Anim. Reprod., v.5, n.3/4, p.67-82, Jul./Dec. 2008

New assisted reproductive technologies applied to the horse industry: successes and limitations

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Abstract

The development of assisted reproduction technologies (ART) in the horse has been slow compared with that in other large domestic animals. Besides artificial insemination and embryo transfer, other technologies based on *in vivo* and *in vitro* procedures of embryo production (IVP) have appeared, but the success rates of equine IVP are still far from allowing their use in routine protocols. Intracytoplasmic sperm injection (ICSI) is one of the most promising techniques applicable to the horse industry. With ICSI just one spermatozoon is injected into a mature oocyte, allowing the use of poor quality semen that could not otherwise be used for artificial insemination. Moreover, ICSI, followed by *in vitro* culture to the blastocyst stage, may be used in cases where multiple oocytes are available (e.g. when oocytes are obtained post-mortem). Those are just some examples to highlight the importance of ICSI in preserving genetic material. Cloning by Nuclear transfer (NT) can also be used for salvaging valuable equine genetics. The cloning process utilizing somatic cells is a powerful instrument for the preservation of animals with a unique genotype. Although recent reports on horse cloning show that it can be performed relatively efficiently, compared with other species, blastocyst production and thus live foal production is still low with this technique.

Keywords: biotechnology, cloning, equine, ICSI, oocyte transfer.

Introduction

The fertilization process as it occurs in the oviduct is very complex, and involves the interaction between a female and male gamete to form a diploid zygote, which in time can develop into a new individual. The term "*in vitro* fertilization" (IVF) refers to fertilization of the oocyte outside of the body; IVF typically is used to refer to standard methods (incubation of sperm and egg together in media) as opposed to ICSI. The term *in vitro* production (IVP) is used to define methods to produce embryos exclusively *in vitro*, that is, by *in vitro* maturation (IVM) of oocytes, IVF or ICSI, and *in vitro* culture (IVC) of embryos to a transferable stage. *In vitro* production of embryos has been conducted in humans and cattle worldwide to circumvent both male and female infertility, to produce embryos for research, and as an alternative to recovering *in vivo* fertilized embryos.

In horses, the progress of the study of the early events of fertilization has been slow compared with that in other large domestic animals. The development of assisted reproduction technologies (ART) in the horse dates back to the late nineteenth century with the establishment of the first equine pregnancies obtained by artificial insemination (Heape, 1898). However, progress in assisted reproduction in the horse has been rapid in the last decade. ART covers a range of procedures (IVP, oocyte pick-up (OPU), oocyte transfer (OT), ICSI and cloning), all of which have the ultimate aim of assisting the 'infertile' mare to become pregnant and deliver a live offspring (Galli *et al*., 2007).

The development of techniques such as ultrasound-guided aspiration of follicles (Brück *et al*., 1992; Bracher *et al*., 1993; Cook *et al*., 1993; Dippert *et al*., 1994), which allowed the utilization of live mares as donors of *in vivo* matured oocytes, and immature oocytes destined to *in vitro* maturation, has lead to some clinical interest. However, standard IVF, is not efficient in the horse. Although OT can be used as an efficient method for production of foals from isolated oocytes, attempts to fertilize equine oocytes *in vitro* have resulted in only limited progress. Successful IVP depends on a number of factors: availability of healthy immature oocytes, efficient and repeatable methods for IVM, physiological sperm capacitation, and subsequent optimum embryo *in vitro* culture systems. The success rates of some individual steps involved in equine IVP are still far from allowing their use in routine protocols as in cattle.

Recent progress and difficulties in assisted reproduction in the horse is the focus of this review, with the emphasis on the procedures of OPU, OT, ICSI and cloning.

Oocyte collection

The good results obtained from *in vitro* production of bovine embryos is, in great part, related to the unlimited availability of abattoir-collected bovine

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Received: January 9, 2008
Accepted: May 13, 2008

oocytes and the total freedom to conduct research with such material. By contrast, in the case of the horse there was a scarce availability of abattoir ovaries which limited the rate of the advances in research that depends on oocyte utilization. Moreover, the anatomy of oocyte attachment to the follicle wall in the horse interferes with the efficiency of recovery methods both *in vivo* and *in vitro*. In horses, the follicle presents a thecal pad beneath the cumulus cells attachment. One notable anatomic feature of this pad is the extension of cumulus granulosa cells processes into the thecal pad. The position of the pad, the granulosa cells processes that extend into it and the acid polysaccharide component of the pad seem to act as an anchor for the cumulus attachment (Hawley *et al*., 1995). Another feature of the equine ovary is the lower number of visible follicles compared to other species. The average number of visible follicles on horse's ovaries is 6 per ovary (Hinrichs and DiGiorgio, 1991; Hinrichs *et al*., 1993, Galli *et al*., 2007), compared to 12, 4 in cows (Seneda, 2001).

Use of abattoir ovaries

Although, all around the word just a small number of equine abattoirs exist, the use of oocytes obtained from slaughterhouse ovaries is a good alternative for use in research and represent an important tool in the development of several technologies applied to the equine industry. Oocytes from slaughterhouse ovaries can be collected by follicle aspiration with or without flushing the follicle, dissection and rupture of the follicle with scraping to recover the mural granulosa and slicing the ovaries.

Recovery of oocytes by aspirating the follicle with a needle and syringe, as performed in cattle, results in a low recovery rate in the horse, with a large proportion of the oocytes being stripped of the majority of their cumulus cells. The recovery rate obtained with the aspiration of follicles without flushing is 1.5 (Okolski *et al*., 1987) to 2.7 oocytes/ovary (25 – 30%) in horses compared to 10 oocytes/ovary in the cow (Hinrichs, 1991). Alm and Torner (1994) improved this method by isolating the follicle before aspiration, obtaining 50% of recovery rate.

Better recovery rates (50 – 80%) of cumulus intact oocytes are obtained when follicles are opened with a scalpel and the granulosa layer scraped from the follicle using a curette (Hinrichs *et al*., 1993; Del Campo *et al*., 1995). Unfortunately, this increases exponentially the time necessary to collect oocytes from a given number of ovaries. As mentioned previously, with the aspiration technique 1.5 (Okolski *et al*., 1987) to 2.7 (Hinrichs, 1991) oocytes per ovary can be collected. This number increases to 3.1 (Zhang *et al*., 1990) when the follicular wall is scraped. Because of the low number of oocytes obtained, Choi *et al*. (1993) sliced and washed the ovaries obtaining 4.14 oocytes per ovary.

In vivo collection of oocytes

Vogelsang *et al*. (1983) were the first to describe a standing aspiration technique. Later Palmer *et al*. (1987) obtained 63% oocyte recovery washing the pre-ovulatory follicle with 20 ml of Dulbecco's PBS + heparin. McKinnon *et al*. (1988) reported a recovery rate of 71.4% when using a trochar cannula and a larger needle (9.8 mm) to aspirate pre-ovulatory follicles via the flank. A similar procedure was described by Hinrichs (1991) with a recovery rate of 73%. In addition to the flank technique, an incision was made in the cranial vagina enabling the operator to introduce his hand in the peritoneal cavity and to hold the ovary directly against the trochar cannula.

Vogelsang *et al.* (1988) collected equine oocytes transcutaneously through the flank and compared with the use of laparotomy and total exposition of the ovary. The recovery rate was 10 to 38% via flank and 14 to 60% via laparotomy. Although an increase in the recovery rate was achieved, the use of a surgical intervention is not desired, since it leads to a limitation in the number of times the procedure could be repeated.

The first group of researchers to describe the transvaginal ultrasound-guided technique (TVA) in standing mares to harvest oocytes was Brück *et al*. (1992). Based on the same idea used in human and cattle IVF programs, they used a finger shaped transducer connected to an ultrasound console that showed a puncture line on the screen, to aspirate pre-ovulatory follicles. A single lumen needle attached to a 50 ml syringe was used to flush the follicular cavity up to 3 times with Dulbecco's PBS. One oocyte was recovered out of four follicles.

It is impossible to compare recovery rates between different groups of workers (14 to 79%) because the technology used is different and many factors influence these results. One of these factors is the puncture system including double or single lumen needle, continuous flushing or not, scraping or not of the follicular wall with the needle and the size of the follicle. In addition, another important factor is the technician's experience.

The use of a vacuum pump significantly increased the recovery rate after follicle aspiration in the mare (Vogelsang *et al*., 1988; Kanitz and Berger, 1995). The vacuum pressure utilized ranged from 150 mmHg (Carnevale *et al.,* 2005; Purcell *et al*., 2007) to 300 mmHg (Duchamp *et al*., 1995, Squires and Cook, 1996; Bogh *et al*., 2002).

Cook *et al*. (1993) performed a study comparing single versus double lumen needles. The 12 g double lumen resulted in the highest recovery rate (84%) of pre-ovulatory follicles compared to the single lumen needle (52%). The double lumen needle allowed the fluid to drip continuously into the follicle while suction was being applied. The turbulence created by

the continuous flux into the follicle helps with the detachment of the oocyte from the follicular wall, after the gonadotrophical stimulation, increasing the recovery rate (Cook *et al*., 1992).

The aspiration of immature follicles allows for an increase in the number of follicles punctured, however the recovery rate is much lower than the one obtained in the pre-ovulatory follicle. Cook *et al*. (1992) aspirated oocytes on days 7 to 9 of diestrus when four follicles measuring 10 to 25 mm were detected. A total of 135 diestrus follicles were aspirated and 25 oocytes (18.5%) were recovered. While in the pre-ovulatory follicle aspiration alone is enough for the detachment of the oocyte, in small follicles it is also necessary to flush the follicle and to scrape the follicular wall to remove the oocyte. This procedure is particularly difficult in follicles between 20 and 35 mm, due to the large diameter. More oocytes are collected from small follicles 5 to 15 mm (52%) than from follicles 20 to 27 mm (22%; Bezard *et al*., 1997). In a follicle smaller than 20 mm, the scraping is more easily performed. However, the oocytes obtained from follicles smaller than 10 mm have a lower ability to mature *in vitro*.

The use of superovulatory treatments did not increase the recovery rate of oocytes obtained from pre-ovulatory and/or diestrous follicles. Brück *et al.* (2000) observed a similar recovery rate after aspiration of follicles ≥4 mm from non-treated mares (48%) and from mares stimulated to superovulate with 25 mg of equine pituitary extract (EPE) once a day (40%). When pre-ovulatory follicles were aspirated, MacLellan *et al.* (2002) obtained higher recovery rates from non superovulated mares (69%) compared with mares superovulated with EPE twice a day (20%). Also, the use of equine FSH did not increase the recovery rate of oocytes obtained from superovulated mares (21% and 23% for superovulated and control mares, respectively; Purcell *et al*., 2007)

Pregnant mares provide an additional potential source of oocytes (Meintjes *et al*., 1995). Ginther (1992) and Ginther and Bergfelt (1992) have described the occurrence of follicular waves during early pregnancy in mares. Follicular activity is variable among early pregnant mares, ranging from periodically to sporadically occurring major waves (largest follicle ≥ 30 mm) in some mares, to only minor waves (largest follicle ≤ 30 mm) in others.

Oocyte recovery rates from pregnant mares seem to be higher compared to cycling mares. Goudet *et al*. (1998c) reported 54% oocyte recovery rates in pregnant mares versus 47% in cyclic mares. Meintjes *et al*. (1995) reported 75.8% oocyte recovery for pregnant mares versus 42.9% for the control group (pre-ovulatory follicles stimulated with hCG). Eleven aspirations were performed per mare on a 44-day period (from day 22 to day 66 of gestation) with a maximum of 7 oocytes recovered per aspiration section. In a second study (Meintjes *et al*., 1997), follicular aspirations were extended until day 150 of gestation. The mean number of aspirations per mare was around 7.6 and the average number of oocytes obtained was 18.9 per mare. They concluded that an average of 2.5 oocytes could be collected every 7 to 10 days from pregnant mares. It was estimated that 19 oocytes could be retrieved from a mare between days 21 to 150 of gestation compared to 12 collected during 130 days in cycling mares without any hormonal treatment. Cochran *et al*. (1998) reported an average of 13 follicles per procedure, and an oocyte recovery rate of 66% for 20 aspirations performed during days 14 to 70 of pregnancy.

Oocyte maturation

After the oocytes are collected and selected on the basis of cumulus morphology, they are transferred to maturation medium and allowed to mature for 24 to 36 hours *in vitro*. Oocyte maturation consists of nuclear and cytoplasmic modifications that prepare the oocyte for fertilization. *In vivo*, maturation is a process coinciding with follicular development, changing hormone levels, and meiotic progression. Previous studies have shown that the concentration of estradiol-17β (E2) in follicular fluid remains high in equine preovulatory follicles (Kenney *et al*., 1979; Watson and Hinrichs, 1988). The ovulatory LH surge occurs as a progressive rise that takes several days, with a maximum concentration occurring one day after ovulation (Whitmore *et al*., 1973; Irvine *et al*., 1994). Regarding follicular fluid concentrations of progesterone (P4) in equine pre-ovulatory follicles, Fay and Douglas (1987) observed slightly higher levels in follicles 25 mm in diameter as compared to smaller ones. Watson and Hinrichs (1988) reported a 20-fold rise in P4 in follicles approaching ovulation after hCG treatment. All those changes may be important for the complete maturation of the oocyte.

Nuclear maturation is referred to as the resumption of the meiosis process through to MII, with the aim of reduction of the DNA content in the oocyte. Oocyte nuclear maturation is marked by extrusion of the 1st polar body and formation of a Metaphase II plate. In most species, the resumption of meiosis is triggered by LH, and ultimately results in activation of the cyclin B/cdc2 complex (Maturation Promoting Factor - MPF) in the ooplasm (Lee and Nurse, 1988; Cran and Moor, 1990).

A detailed description of meiotic changes in horse oocytes is presented by Grondahl *et al*. (1995) who used transmission electron microscopy to visualize the process. They observed a flattening of the spherical equine oocyte nucleus, followed by an increased undulation of the nuclear envelope, formation of the metaphase plate of the 1st meiotic division, and finally the extrusion of the 1st polar body and subsequent formation of the metaphase plate of the 2nd meiotic division. According to the stage of nuclear maturation

observed using light microscopy, oocytes were classified as: GV (germinal vesicle), a spherical oocyte nucleus is located centrally or peripherally in the ooplasm; GVBD (germinal vesicle breakdown), the oocyte nucleus presents an irregular envelope surrounding disperse condensed cromatin; MI (metaphase I), characterized by the presence of metaphase chromosomes peripherally in the ooplasm; and MII (metaphase II), presence of metaphase chromosomes peripherally in the ooplasm and presence of a polar body in the perivitelline space.

The occurrence of meiotic maturation is accompanied and probably regulated by changes in the phosphorylation patterns of various cellular proteins. An important component of this activity is the Maturation Promoting Factor (MPF; Masui and Makert, 1971), which was found to be a universal cell cycle regulator of both mitosis and meiosis (Nurse, 1990). The MPF is a serine/threonine protein kinase composed of the catalytic sub-unity cyclin-dependent kinase 1 (cdk1, also known as p34cdc2) and its regulatory sub-unity, cyclin B (Downs, 1993; Taieb *et al*., 1997). Active MPF induces chromosome condensation, nuclear envelope breakdown and cytoplasmic reorganization with entry into M-phase of either mitotic or meiotic cell cycle (Murray, 1989; Murray and Kirschner 1989; Motlik and Kubelka, 1990). During oocyte meiotic maturation of mammalian species, MPF activity is very low in the germinal vesicle stage, and peaks at metaphase I and II stages (mouse: Hashimoto and Kishimoto, 1988; Choi *et al*., 1991; rabbit: Naito and Toyoda, 1991; goat: Jelinkova *et al*., 1994; pig: Dedieu *et al*., 1996; bovine: Wu *et al*., 1997).

Other kinases are also involved in the regulation of meiotic events, such as mitogen-activated protein kinase (MAPK). In horses, Goudet *et al.* (1998a) investigated the presence of p34cdc2, cyclin B and MAPK in oocytes matured *in vivo* and *in vitro*. The results showed that the incompetence of equine oocytes to resume meiosis *in vitro* is not due to the absence of these proteins. It may be due to a deficiency of regulators of MPF and/or to an inability to phosphorylate MAPK. The MPF activity could be measured by phosphorylation of histone H1 kinase and is associated with equine oocyte competence. MPF activity was not different in equine oocytes that reached metaphase I and II. However, the histone H1 kinase activity was significantly higher in the pre-ovulatory oocytes that reached metaphase II *in vivo* then in oocytes that reached metaphase II after *in vitro* culture (Goudet *et al*., 1998b).

Cytoplasmic maturation is characterized by several changes in the shape and localization of the organelles. Grondahl *et al.* (1995) described a breakdown of the intermediate junctions between the cumulus cell projections and the oolema with an enlargement of the perivitelinic space. In the same paper he described the formation and arrangement of a large number of cortical granules immediately beneath the plasmic membrane and the structural change of the mitochondria to a round shape. The migration of the cortical granules is believed to be an important step in cytoplasmic maturation (Cran, 1989) and has been used to assess the oocyte maturity (Long *et al*., 1994; Goudet *et al*., 1997). The redistribution of the cortical granules provides the ovum the capacity to maintain the block of polyspermy after sperm penetration (Cran, 1989).

Factors that may affect cytoplasmic maturation include presence of cumulus cells, addition of gonadotropins to the maturation media and time of culture. The structure of the oocyte and its associated granulosa cells is collectively called the cumulus oocyte complex. The presence of these cells during maturation has proven to be beneficial in many species including humans (Kennedy and Donahue, 1969), rabbits and cows (Robertson and Baker, 1969). The cells from the *corona radiata* are connected with the oocyte membrane, permitting the transfer of molecules from the granulosa cells to the oocyte (Okolski *et al*., 1991). The association between germinal cells and somatic granulosa cells regulates the level of synthesis of specific proteins and the pattern of protein phosphorylation in growing oocytes, thus directly regulating their metabolism.

The rate of *in vitro* maturation of equine oocytes ranges from 20 to 85% (reviewed by Galli *et al*., 2007). Comparisons between *in vitro* maturation procedures in horses are difficult because each study has a different protocol. In other species such as ruminants and pigs, the presence of expanded cumulus is linked to the collection of oocytes from atretic follicles, and these oocytes are generally discarded immediately because of their extremely low developmental capacity (de Loss *et al*., 1989). However, in horses, Hinrichs *et al*., in a series of studies demonstrated that oocytes with expanded cumulus were more capable of complete maturation than oocytes with compact cumulus (Hinrichs and Williams, 1997; Hinrichs and Schimidt, 2000; Hinrichs *et al.,* 2005b). Like in bovine, oocytes classified as expanded originate from follicles in the early stages of atresia, which appears to induce the meiotic competence (Hinrichs and Williams, 1997). The classification as expanded or compact cumulus oocytes complex must include not just the aspect of the cumulus cells, but also the aspect of the mural granulosa cells. Oocytes collected from follicles with any individual group of granulosa cells presenting signals of expansion until total expansion of the cumulus cells are classified as expanded while oocytes with either cumulus and/or granulosa compact cells are classified as compact (Hinrichs *et al*., 2002; Fernandes *et al*., 2006).

Although the majority of expanded oocytes presented an intact germinal vesicle just after harvest from the follicles (Hinrichs *et al*., 1993), those oocytes matured faster *in vitro* (Hinrichs *et al.,* 1993; Zhang *et al*., 1989) and presented a higher maturation rate (Alm

and Hinrichs, 1996; Hinrichs and Schmidt, 2000) when compared with compact oocytes submitted to the same conditions. However, according to Hinrichs (2005) and Galli *et al*. (2007) no statistical differences were observed in developmental competence of compact or expanded oocytes submitted to ICSI and cultured into sheep oviducts.

The majority of studies in equine oocyte IVM use TCM 199 as a culture medium with the addition of serum, follicular fluid or hormones (LH, FSH and estradiol 17β; Willis *et al*., 1991; Dell'Aquila *et al*., 1997; Hinrichs and Schmidt, 2000; Galli *et al*., 2002; Lagutina *et al*., 2005). However, the source and levels of LH and FSH have not been optimized. Luteinizing hormone of ovine (Dell'Aquila *et al*., 1997), bovine (Willis *et al*., 1991; Shabpareh *et al*., 1993; Squires *et al*., 1996), and equine origin (Bezard *et al*., 1997; Goudet *et al*., 1997), and equine pituitary extract (EPE) (Landim-Alvarenga and Choi, 1999) have been used but none of these has increased the efficiency of conventional IVF.

In vitro fertilization and ICSI

In the mare, the fertilization process *in vivo* has been described (Betteridge *et al*., 1982; Enders *et al*., 1987; Bezard *et al.,* 1989; Grondahl *et al.,* 1993) and blastocyst formation and establishment of pregnancy have been achieved following transfer of IVM oocytes to oviducts of mated recipient mares (Zhang *et al*., 1989. Fernandes *et al*. 2006). Conversely, reports on conventional IVF of *in vitro* or *in vivo* matured equine oocytes are few, and the data is difficult to interpret due to variations in techniques used by different laboratories. Parameters for assessing fertilization include: the presence of swollen sperm heads in association with sperm tail or mid piece (Zhang *et al*., 1990), the presence of two pronuclei (Del Campo *et al*., 1990), or cleavage. However, in the mare the success of producing embryos using IVF procedures remains extremely low.

The first cleavage after IVF of *in vivo* matured oocytes has been reported by Bezard *et al*. (1989). The fertilization rate was low, with 26% of oocytes fertilized and only 18% cleaved. However, two successful pregnancies were obtained after 14 surgical transfers of these fertilized oocytes (Palmer *et al*., 1991).

The reason(s) for poor IVF rates of equine oocytes remains unclear. Sperm cell capacitation (Alm *et al*., 2001; Hinrichs *et al*., 2002), oocyte maturation (Li *et al*., 2001), and changes in the zona pellucida (Dell'Aquila *et al*., 1999; Landim-Alvarenga *et al*., 2001) have all been offered as possible reasons for the poor IVF rates. The most encouraging method for capacitation induction and acrosome reaction of equine sperm cells is the use of calcium ionophore A23187, reaching 17 to 36% fertilization rates (Del Campo *et al*., 1990; Zhang *et al*., 1990; Grondahl *et al.*, 1995; Alm *et al*., 2001). The use of caffeine or heparin for sperm capacitation did not improve the fertilization rate (<17%; Del Campo *et al*., 1990; Grondhal *et al*., 1995; Dell'Aquila *et al*., 1996). The question of whether stallion spermatozoa are unique in their requirements for these agents remains open.

In order to increase the IVF rates in the horse, fertilization techniques such as partial zona dissection or removal, subzonal sperm injection, zona drilling, and intracytoplasmic sperm injection (ICSI) have been used. Choi *et al*. (1994) used partial zona dissection or dissection in an attempt to fertilize equine oocytes *in vitro*. For partial zona dissection, a slit in the zona pellucida of the oocyte is made; for partial zona removal, a piece of the zona pellucida is removed facilitating access of the spermatozoa to the membrane of the oocyte. Sperm penetration rates evaluated by staining were 52% for partial zona removal oocytes and 12% for partial zona dissection oocytes. Although polyspermy was evident in some of these oocytes with a large slit in the zona pellucida, monospermic penetration rates were found to be between 57% and 58%.

For zona drilling, a small hole is made in the zona pellucida with a drop of acidic Tyrode's solution, facilitating the mobile sperm cells to overcome the zona barrier. Li *et al*. (1995) obtained 33 to 79% of cleavage after zona drilling of *in vitro* matured oocytes obtained from pregnant mares. The best results were achieved after sperm exposure to 1.0μM concentration of Ca^{++} ionophore A23187. Although polyspermy was not examined, 45.5% of the oocytes that cleaved developed to the morula and blastocyst stages.

At the end of the 20^{th} century, the intracytoplasmic injection of a single spermatozoon (ICSI) had been introduced in human IVF with great success (Palermo *et al*., 1992). For ICSI, a direct injection of one spermatozoon, after crushing its tail, is made with a fine pipette into the cytoplasm of a mature oocyte exhibiting the first polar body in the perivitelinic space. While other methods depend on the presence of a functional capacitated and acrosome reacted spermatozoon for fusion with the membrane of the oocyte, ICSI does not seem to require capacitation or acrosome reaction. Sperm injection circumvents the problem of having sperm able to bind to the oocyte, penetrate the oocyte and initiate fertilization. With ICSI, spermatozoa are injected directly into the oocyte, initiating fertilization.

The first horse pregnancy from ICSI was reported in Colorado in 1996 (Squires *et al.,* 1996). In this pioneering work, the authors injected four *in vitro*-matured oocytes with sperm, and transferred them to the oviducts of recipient mares. Although several other researchers have successfully produced foals from ICSI (McKinnon *et al*., 1998; Cochran *et al*., 1998; Choi *et al*., 2002a; Galli *et al*., 2007), subsequent studies showed that this success rate was difficult to repeat.

Initially, laboratories working with ICSI in the horse had difficulty in achieving good rates of embryo development after sperm injection. However, in 2002, the use of the Piezo drill for ICSI was reported to increase cleavage rates to 69 to 89% (Choi *et al*., 2002a; Galli *et al*., 2002). The Piezo drill is a device that causes minute vibrations in the injection pipette; these not only facilitate penetration of the zona pellucida but also ensure breakage of the sperm and oocyte plasma membranes.

One of the main advantages on the use of ICSI over IVF or OT is the possibility of utilization of semen with very low fertility rates and poor quality. For ICSI in equine, three different preparations of sperm have been used: fresh (Kato *et al*., 1997; Schmid *et al*., 2000), cooled (Cochran *et al*., 1998; Li *et al*., 2000), and frozen-thawed (Dell'Aquila *et al*., 1997, 1999; Grondahl *et al*., 1997; Li *et al*., 2001). Researchers working with fresh semen achieved minimal pronucleus formation without chemical activation of the oocyte after ICSI (Kato *et al*., 1997; Guignot *et al*., 1998; Schmid *et al*., 2000). The use of cooled semen either required (Li *et al*., 2000) or did not (Cochran *et al*., 1998) chemical activation for pronucleus formation. When frozen thawed semen was used, good rates (around 50%) of pronucleus formation were obtained without activation (Grondahl *et al*., 1997; Dell'Aquila *et al*., 1997, 1999). Because spermatozoa injected into the oocyte by ICSI are still surrounded by their plasma membrane, it is possible that the process of freezing, which may result in changes in sperm membrane, has a beneficial effect on the diffusion of sperm factors into the cytoplasm of the oocyte leading to activation. However, according to a review from Choi *et al*. (2002b) the controversy on the use of each type of sperm preparation appears to be related to ineffective ICSI technique, since the use of Piezo drill has shown that fresh or frozen semen is equally and highly effective for fertilization.

A study from Lazzari *et al*. (2002) compared the developmental capacity of *in vitro* matured oocytes fertilized by ICSI with frozen-thawed stallion semen of different motility and/or fertility. No difference in either cleavage or advanced embryo development rates among oocytes injected with spermatozoa from stallions of good, poor and no fertility was observed, as long as a motile spermatozoon was selected for ICSI. In contrast, when an immobile sperm from semen having very poor motility post-thawing and no fertility was used, a significantly lower cleavage rate was observed and no embryos were capable of developing to the compact morula or blastocyst stages. However, in a study performed by Choi *et al*. (2006), a 13% blastocyst rate was achieved with non-motile sperm (8/63 injected oocytes) without extra activation stimulus, thus it appears that motility is not an absolute requirement.

Before the injection of the spermatozoa into the oocyte, the semen needs to be prepared by selection of viable sperm using Percoll gradients or Swim up procedures. The sperm suspension is then diluted in a TALP solution with 10% (w/v) polyvinylpyrrolidone to slow down the flagellar beating and allows the capture of the sperm cell. The injection pipette used for ICSI in horses has to be 7 - 8μm in diameter (outside) and a 120 - 140 μm diameter (outside) pipette is used to hold the oocyte in the proper position. The entire procedure must be performed under oil in an inverted microscope with a micromanipulator. Each sperm is immobilized by applying a few pulses with the Piezo drill to the sperm tail immediately before injection.

Initially the ICSI procedure presented limited clinical use because of the lack of standardization in the technique. Due to the absence of a standard protocol for culture of fertilized equine oocytes to the blastocyst stage, it was necessary to transfer the zygotes directly to the oviduct of a recipient mare immediately after injection. Choi *et al.* (2004b) obtained a blastocyst recovery rate of 36% after collection of ICSI injected IVM oocytes transferred immediately after injection showing that ICSI can result in efficient embryo production if embryos are cultured in an optimal environment. However, the direct transfer of an ICSI embryo to the oviduct of a recipient mare seems to have very little benefit over performing OT. Although some advantages can be noted, especially concerning the use of poor quality semen or semen with low numbers of spermatozoa, the ICSI procedure includes a very high cost in equipment and technician. Moreover, the inefficiencies associated with the ICSI procedure, including the time needed for micromanipulation, the risk of lyses of the oocyte during the injection procedure, and the reduced rate of embryo cleavage, render ICSI a less efficient method than oocyte transfer when one oocyte and semen of normal fertility is used. (Hinrichs, 2005).

For the ICSI procedure to become clinically available, the development of suitable culture systems was necessary. In cattle, 25–35% of fertilized oocytes typically develop to blastocysts *in vitro*. In contrast, most of the work with *in vitro* culture of equine embryos has been disappointing; with blastocyst rates remaining low, ranging from 4 to 16%. However, the progress in IVM and ICSI technologies has increased efforts to design suitable culture systems for early cleavage stage embryos. Many different culture conditions have been reported for preimplantation development of ICSI fertilized horse oocytes, including defined media such as G1.2 (Choi *et al*., 2002a), DMEM-F12 and CZB (Choi *et al*., 2004a) and modified SOF (Galli *et al*., 2002).

Recently, the culture of ICSI-produced equine embryos in medium DMEM/F-12 in a mixed-gas environment, was able to support >35% blastocyst development (Hinrichs *et al*., 2006; Choi *et al*., 2006), similar to the rates obtained with *in vivo* culture. The range of blastocyst formation using this system

increased from 27 to 44% (Choi *et al*., 2006), with pregnancy rates of 50%. This exciting result allows the technique to now be offered commercially in some laboratories around the world. However, when cell number counts were compared (Tremoleda *et al*., 2003) among *in vivo* produced embryos and those produced by *in vitro* culture in SOF-aa-BSA media, both on day 7 of development, the *in vitro* produced embryos had significantly fewer cell numbers, resembling a day 5 rather than a day 7 embryo. It is important to take this into account difference when embryos are transferred to synchronized recipients.

The clinical use of ICSI is mainly for production of foals from stallions that have very few sperm or perhaps stallions that have died and a limited quantity of frozen semen is available. However, another promising application is to obtain embryos from mares post-mortem. That is, when a mare dies and the owner wishes to attempt to obtain foals from the mare's oocytes. In this case, multiple immature oocytes are recovered from the follicles present in the ovaries and are matured *in vitro*. Use of ICSI under these circumstances has a major advantage over OT, since with ICSI every oocyte that is capable of making a blastocyst has a chance to produce a foal. After the oocytes have been collected and matured *in vitro*, those in metaphase II are fertilized by ICSI and placed into embryo culture. Blastocysts are identified after 7–8 days of *in vitro* culture, and each blastocyst may be transferred separately to a recipient mare.

Those recent progresses obtained with ICSI in the horse leads to the appearance of some commercial clinical programs. Carnevale *et al.* (2007) reported the use of frozen, cooled or epididimal sperm to inject pre-ovulatory oocytes collected from donor mares in a commercial program held by Colorado State University. Injected oocytes were cultured *in vitro* until cleavage and early embryos were transferred into recipient mare's oviduct. Of 90 oocytes, 68 cleaved and were transferred approximately 1.5 days after ICSI. Pregnancy rates were 44 and 31% at 16 and 50 days of gestation, respectively, and the foals are expected to be born in 2008.

Obviously, ICSI is the ultimate in low-dose insemination, because only a single spermatozoon is injected into the oocyte. Studies are being conducted on frozen semen with few numbers of spermatozoa per straw for subsequent sperm injection, and also to determine the effect of thawing, re-dilution and re-freezing of semen on embryo development after ICSI (Squires, 2005). Other possibilities include cutting a piece of the straw while under liquid nitrogen, thawing the semen, then refreezing the extra sperm that are not needed for the ICSI procedure. Choi *et al*. (2006) demonstrated that thawing one semen straw, diluting 1:100 and refreezing does not lower blastocyst formation rate after ICSI. This technique would allow one to conserve genetic material for a long time period and extend the use of valuable semen several orders of magnitude compared with its use in conventional breeding methods (Squires, 2005). Moreover, sperm injection is a powerful tool that can be used to evaluate *in vitro* maturation systems for oocytes, study fertilization, and provide *in vitro*-produced embryos for subsequent studies.

Cloning

Cloning is the production of genetically identical individuals by non-sexual means (Seidel, 1983). The first results for mammalian cloning were obtained by Willadsen (1986), when cloned sheep were born after the split of 8 to 16 cell embryos. At the end of the 20th century, Wilmut *et al*. (1997) surprised the world by producing the first clone obtained from a differentiated cell. Since then, the production of clones by nuclear transfer has been a success in many mammalian species including the horse.

The cloning process utilizing somatic cells is a powerful instrument for the multiplication of animals with a unique genotype as well as for the preservation of endangered species, representing of the most extraordinary conquered feat in developmental biology research (Wilmut *et al*., 1997). However, the efficiency of the technique is still low (often less then 1%) due to the highly complex process that involves a combination of biological and technical factors, all of which are not fully understood (Solter, 2000). Moreover, nuclear transfer leads to several developmental problems in embryos of most species, with a high rate of miscarriage and perinatal death (Wilmut *et al*., 1997; Hill *et al*., 1999; Heyman *et al*., 2002). However, it is not clear if the developmental failure in embryo development is linked with the re-programming of the somatic nuclei or is intrinsic on the cloning process itself (Han *et al*., 2003).

The nuclear transfer technique involves obtaining somatic cells from the genetic donor, as well as *in vivo* or *in vitro* matured oocytes to be used as recipient cytoplasm (oocyte donor genetics are not important). By micromanipulation, the area of cytoplasm containing the chromatin of the oocyte is removed, creating an enucleated oocyte or cytoplast. The selected somatic cells, grown *in vitro* from a tissue sample (e.g. a skin biopsy), are combined with the cytoplast either by fusing the two cells by electric pulse, or by breaking the donor cell membrane and injecting the cell directly into the cytoplasm of the oocyte. The recombined oocyte, now containing the nucleus of the genetic donor, needs to be artificially activated to start embryo development, and finally the embryo is transferred to a recipient mare (Hinrichs, 2005). If a sub-optimal condition happens in any of these steps the production of the cloned embryo will be influenced.

The first equids cloned by somatic cell nuclear transfer were three mules obtained from cells from a

45 day old fetus. *In vivo* matured oocytes were utilized as cytoplast and the embryos were transferred directly to the oviduct of the recipient mares. This same group reported establishment of seven pregnancies from transfer of 62 oocytes subjected to adult somatic cell nuclear transfer, but all pregnancies were lost before 80 days of gestation (Vanderwall *et al*., 2004). The same year Galli *et al*. (2003) reported the birth of a cloned horse from an adult somatic cell transferred to an *in vitro* matured oocyte. The embryo was then cultured to the blastocyst stage before transcervical transfer to the uterus of the recipient mare. One of the most exciting aspects of the birth of this cloned horse is that the recipient mare who became pregnant and delivered the clone was also the somatic cell donor. In that report, 841 recombined oocytes were cultured, and 22 blastocysts developed (3%). Seventeen blastocysts were transferred, and four pregnancies resulted (24% pregnancy rate after transfer). Of the four pregnancies, two were lost around 30 days, one was lost at 6 months of gestation and one was carried to term for a normal birth. More recently, another group reported the birth of a cloned horse at Texas A&M University (Hinrichs *et al*., 2005a. In this study, 567 recombined oocytes resulted in 11 blastocysts (1.9%) and 4 pregnancies (36% pregnancy rate), which produced 2 viable foals.

The effect of the donor cell on cloning success was also studied. Lagutina *et al*. (2005) obtained 24.3% pregnancy rates (9/37 mares transferred corresponding to 9/101 blastocysts transferred) when adult fibroblasts were used for nuclear transfer. The same authors describe that when fetal cells were used the rate of success was only 5.6% (1/18 mares transferred corresponding to 1/33 blastocyst transferred).

At this point is clear that the proportion of cloned horse pregnancies that are carried to late gestation or term vary among laboratories. So the increasing efficiency of equine cloning could make it possible for clinical applications. In fact, there is at least one commercial company that is attempting equine cloning for clients. Clinically, cloning can be used to prolong the use of genetically exceptional individuals with acquired infertility, males that have been castrated and/or animals that die before being able to reproduce. However, it is important to point out that nuclear transfer will never become a common clinical procedure, but can be used to solve exceptional problems.

Equine cloning can be particularly difficult due to the low availability of cytoplast suitable for nuclear transfer. Since one major factor on the success of the technique is the quality of the cytoplast, it needs to be obtained from healthy, good quality Metaphase II oocytes, which undergo adequate cytoplasmic maturation. In mammalians, during oogenesis the oocytes accumulate mRNA and their precursors into the cytoplasm. This reservoir is necessary at the beginning of embryo development, after fertilization, in the period just before the embryonary genome activation, which in bovine occurs at the 8 to 16 cell stage (Barnes and Eyestone, 1990). It is very well documented that the embryo development on the first cell cycles depends on the maternal transcript storage during oogenesis and final maturation of the oocyte.

The production of cytoplast requires the removal of the nuclear material from a mature oocyte. This procedure is essential for the maintenance of the normal number of chromosomes of a given species. In horses, the visualization of the metaphase plate containing the chromosomes may be difficult due to the high amount of lipids present in the oocyte cytoplasm. Moreover, the metaphase plate is not always located next to the released polar body. These features make the enucleation of the oocyte, in absence of a specific DNA stain, almost impossible. The main stain used to mark DNA in nuclear transfer procedures is the Hoechst 33342 which allows the visualization of the chromosomes under UV light during enucleation.

Although the best results were obtained using the technique of DNA staining, there are some concerns related to the exposition of the oocyte to the UV light which could lead to plasma membrane damage (Smith, 1993), nuclear and mitochondrial DNA breakage (Dominko *et al*., 2000), and impairment of the embryonary development (Prather *et al*., 1987; Smith, 1993). The removal of part of the cytoplasm next to the metaphase plate during enucleation may also be detrimental to the cytoplasm since important organelles, proteins and mRNA are taken along with the chromosomes (Barnes and Eyestone, 1990).

Since the removal of the chromatin without previous staining is not safe due to the variable position of the metaphase plate in relation with the polar body (Dominko *et al*., 2000) an alternative to that may be the use chemical enucleation. This technique is less invasive and leads to minimal removal of cytoplasm contents (Russel *et al*., 2005). Basically, the chemical enucleation involves the use of drugs that impair the organization of the cell cytoskeleton, like colchicines or demecolcine (Ibáñez *et al*., 2003; Russel *et al*., 2005), resulting in the loss of the microtubules that hold the metaphase plate. Microtubules and microfilaments are responsible for structural changes in the oocyte during maturation, resulting in the organization of the metaphase plate and extrusion of the first polar body (Li *et al*., 2005). The oocytes submitted to chemical enucleation presented a protrusion on the cortical region of the oocyte containing the nuclear material, and the mechanical removal of this protrusion is enough to provide the enucleation. Although the use of demecolcine has been reported in domestic animals such as cows and pigs, in horses there are few reports concerning chemical enucleation methods. Using this technique, Fernandes *et al*. (2007) found that observation of the protrusion was possible in 23.6 and 28.3% for expanded and compact equine oocytes,

respectively, indicating that although enucleation rates of equine oocytes using demecolcine were low, the technique may be used as an alternative method for preparing oocytes for nuclear transfer in the absence of a fluorescent microscope.

While the cytoplast is always represented by an enucleated oocyte, different cell types can be used as nuclear donors. Blastomeres, embryonic stem cells, mammary gland cells (Wilmut *et al*., 1997), oviductal cells (Kato *et al.*, 1998), cumulus cells, (Kato *et al*., 1998), muscular cells (Shiga *et al*., 1999) and fibroblasts (Shin *et al*., 1999) were used as nuclear donors in different species. The synchronization of the cell cycle of the nuclear donor cells and their nuclear integrity is important for the success of the nuclear transfer programs. According to Campbell *et al.* (1996), the induction of nuclear donor cells to G0-G1, a quiescent stage of the cellular cycle is beneficial. The structural modification of the chromatin and the reduction of the transcriptional activity in G0-G1 cells are thought to facilitate the re-programming of the differentiated nuclei of the donor cells to the totipotent status necessary for the normal development of the reconstituted embryos (Wilmut *et al*., 1997; Kato *et al*., 2000). The synchronization of cells in G0-G1 can be induced in somatic cells by serum starvation during culture, or by letting the cells grow until confluence (inhibition of growth by cellular contact). Cell cycle inhibitors like roscovitine can also be used to prepare the nuclear donor cell before nuclear transfer (Hinrichs *et al*., 2006). Although cloning using adult cells is a desirable technique in the horse industry, Vanderwall *et al*. (2004) and Lagutina *et al*. (2005) reported high rates of embryonic loss in mares that receive a nuclear transfer embryo. Embryonic and fetal losses in cloning programs are often attributed to failure in genomic reprogramming, which may be in part due to inefficient synchronization of the cellular cycle before transfer.

Cloning by nuclear transfer involves the fusion of the donor cell with the enucleated cytoplast which is performed by fusing the two cells by electric pulse, or by breaking the donor cell membrane and injecting the cell directly into the cytoplasm of the oocyte. The information on electrofusion in horses has been conflicting, with rates of success ranging from 20 to 67% (Choi *et al*., 2001; Hinrichs *et al*., 2002). The fusion rates were improved (82%) when the electrofusion was associated with the use of the Sendai virus (Li *et al*., 2001). Conversely, the direct transfer of the donor cell into the cytoplast through the use of a micropipetet, similarly with the ICSI procedure, leads to 13 to 40% reconstruction success. Choi *et al*. (2002a) obtained reconstruction rates of 20 to 40% with electrofusion using the Piezo drill for the injection of the donor cell directly into the cytoplast. However, when the direct transfer of the donor cell is performed, there is a risk of breakage of the ooplasm membrane which leads to failure of the nuclear transfer. Moreover, when compared with the electrofusion technique, the direct transfer of the donor cell into the ooplast leads to the injection of some media components which may be deleterious (Choi *et al*., 2002a).

The next step in the nuclear transfer procedure is the activation of the reconstructed embryo. This step, essential for the success of nuclear transfer, is performed by using a combination of several chemicals that interfere with the cellular cycle. In the MII oocyte, the arrest of meiosis is mainly due to the high levels of MPF, the mitogen activated kinase (MAPK) and the cytostatic factor (CSF) or Mos (product of the proto-oncogen c-Mos). After fertilization, the levels of MPF drop in response to the elevation of intracellular calcium (Nurse, 2000). The blockage of the MPF and the MAPK allows the occurrence of the initial events that result in embryo development. The increase in the intracellular calcium inactivates the CSF, leading to a decrease in the MPF activity (White and Yue, 1996). The blockage of the MPF allows the transition anaphase/thelophase, extrusion of the second polar body and extrusion of the cortical granules. However, if the activation was not efficient, the MAPK activity will still be present and the MPF will rapidly increase again (Liu *et al*., 1998).

Most of protocols for oocyte activation in horses used combination of drugs that increase the intracellular calcium, like ionomycin, and reduce the activity of the MPF and MAPK like 6-dimethylaminopurine (6-DMAP) and/or cycloheximide (Wells *et al*., 1998, 1999; Galli *et al*., 1999). Galli *et al*. (2007) found that either of the two used alone, after ionomycin exposure, did not provide satisfactory results. However, by using a combination of the two, the activation rate was around 90%. Hinrichs *et al.* (2006) have used a combination of injection of sperm extract and culture in 6-DMAP to produce embryos resulting in successful foaling of cloned offspring with great success.

Another drug that recently has been used in equine cloning is roscovitine, which inhibits the adhesion of ATP to the catalytic subunity of MPF ($P34^{cdc2}$). Roscovitine is effective in suppressing meiosis in bovine and equine in a reversible way (Franz *et al*., 2003; Choi *et al*., 2006).

There is no doubt that the nuclei of an already differentiated somatic cell, when transferred to a cytoplasm needs to be reprogrammed. The re-programmation of the somatic cell nuclei includes the removal of several epigenetic factors. The mechanism by which this occurs is still not totally understood. Several studies have been performed to better understand the molecular events of nuclear re-programmation including: switch of nuclear proteins and/or histones (Bordignon *et al*., 1999) and modification of the nuclear morphology (Kanka *et al*., 1999). The reprogrammation of the somatic H1 histone in the reconstructed embryo is influenced by the cell cycle and by the kinase enzymes (Bordignon *et al*.,

1999, 2001). Enzymatic modifications of the histones including acetilation, mutilation, phosphorylation and ubiquitilation are examples of dynamic modifications of the chromatin structure which controls gene expression (Li, 2002; Jenuwein and Allis, 2001). In all animals the control of which gene (from maternal or paternal side) will be active is controlled largely by methylation and demethylation of the DNA. The methylation status of the genes changes throughout embryonic and fetal life and also depending upon the tissue in which the cell resides. The complete reprogrammation of the somatic cell nuclei by the cytoplast should result in a gene transcription pattern similar to the one observed in *in vivo* produced embryos. However, since the nuclear donor cell goes through a series of complex modifications in a very short period of time, which are very different from the ones occurring during gametogenesis, it is believed that the high mortality rate observed in cloning is due to errors in the nuclear reprogrammation (Rideout *et al*., 2001). The complete reprogrammation of the somatic cell nuclei by the cytoplast should result in a gene transcription pattern similar to the one observed in *in vivo* produced embryos. Moreover, although the genes of the clone will be exactly the ones of the donor, within the genome certain genes may be "turned off", while the transcription of others is enhanced. The differences in which gene will be active results in the reprogramming of the clone nuclei which may not be perfect. The state of activity of the DNA during fetal life may affect the phenotype of the animal at birth and after birth. However, what is important is that the offspring of the cloned embryos will carry the exact same genetics as the original donor (Hinrichs, 2005).

The increasing efficiency of equine cloning makes it potentially clinically applicable at this time, and there is at least one commercial company that is attempting equine cloning for clients. However, although animals from different species have been cloned, the fate of these cloned individuals is still uncertain. Until now, the low efficiency of the method is a consequence of the technical problems that affect the embryos during their development (Bordignon *et al.*, 2003). The main abnormalities found are dysfunction of the mitotic fibers (Simerly *et al*., 2003), chromosomal abnormality (Booth *et al*., 2003), and low number of cells in the inner cell mass. Although a great number of reconstructed embryos are capable of beginning cleavage and develop until the blastocyst stage, the pregnancy rate is much lower and the embryonic death is much higher than the ones observed in normal pregnancy. Although Hinrichs *et al*. (2006) have found a pregnancy rate of 50% after transfer of *in vitro*-produced embryos, half of these are lost in early gestation. In other species the main problems with cloned animals are respiratory dysfunction, immunological deficiency, cardiac and vascular abnormalities, renal failures and hepatic congestion and fibrosis (Hill *et al*., 1999; Renard *et al*., 1999; Chavatte-Palmer *et al*., 2002 Cibelli *et al*., 2002).

Besides the many clinical options, the possibility of cloning opens up many new areas for study and raises ethical questions. One very important aspect that should be understood is that a cloned foal will not be an exact copy of the original horse. Based on spontaneous twins and the few split-embryo identical twins produced, it is known that the intrauterine environment affects not only the size of the foal at birth, but also the adult size and phenotype especially concerning the distribution of the white hair areas. Because of that, the cloned animal frequently does not have the exact same coat as the donor animal.

Another important aspect is that although the reconstructed embryo will have the nuclear DNA of the genetic donor, the mitochondrial DNA will be from the recipient oocyte. A very small proportion of the donor cell mitochondria will also be present but proportionately in much lower numbers. The impact of the source of mitochondria, or a mixture of mitochondria, on the traits of the progeny is currently unknown (Hinrichs, 2005). In a cloned female horse all their oocytes will have a heterogeneous mitochondria population and it will be passed down to her offspring. However, in the case of a male clone, since the mitochondria present in the sperm do not contribute to the mitochondria of the embryo after fertilization, the cloned colt could be considered to produce the same progeny that its genetic donor would have produced (Hinrichs, 2005).

While cloning is not yet clinically efficient, "banking" of tissue from animals, including horses, is currently being done commercially by a number of companies at a reasonable cost. A skin or lip biopsy is taken from the animal and sent to the company in a transportation package (supplied by the company). At the laboratory, cells are grown from the tissue in culture and the company stores the cells in liquid nitrogen. It is possible to obtain tissue from animals hours to days post-mortem (especially if tissue has been cooled but not frozen) and still support successful tissue culture. Thus, cell banking is an option that can be offered to clients that are extremely concerned about the loss of genetic potential when a horse dies or becomes infertile; the decision on whether to use the cells to produce a cloned foal can be made in the future.

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