastocyst stage was seen in the G4 group, with the additional chemical activation reducing development even further, as observed in the G1 SA/CA subgroup. Both the MII and IVF hemi-cytoplast components, along with the sperm-mediated activation, had indeed an important role in development, as seen in the G1 SA subgroup. When both cytoplasts used for reconstruction were IVF hemi-cytoplasts, as in G4, the lower developmental potential was

observed perhaps due to a less efficient chromatin remodeling process, i.e, as the IVF hemi-cytoplasts were at a G1-like stage of the cell cycle by the time of embryo fusion and/or activation (Table 4), the donor nucleus was not exposed to cytoplasmic remodeling factors likely due to the maintenance of the nuclear envelope upon fusion, compromising reprogramming and development, being events both dissociated from the activation pathway related to cleavage.

It was anticipated that the fusion of two IVF hemi-cytoplasts (group G4) would have better developmental potential than the the cytoplastic association in group G1, yet lower than controls, as seen by others (Shurmann *et al*. 2006). The blastocyst rate for the SCNT group was higher (28.8%) than for G4, irrespective of the additional chemical activation (G4 SA/CA, 5.6%) or not (G4 SA, 8.0%), what is in agreement with a few previous studies regarding MII cytoplasts as recipients of choice for nuclear transfer (Willadsen 1986, Robl *et al*. 1987, Tani *et al*. 2001), although the opposite was also reported by others (Campbell *et al*. 1994, Bordignon & Smith 1998; 2006, Wakayama *et al*. 2000. However, the lower rate of development may have been caused by the manipulation of the early zygotes, since such structures were more sensitive to certain procedures than MII oocytes (e.g., higher sensitivity to the protease) and were more intensively manipulated (i.e., longer exposure to UV light). In addition, timing for zygote splitting and fusion has also been seen to affect development after cloning. Schurmann *et al*. (2006) demonstrated that SCNT cloning using IVF cytoplasts attained the same developmental competence as MII oocytes when enucleation was performed 4 h after the onset of IVF, for higher pregnancy and delivery rates. A significant distinction between our study and the work by Shurmann *et al*. (2006) is that the time for sperm-mediated putative activation (time = 0), and the fusion and chemical activation interval for our IVF Cytoplasts were larger than that previous report, which was not longer than 4.5 h. When an interval more similar to ours was used (up to 7 h, being ours between 11 and 13 h),

blastocyst yield was lower than 10%. The authors argued that such positive or negative contributions were likely due to the sperm-mediated activation events, promoting a more physiological pattern of epigenetic reprogramming than conventional chemical activation. We suggest that the events, at such extended period of time after the onset of IVF, are mostly due to the indirect effect of the activation process *per se*, with less impact of spermatic factors than of zygotic events. Such proposition still needs to be further evaluated.

The experimental groups containing IVF karyoplasts (groups G2 and G3) behaved in a completely distinct pattern when compared with all the other groups, including groups using IVF cytoplasts. Despite cleavage rates being not so different than other experimental groups, embryo development beyond the 8- to 16-cell stage was impaired. It is highly possible that the intense manipulation of zygotes was very crucial for development, as as many important biological events are taking place at the time (Figure 6), with most structures undergoing chromatin decondensation or even pronuclear formation during the interval after the onset of IVF (6 to 19 h). Any detrimental effect caused by the manipulation steps might be a reason for their low development. Results of *in vitro* development for the remaining IVF and MII hemi-karyoplasts (Table 4) reinforce that concept, as cleavage rates were lower in IVF hemi-embryos, in special in sperm-activated halves (1 x 50%) than aggregates and/or chemically-activated embryos, with embryo development beyond embryo blockage being impaired, exactly as observed in groups G2 and G3 (Table 3). This fact might also be explained by the deterimental effect of the UV light at a very critical cell cycle phase of the zygote (Tsunoda *et al*. 1988, Smith 1993), being this effect avoided by McGrath & Solter (1983) and Smith & Wilmut (1989) with mouse zygotes without UV light exposure.

In an attempt to elucidate the causes of such poor embryo development in groups G2 and G3, a pilote study was carried out to determine the effects of the manipulations procedures used for zygote splitting on subsequent development, including zona removal by

protease, CCBH and/or Hoechst 33342 stain incubation, and/or UV-light exposure for distinct periods of time. Exposure to those chemicals did not affect cleavage and blastocyst rates, despite a previous report demonstrating the detrimental effect of Hoechst 33342 stain on embryo development (Smith 1993). Nevertheless, the UV-light exposure for only 10 s was detrimental for development up to the blastocyst stage, in spite of the lack of negative effect on cleavage, in a similar pattern as observed in the experimental groups G2 and G3. As the experimental conditions were the same, developmental failure for embryos reconstructed with IVF Karyoplasts was likely due to UV-induced DNA breaks and fragmentation during zygote splitting. The detrimental effects on IVF Cytoplasts cannot be minimized, as it may have also played a part on the lower blastocysts rates in some related experimental groups in this study.

Studies with mouse and human embryos have previously shown that embryos may show blastomere and nuclei fragmentation following cell cycle arrest (Jurisicova *et al*. 1996, Warner *et al*. 1998). Reports of bovine embryos describe the same characteristics (Yang & Rajamahendran 2002, Paula-Lopes *et al*. 2003). Although well described, nuclear fragmentation does not arise before a certain number of hours in culture. For instance, 70 h post-insemination in cattle is usually the time when nuclear fragmentation becomes evident (Yang & Rajamahedran 2002, Paula-Lopes *et al*. 2003, Meirelles *et al*. 2004). This is one possible explanation for the cleavage rates observed on groups G2 and G3, followed by no further development beyond the embryo blockage stages. At our fusion time-point, approximately 11 after the IVF, a higer percentage of the zygotes was at the pronucleus stage, with some already migrating towards one another. In such conditions, some zygotes may be even further into the S-phase. Then, in our experimental group G2, which combined an IVF hemi-karyoplast and an MII cytoplast, the correct ploidy could have been impaired, or the

DNA may have undergone a pulverization if in S phase, as suggested by Campbell *et al*. (1996a).

Blastocyst rate is the golden standard criterion used to evaluate *in vitro* developmental capacity. However, similar blastocyst rates *in vitro* do not necessarily faithfully translate the effect of an improved production system. Due to distinct activation processes, the variable degree of manipulation imposed to the cytoplasts, and the variation in cleavage rates, a more reliable strategy to evaluate embryo development was also to base the blastocyst rate on cleavage. The IVF control group had lower cleavage and blastocyst rates likely due to the absence of any oocyte selection criteria prior to IVF (i.e., maturation, fertilization rates), as also seen in our previous study (Ribeiro *et al*. 2009). However, if the mean *in vitro* maturation rate observed in oocytes selected in the other groups is taken into account, especially for those used for the production of IVF Cytoplasts and IVF Karyoplasts (65.7% maturation rate 6-7 h after IVF), it is possible to infer that approximately a third of the *in vitro*-fertilized oocytes used in the IVF control group were not viable. Consequently, cleavage and blastocyst rates, after correcting or adjusting to the selection factor, were similar to parthenote controls and higher than the TNCS group, demonstrating the competence of oocytes used in this study. Along with the other groups of parthenote controls (PC and ZFPC), not submitted to zona removal (group PC only), manual bisection, UV-light exposure and electrofusion, the experimental group G5 had also an important scientific value as a control for the manipulation and activation process and culture conditions. Therefore, the parthenote controls were more reliable as controls for oocyte quality, MIV and the early manipulation processes, whereas the TNCS control group and subgroups G5 were additional controls for any possible experimental error and biases during the whole cloning procedure. In effect, in our production system, for logistical reasons, and due to the large number of oocytes to be processed in each replication, the cytoplasts derived from MII-oocytes were

maintained in the incubator for up to 2 h after enucleation prior to embryo reconstruction by nuclear transfer. The time in which MII-cytoplasts remained enucleated in our study may have affected viability in the TNCS and G5 groups, as previously suggested occurring (Ledda *et al*. 2001, Simerly *et al*. 2003, Lee & Campbell *et al*. 2006).

Results in the chemically-activated G5 subgroup were likely due to the negative effect imposed by the manipulation process *per se*, or even due to the level of cytoplasmic mosaicism, for instance, impairing development, when this group was compared with the parthenote controls. Also, the relative manipulation intensity and the exposure time to lower temperatures during handling may be possible explanations for a lower developmental rate in parthenote control groups.

The lack of blastocyst development in the non-activated G5 subgroup was expected, since the absence of chemical activation stimulus would not provide a considerable rate of spontaneous activation due to the manipulation conditions (Kono *et al*. 1989, Procházka *et al*. 1993). Moreover, cleavage in the non-activated G5 subgroup may be a reflection of either the fragmentation rate or the manipulation conditions, through the induction of weak activation stimulus, yet insufficient to support further embryo development. It is possible that such effects, in addition to other factors (e.g., nuclear reprogramming), may have caused a lower blastocyst rate in the SCNT control in comparison with PC and ZFPC parthenote controls, standards for oocyte competence.

Fusion rates were different among groups of reconstructed embryos likely due to the biological nature of the structures being fused. Data presented in Table 2 can be combined in two distinct blocks of fused structures: (a) structures composed of two hemi-cytoplasts and a somatic cell (TNCS, G1, G4); and (b) structures composed of a hemi-cytoplast and a hemi-karyoplast (G2, G3, G5). Pooled fusion rate for structures in block ‘b’ was higher than for block ‘a’ (906/1,084, 83.6%, *vs.* 909/1,210, 75.1%; $P<0.05$). This results is not surprising,

since probability for failures in membrane fusion in structures in block ‘a’ is higher, as failures may occur between both hemi-structures or between the somatic cell and the hemi-cytoplast (majority of cases, data not shown), whereas in structures in block ‘b’, failures in membrane fusion occur solely between hemi-structures. In general, fusion failures between hemi-structures were associated with reduced viability or degeneration of one or both structures, as assessed by morphological evaluation following fusion, for which IVF hemi-structures appeared to be more sensitive to the whole manipulation procedure *per se* than MII hemi-structures, as mentioned above, justifying the lower fusion rates observed in groups G2 and G3 compared with G5. Also, fusion was even lower in G3 in comparison with G2, as group G3 combined two hemi-zygotes, whereas structures in group G3 were reconstructed with a hemi-zygote and a hemi-oocyte in MII.

The WOW system was necessary in this study since zona-free embryos were used for *in vitro* culture. When in culture, paracrine factors may be released by fitter embryos or blastomeres, supposedly assisting less competent embryos or blastomeres, boosting development (Boiani *et al*. 2003); such interactions may present in the WOW system. Moreover, the WOW system prevents zona-free embryo aggregation or disaggregation, also allowing the collection of detailed data on individual embryos during development, such as during assessment of cleavage rate. As expected, Day-7 blastocysts of lower morphological grade or in earlier stages of development had lower cell density as a whole and in the cell lineages, as also previously described in our previous study (Ribeiro *et al*. 2009). The assessment of embryo quality is a good predictor of post-implantation development (Misica-Turner *et al*. 2007) and for the correct pattern of expression for some genes (Kurosaka *et al*. 2002). However, the *in vivo* developmental potential of cloned embryos from the experimental groups in this study still needs to be verified.

The kinetics of early cleavage stage embryos is crucial to the success of embryo development and has also been studied previously (Langendonckt *et al*. 1997, Lonergan *et al*. 1999; 2000, Gutiérrez-Adan *et al*. 2001; 2004). It has been well established that embryos with more rapid rates of development have greater developmental potential when compared with the co-cultured embryos with slower developmental rates (Meirelles *et al*. 2004). This characteristic is probably due to the quality of the inherited cytoplasm and may correlate to a more functional maternal-zygotic transition. A close positive relationship exists between the onset of the first cleavage and the embryo developmental competence (Langendonckt *et al*. 1997, Lonergan *et al*. 1999; 2000, Gutierrez-Adán *et al*. 2001; 2004). Zygotes that cleaved the earliest after IVF were more likely to reach the blastocyst stage than late-cleaving embryos (Lonergan *et al*. 1999). In our study, the evaluation of the specific stage of development at cleavage (Day 2) allowed us to perform an analysis regarding individual embryo developmental potential per group. Interestingly, the number of blastomeres per embryo assessed on Day 2 of development was a good predictor for stage of development on Day 7, since the embryos with more blastomeres on Day 2 reached more advanced stages of development, with the opposite also being valid. However, the number of blastomeres at cleavage was not a good predictor of the blastocyst rate, because the overall mean blastocyst rate was similar among the stages of development. Cloned embryos tended to express such behavior in a more obvious pattern, being usually more developed on Day 2 of development than IVF and parthenote control embryos. In fact, a faster kinetics of development had already been reported for SCNT embryos (Bhak *et al*. 2006). Nonetheless, despite the role that embryo density plays in successful development, the proportion of embryos that reach the blastocyst stage seems to be determined by a biological clock mechanism (Evsikov *et al*. 1990), not by the number of cell divisions. The significance of such findings, and the association with the level of genomic reprogramming after cloning, still need to be verified.

In summary, the use of IVF cytoplasts for the reconstruction of cloned embryos was as effective as controls only when associated with an MII cytoplast, without further chemical activation. In fact, the additional chemical activation in sperm-mediated activated structures compromised development to the blastocyst stage. Also, IVF cytoplasts, when combined for cloning, did not provide any improvement in development, with the use of IVF karyoplasts, under our experimental conditions, haltering development, but not cleavage, after embryo reconstruction. Certainly, intrinsic (biological causes) and extrinsic (manipulation procedures) factors played significant roles in the rates of embryo development observed in each group in this study, with the separation of the contributing effects of each factor within each group or sub-group being very difficult to attain. However, it is important to keep in mind that the way to enhance embryo viability after *in vitro* embryo manipulations, such as cloning by SCNT, is trying to mimic physiological events or to minimize sub-optimal conditions. Further studies are still need to better understand the remaining open questions, also evaluating the *in vivo* developmental potential of cloned embryos reconstructed using distinct cytoplast types and activation protocols.

6 CONCLUSIONS

Based on the results observed in this study, we concluded that:

a) The use of an IVF cytoplast (pre-activated by the sperm) provided *in vitro* viability similar to SCNT controls only with the association of one IVF and one MII cytoplast, in the absence of additional chemical activation, partially proving our first hypothesis.
b) The association of two pre-activated cytoplasts did not provide blastocyst rates higher than, nor similar to controls after SCNT, denying our second hypothesis.
c) The additional chemical activation did not enchance blastocyst rates for embryos reconstructed using IVF cytoplasts, being this detrimental to *in vitro* development, denying our third hypothesis.

In addition to the main conclusions above, we further concluded that:

d) The use of IVF cytoplasts for the reconstruction of cloned embryos supported development to the blastocyst stage, depending on the embryo reconstruction scheme (cytoplast/karyoplast type) and on the activation protocol.
e) IVF cytoplasts, when combined for cloning using a somatic cell as karyoplast, did not provide any improvement in embryo development, regardless of the activation protocol (group G4).
f) The use of IVF karyoplasts did not support embryo development to the blastocyst stage, after embryo reconstruction, despite normal cleavage rates, and irrespective of the reconstruction scheme or activation protocol (groups G2 and G3).
g) The additional chemical activation imposed to structures reconstructed with sperm-mediated activated structures generally reduced development to the blastocyst stage.
h) The manipulation procedures for cloned embryo production significantly affected embryo development, as seen when MII cytoplasts were fused to MII karyoplasts, by the reconstruction of a parthenote cloned embryo that was exposed to most manipulation steps during HMC and to the chemical activation protocol (group G5 CA).
i) The activation process must be as close to the physiologic as possible, both spatially and temporally, being apparently as significant to embryo development as the level of cytoplasmic mosaicism and types of cytoplast used for embryo reconstruction.

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