Review Articles

Assisted Reproduction Techniques in Horses: The Challenge

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Abstract

Assisted reproduction techniques (ARTs), such as artificial insemination and embryo transfer are well established and have been used in a wide range of species. To date, the economic impact of these ARTs in horses has rapidly increased. While many studies have attempted to improve the efficiency of ARTs, overall success remains relatively restricted due principally to a limited knowledge of the unique reproductive physiology of this species. Improving efficiency of particular techniques has become an affordable milestone in the development of ARTs in equine reproduction

Keywords : biotechnology, reproduction, horse

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บทคัดย่อ

เทคโนโลยีช่วยในการเพิ่มประสิทธิภาพการสืบพันธุ์ในม้า: สิ่งท้าทาย

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การใช้เทคโนโลยีชีวภาพเพื่อเพิ่มประสิทธิภาพทางการสืบพันธุ์เช่น การผสมเทียมและการย้ายฝากตัวอ่อน เป็นเทคนิคที่ได้รับการพัฒนาและสามารถนำไปใช้ได้ในสัตว์หลายประเภท เทคโนโลยีเหล่านี้ถูกนำมาใช้อย่างแพร่หลายและมีความสำคัญอย่างยิ่งในการเลี้ยงม้า ถึงแม้ว่าในปัจจุบันจะมีการศึกษาการใช้เทคโนโลยีนี้มากมาย ความสำเร็จและประสิทธิภาพโดยรวมของเทคโนโลยีเหล่านี้ในม้ายังอยู่ในระดับที่จำกัด ทั้งนี้เนื่องจากลักษณะสรีรวิทยาทางระบบสืบพันธุ์ของม้ามีความแตกต่างกับสัตว์ประเภทอื่นๆ ดังนั้นการพัฒนาประสิทธิภาพการใช้เทคนิคต่างๆ ทางเทคโนโลยีชีวภาพจึงเป็นสิ่งที่จำเป็นในงานด้านระบบสืบพันธุ์ม้า

คำสำคัญ : เทคโนโลยีชีวภาพ การสืบพันธุ์ ม้า

ภาควิชาสูติศาสตร์ เธนุเวชวิทยาและวิทยาการสืบพันธุ์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
***ผู้รับผิดชอบบทความ**

Introduction

Within the past two decades, assisted reproductive techniques (ARTs) such as artificial insemination (AI), *in vitro* embryo production and embryo transfer (ET) are the powerful tools for contributing of the most genetically desirable animals to the next generation. Although these techniques have been well integrated to clinical exploitation in the improvement of reproductive efficiency and for the safeguarding of irreplaceable genetic lines in endangered species or rare breeds, overall success of these ARTs in horses has, however, been hampered by many factors including the breeding associations (i.e., AI and ET are officially prohibited in Thoroughbred race horses) and a relatively limited knowledge of reproductive physiology. In addition to these ARTs, *in vitro* embryo production and stem cells technology are emerging techniques and have become increasingly important means of treating a number of incurable diseases in man and animals.

This article reviews the state-of-the-art regarding assisted technologies in equine reproduction. These include techniques for artificial insemination, the induction of multiple ovulation, *in vitro* embryo production, cryopreservation of oocytes/embryos, embryo transfer and stem cell technology.

Artificial insemination

Artificial insemination (AI) technique was first established in horse in 1322, but overall success of AI remains poor especially when frozen-thawed semen is used. Semen, collected by artificial vagina is usually diluted with semen extender in order to increase the longevity of the sperm *in vitro* and to adjust the total number of sperm used for the AI (approximately 250-500 million progressively motile sperm per an insemination). Equine semen from çcool-toleranté stallions can be stored at 4°C for up to 48-72 h while pregnancy rates remained approximately 60-70% (Squires et al., 1998). Indeed, sperm from some stallions, stored at 4°C in a non-fat dry milk glucose-based extender, survived with a progressive motility of approximately 40% for 4-5 days (unpublished data). In addition to cooled semen, cryopreservation of stallion's semen has been considerably difficult. The freezability of stallion semen is unfortunately variable among individual stallions. The technical terms of "good-freezer" and "bad-freezer" are,

therefore, generally applied. For good freezer stallion, pregnancy rates similar to cold storage semen can be achieved, while the pregnancy rate is critically low for " poor-freezer" stallions (Samper, 2001). Another obstacle is the short lifespan of post-thaw stallion semen within the mare's reproductive tract, most likely because cryopreservation induces capacitation-like reaction (Watson, 2000). It is therefore important to inseminate the mare close to the time of ovulation. This is labour-intensive because the mares exhibit oestrus signs for almost one week and the mare's ovaries need to be examined every 6-8 h, using ultrasonography, to ensure the exact time of an ovulation. To prevent the evacuation of deposited semen and to improve the efficiency of sperm transport, a little amount of semen containing low-dose sperm can be deposited via hystero-videoscopy to the utero-tubule junction or to the tip of uterine horn by deep intrauterine insemination (Morris et al., 2000). These techniques have broken through the limitation of sperm numbers, in particular when sex-sorted sperm or poor quality semen are used. By this technique, the number of spermatozoa can be reduced to as low as 1 million, while the conception rates per cycle remain at approximately 50-60% per cycle (Morris et al., 2000).

Induction of multiple ovulation

Follicle development in the mare occurs in a wave-like fashion, with 1-2 follicular waves per 21-22 days of oestrous cycle (for review see Ginther, 2000). The exact mechanism that controls the ovarian function and folliculogenesis in the horses is not entirely clear, but it has been suggested that deviation in growth between the two largest follicles occurs soon after follicle stimulating hormone (FSH) levels decrease (Gastal et al., 1997), and it is controlled by insulin-like growth factor (IGF-1) system (Ginther et al., 2004). Interestingly, follicle development in equids is unique because equine ovaries are anatomically "inside-out", with the follicles developing within a central ovarian cortex surrounded by a vascularised, soft tissue medulla. As a result, ovulation can only takes place in one specific area (the so-called ovulation fossa). This anatomically restricted area for follicle development has been proposed to limit multiple follicle development and ovulation when mares are treated with gonadotrophin preparations to induce superovulation (Ginther, 1979). Paradoxically, most gonadotrophin preparations used successfully for superovulating cows, such as porcine follicle stimulating hormone and equine chorionic gonadotrophin (eCG), fail to induce multiple follicle development in mares, even when large amounts are used (McCue, 1996). To date, crude equine pituitary extract (EPE) and equine FSH-enriched pituitary extract are the only gonadotrophins shown to usefully stimulate multiple follicle development in mares, with a mean of 1.7-3.8 ovulations per oestrous cycle (reviewed by McCue, 1996). However, a variable of LH and FSH in EPE, as crude protein extract, has contributed to the variability of superovulatory responses in mares. In this regard, using recombinant FSH without LH activity would fit to the bill for superovatory inducer since it is highly potent and bio-secured from "brain-carrying" diseases. Unfortunately, treating mares with human recombinant FSH even at high amounts gave no more than 2 ovulations per oestrous cycle (Tharasanit et al., 2006[a]). Many attempts have been made aimed particularly to increase the efficiency of superovulation techniques in mare, in terms of ovulation and embryo recovery rates. These include types of gonadotrophin preparations, day of initial treatment, dose of gonadotrophin used and the frequency of injections. For example, Alvarenga et al. (2001) achieved mean ovulation and embryo recovery rates of 7.1 and 3.5 per oestrous cycle, respectively, after treating mares twice daily with 25mg of EPE starting in early dioestrus (day 6-8 post ovulation). Very recently, highly purified equine pituitary extract (equine FSH) has become commercially available and clinically useful for superovulating mare with a mean of ovulation and embryo recovery rates similar to the EPE regime (Niswender et al., 2003).

***In vitro* embryo production**

Development of *in vitro* oocyte maturation, *in vitro* fertilisation, and the culture of zygotes/embryos has resulted in dramatic improvements in the success of these techniques (*in vitro* embryo production, IVP). Although knowledge regarding the mechanism that controls the process of equine oocyte maturation (both nuclear and cytoplasmic maturation) is primarily restricted, many laboratories have been capable of producing live offspring from IVP equine embryos. The restricted development in this IVP technique has contributed to the difficulties in obtaining equine oocytes because the slaughterhouses for horses are usually far from laboratories and the recovery rate of good quality oocytes per ovary is extremely low (~3 oocytes per ovary). While the oocytes may alternatively be retrieved from live donors via transvaginally ultrasound guided ovum aspiration (OPU), however this technique requires skilled operatives and is labour intensive. More importantly, the recovery rate of good quality oocytes is usually low (Bruck et al., 1997).

Indeed, immature equine oocytes are capable of resuming and reaching metaphase II (MII) stage at a reasonable rate of 50-80% (Hinrichs, 1998) but only a few of these MII oocyte can develop to the blastocyst stage. To make matters worse, the success of conventional IVF is usually poor (Zhang et al., 1990), and only two foals have ever been born from conventional IVF (Palmer et al., 1991; Bezard, 1992). It has been suggested that poor success of equine IVF is the result of an inadequate oocyte's cytoplasmic maturation and culture environment. In addition, because *in vitro* fertilisation by means of intracytoplasmic sperm injection (ICSI) yields a reasonable fertilisation rate, the failure of conventional IVF is, in turn, believed to involve inadequate sperm activation, even though equine sperm cells have been shown to undergo capacitation and acrosome reaction when incubated *in vitro* (Rathi et al., 2001). As stated previously that conventional IVF is poorly successful in horses, ICSI has therefore become the technique of choice for fertilizing equine oocytes *in vitro* (Galli et al., 2002; Choi et al., 2004). Although suboptimal culture conditions partly explain poor embryo development following ICSI of equine oocytes (Galli et al., 2002; Tremoleda et al., 2003), factors intrinsic to the oocyte also critically affect their developmental potential. For example, the degree of cumulus expansion at oocyte recovery appears to affect the developmental capacity such that the oocytes with an expanded cumulus at recovery exhibit better developmental competence (Hinrichs and Schmidt, 2000). Recent improvements in culture techniques have led to respectable rates of blastocyst formation in vitro (20-24% blastocyst rate) from ICSI oocytes (Choi et al., 2004). However, the quality of blastocyst produced *in vitro* is far from those derived *in vivo* (i.e size and cell numbers, Fig. 1)

In addition to ICSI, somatic cell nuclear transfer has been proven and offers a great opportunity for producing live offspring in a wide range of species, including horses. The aim of producing foals by this technique is obviously not to clone champion horses though many of top-competing horses are inevitably incapable of normal fertilization since most of them are gelded (castrated male horse). Six years following the birth of "Dolly", the world's first cloned sheep by somatic cell nuclear transfer, the first cloned horse was produced in Italy (Galli et al., 2003). In the same year, three healthy cloned mules were also born in Idaho, the United State (Woods et al., 2003). Interestingly, the first cloned horse demonstrated convincingly that autologous pregnancy can be produced because the mare provided the donor cell to create a cloned embryo and carried it to term. To date, the overall efficiency of cloning, in terms of the production of healthy live offspring is, however, extremely poor, in that only 0.7-2.7% of reconstructed cloned equine embryos resulted in the birth of life offspring. Fortunately, there has been no obvious evidence in the horses such as a large offspring syndrome or any placental abnormalities being reported in bovine (reviewed by Vanderwall et al., 2006). There are a number of factors

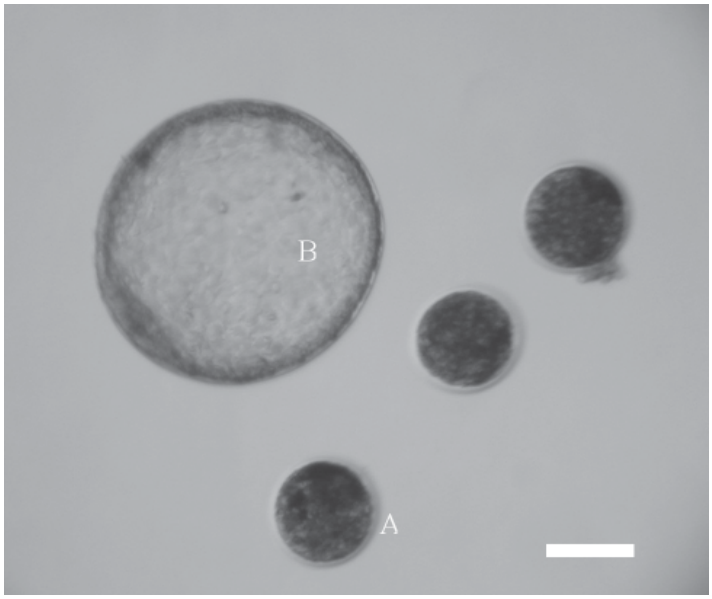

Figure 1 Equine blastocysts (A, 7 days post-intracytoplasmic sperm injection) produced by *in vitro* maturation, fertilization and embryo culture. These embryos are typically small when compared to *in vivo* produced blastocyst (B, 7 days post-ovulation). Scale bar represents 100 μm (Tharasanit, unpublished data).

that contribute to the relatively low success of cloning in horses. The number of equine oocytes to be used as recipient cytoplasm in nuclear transfer program is remarkably limited. Furthermore a limited knowledge of *in vitro* embryo production technique in this species, in particular *in vitro* oocyte maturation and culture of embryos prior to transfer of the blastocyst stage embryo to the recipient mare, has contributed significantly to the restriction of success of nuclear transfer in this species.

Cryopreservation of oocytes and embryos and embryo transfer

Cryobiology is the keystone in preserving the potential of reproductive cells for subsequent use in ARTs. However, while a large number of freezing techniques have been tested, the success of preserving the post-thaw developmental competence of equine oocytes and embryos remains limited, presumably because cell structures (such as the plasma membrane and cytoskeleton) are damaged irreparably during freezing and thawing. The ability of oocytes and embryos to survive cryopreservation has been examined in large numbers of empirical and theoretical studies. Techniques for cryopreservation can be split broadly into two categories, "classical" slow-rate freezing and the more recently developed ultra-rapid, ice formation-free vitrification. In recent years, the number of scientific publications on the vitrification of oocytes, embryos or ovarian tissues has increased rapidly, not only because vitrification yields high survival rates compared to slow-rate freezing, but also because it is simple, quick and does not require expensive equipment such as a programmable freezing machine. On the other hand, cells/tissues to be vitrified must be able to tolerate very high concentration of cryoprotectant, which may become toxic (Fahy, 1986).

In general, oocytes are extremely susceptible to damage during freezing and thawing, primarily because the oocyte is an unusually large cell with a complex structure and relatively low plasma membrane permeability to water and cryoprotectants (Leibo, 1980). Although the oocytes can be broadly cryopreserved at either immature (germinal vesicle stage, GV or matured (metaphase II) stages, the result of oocyte cryopreservation at these specific maturation stages are controversial.

In principle, the immature stage oocyte is quiescent, the chromatin may be protected within the membrane-bound nucleus (germinal vesicle) and the meiotic spindle, a cryosensitive structure has yet to form. In contrast to the GV oocyte, the MII oocyte appears to have a higher membrane permeability to water and cryoprotectant compared with the GV stage oocyte. Indeed, equine oocytes cryopreserved at both maturation stages suffered from cryoinjuries. For instance, although the cryopreserved-thawed GV stage oocytes resumed and reached MII stage at a reasonable maturation rate of approximately 30% (60% MII for non-frozen controls), many of these MII oocytes demonstrated poor meiotic spindle configuration and developmental competence in terms of fertilisation and blastocyst formation rates (Tharasanit et al., 2006[b]). In 2002, the first two foals derived from vitrified-warmed *in vivo* matured oocytes were produced following transfer to the oviduct of inseminated mares (Maclellan et al., 2002). This at least demonstrated that in optimal conditions, equine oocytes are capable of retaining their developmental competence following cryopreservation. Alternatively the oocyte may be matured, fertilised and cultured in vitro and then the embryos can be non-surgically transferred directly to the recipient mare or stored frozen in liquid nitrogen for subsequent use.

While embryo transfer (ET) is essentially a successful technique (70-80% pregnancy rates), the success of ET is highly dependent on the adequate synchronisation of the donor and recipient mares; good results are obtainable only with recipients that have ovulated -1 to 3 days after the donor (Carnevale et al., 2000). This dramatically increases the cost of equine ET and, thus limits its commercial expansion. Unfortunately, reasonable pregnancy rates after transfer of frozen-thawed equine embryos (~60%) can be obtained only when small (<300 μm) or early development stage embryos (day 6-6.5, morula to early blastocyst) are used (Slade et al., 1985; Hochi et al., 1996); few frozen-thawed large expanded equine blastocysts (>300 μm) give rise to less than 20% pregnancy rate (Slade et al., 1985; Squires et al., 1989). This is challenging in practice since the equine embryo develops and expands very rapidly after entering the uterus as late as day 6 after ovulation. In addition, the exact timing of equine embryonic transport through the oviduct appears to be influenced by the time of year, the age and reproductive status of the mare and the type of semen used (Meadows et al., 1999; Carnevale et al., 2000). The reason for the intolerance of expanded equine blastocysts to cryopreservation is not entirely clear. However, cryopreservation *per se* induces changes at a cellular level that may result in cell death. For example, freezing causes the disruption of actin microfilament (Fig. 2, Tharasanit et al., 2005). Many intensive attempts have been made to improve the survival rate of large expanded blastocyst. These include the thinning of the embryonic capsule by trypsinization, preserving the microfilament configuration and increasing the freezing rate by means of vitrification. (Legrand et al., 1999; Legrand et al., 2000, Tharasanit et al., 2005). To date, the poor cryopreservability of equine embryos has resulted in limitations to the clinical exploitation of equine embryo transfer.

Stem cell technology

Stem cell culture is one of the emerging bio-techniques, in which stem cells are theoretically capable of self-renewal and differentiation into many specific cell types (for a review see Wobus and Boheler 2005). This technique has dramatically developed and integrated to human and veterinary medicine, in regard to the treatment of some incurable diseases such as type I diabetes and Alzheimer's disease. The stem cells can be broadly divided into two types regarding their origins and different biological properties, adult and embryonic stem cells. In general, the embryonic stem cells demonstrate a higher potential to self-renew and differentiate to a wide range of cell types (germ layers), while those adult stem cells seem to have a restricted potential of differentiation. Nevertheless, although equine embryonic stem cells are

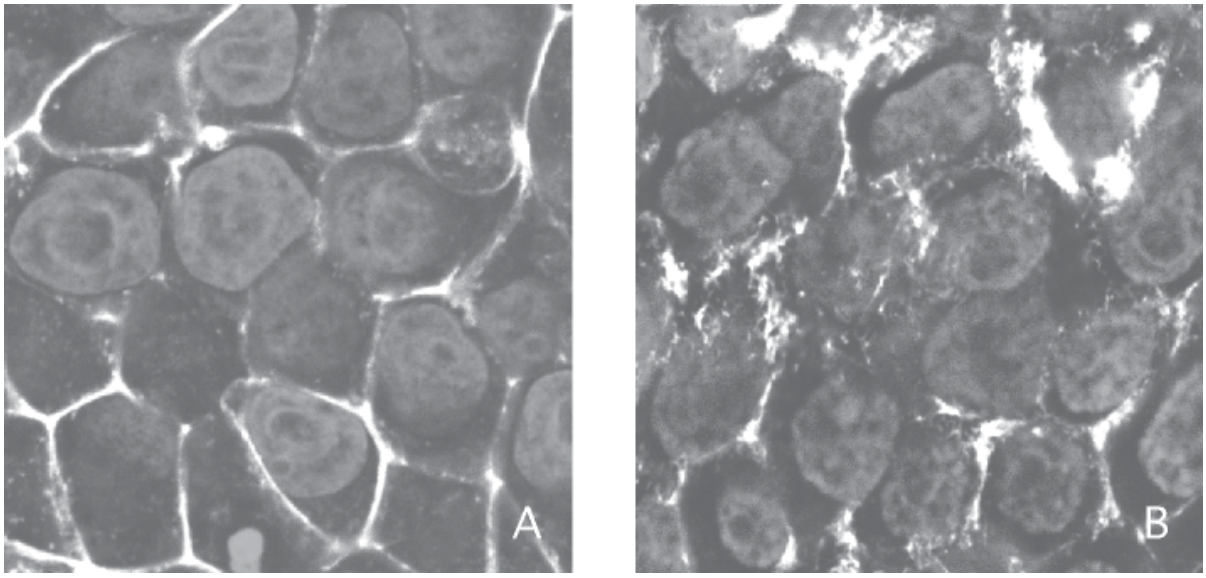

Figure 2 Micrographs demonstrate the effect of cryopreservation on actin microfilament configuration of equine embryos (non-frozen (A) and frozen-thawed (B) embryos). Normal pattern of actin microfilament typifies by an intensely sharp with well-defined actin cytoskeleton (green) beneath cell plasma membrane. The embryos were stained with fluorescently DNA labeling (4' 6' Diamidino-2-phenylindole dihydrochloride; blue) and Alexa Fluor 488-Phalloidin and examined with multiphoton laser scanning microscopy) (Tharasanit, unpublished data)

pluripotent and a few stem cell lines have been produced (Li et al., 2006; Saito et al., 2006), the recovery of only one embryo per non-surgical embryo collection and difficulties of producing *in vitro* equine embryos have markedly contributed to the relatively slow progression of equine embryonic stem cell technology. In contrast, adult stem cells are probably easier to obtain and maybe attract less from ethical or public concern. Currently, the adult stem cells serve as a new tool in equine medicine for treating bone and ligament diseases largely by bone marrow derived mesenchymal stem cells (Taylor et al., 2007). These mesenchymal stem cells have shown the capability of differentiation *in vitro* to adipose tissue, chondrocytes and bone (Vidal et al., 2006) and treating acute tendon injury (Smith and Webbon, 2005). To date, few laboratories can generate equine stem cell lines and the clinical exploitation of these stem cells is very limited. The stem cells however possess a great promise in equine sport medicine especially for racing horses.

Conclusion

To date, assisted reproduction techniques have been intensively used in horses. While moderate success in these techniques has only been achieved, many recent studies have attempted to gain knowledge, aimed specifically to improve efficiency and to overcome some of the unique physiological aspects that have been proposed as restrictive to the development of assisted reproduction in the horses. These include poor freezability of sperm, oocytes and large expanded embryos. Optimisation of the freezing techniques is therefore of importance. In addition, understanding events leading to *in vitro* maturation/ fertilisation of equine oocytes will basically improve the efficiency of *in vitro* embryo production.

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References

Alvarenga, M.A., McCue, P.M., Bruemmer, J., Neves Neto, J.R. and Squire, E.L. 2001. Ovarian superstimulatory response and embryo production in mares treated with equine pituitary extract twice daily. Theriogenology. 56: 879-887.

Bezard, J. 1992. *In vitro* fertilization in the mare. In: Proceeding of the International Scientific Conference on Bioethics in Horse Reproduction, Crakow, Poland (Abs). 12.

Bruck, I., Synnestvedt, B. and Greve, T. 1997. Repeated transvaginal oocyte aspiration in unstimulated and FSH-treated mares. Theriogenology. 47: 1157-l 167.

Carnevale, E.M., Ramirez, R.J., Squires, E.L., Alvarenga, M.A., Vanderwall, D.K. and McCue, P.M. 2000. Factors affecting pregnancy rates and early embryonic death after equine embryo transfer. Theriogenology. 54: 965-979.

Choi, Y.H., Love, L.B., Varner, D.D. and Hinrichs, K. 2004. Factors affecting developmental competence of equine oocytes after intracytoplasmic sperm injection. Reproduction. 127: 187-194.

Fahy, G.M. 1986. The relevance of cryoprotectant "toxicity" to cryobiology. Cryobiology. 23: 1-13.

Galli, C., Crotti, G., Turini, P., Duchi, R., Mari, G., Zavaglia, G., Duchamp, G., Daels, P. and

Lazzari, G. 2002. Frozen-thawed embryos produced by Ovum Pick Up of immature oocytes and ICSI are capable to establish pregnancies in the horse. Theriogenology. 58: 705-708.

Galli, C., Lagutina, I., Crotti, G., Colleoni, S., Turini, P., Ponderato, N., Duchi, R. and Lazzari, G. 2003. Pregnancy: A cloned horse born to its dam twin. Nature. 424: 635

Gastal, E.L., Gastal, M.O., Bergfelt, D.R. and Ginther, O.J. 1997. Role of diameter differences among follicles in selection of a future dominant follicle in mares. Biol Reprod. 57: 1320-1327.

Ginther, O.J. 1979. Reproductive Biology of the Mare: Basic and Applied Aspects, 1st ed, McNaughton and Gunn, Inc. USA. 1-38.

Ginther, O.J. 2000. Selection of the dominant follicle in cattle and horses. Anim. Reprod. Sci. 60: 61-79.

Ginther, O.J., Gastal, E.L., Gastal, M.O. and Beg, M.A. 2004. Critical role of insulin-like growth factor system in follicle selection and dominance in mares. Biol. Reprod. 70: 1374-1379.

Hinrichs, K. 1998. Production of embryos by assisted reproduction in the horse. Theriogenology. 49: 13-21.

Hinrichs, K. and Schmidt, A.L. 2000. Meiotic competence in horse oocytes: interactions among chromatin configuration, follicle size, cumulus morphology, and season. Biol. Reprod. 62: 1402-1408.

Hochi ,S., Kozawa, M., Fujimoto, T., Hondo, E., Yamada, J. and Oguri, N. 1996. *In vitro* maturation and transmission electron microscopic observation of horse oocytes after vitrification. Cryobiology. 33: 300-310.

Legrand, E., Bencharif, D., Battut, I., Taintuier, D. and Bruyas, J.F. 1999. Horse embryo freezing: Influence of thickness of the capsule. Proceeding of 15th Scientific Meeting of the European Embryo Transfer Association. 184-185.

Legrand, E., Krawiecki, J.M., Tainturier, D., Corniére, P., Delajarraud, H. and Bruyas, J.F. 2000. Does the embryonic capsule impede the freezing of equine embryos? Proceeding of the 5th International Symposium on Equine Embryo Transfer. 62-65.

Leibo, S.P. 1980. Water permeability and its activation energy of fertilized and unfertilized mouse ova. J Membr Biol. 53: 179-188.

Li, X., Zhou, S.G., Imreh, M.P., Ahrlund-Richter, L. and Allen, W.R. 2006. Horse embryonic stem cell lines from the proliferation of inner cell mass cells. Stem Cells Dev. 15:523-531.

Maclellan, L.J., Carnevale, E.M., Coutinho da Silva, M.A., Scoggin, C.F., Bruemmer, J.E. and Squires, E.L. 2002. Pregnancies from vitrified equine oocytes collected from super-stimulated and non-stimulated mares. Theriogenology. 58: 911-919.

McCue, P.M. 1996. Superovulation. Vet Clin North Am Equine Pract. 12: 1-11.

Meadows, S.M., Lisa, H. and Welsh, C. 1999. Factors affecting embryo recovery, embryo development and pregnancy rate in a commercial embryo transfer programme. The first meeting of the European equine gamete group, Havemeyer foundation Monograph series 1, Lopuszna, Poland. 61-62.

Morris, L. H., Hunter, R. H. and Allen, W. R. 2000. Hysteroscopic insemination of small numbers of spermatozoa at the uterotubal junction of preovulatory mares. J. Reprod. Fertil. 118: 95-100.

Niswender, K.D., Alvarenga, M.A., McCue, P.M., Hardy, Q.P. and Squires, E.L. 2003. Superovulation in cycling mares using equine follicle stimulating hormone (eFSH). J. Equine Vet. Sci. 23: 497-500.

Palmer, E., Bezard, J., Magistrini, M. and Duchamp, G. 1991. In vitro fertilization in the horse. A retrospective study. J. Reprod. Fertil (Suppl). 44: 375-384.

Rathi, R., Colenbrander, B., Bevers, M.M. and Gadella, B.M. 2001. Evaluation of *in vitro* capacitation of stallion spermatozoa. Biol. Reprod. 65: 462-470.

Saito, S., Sawai, K., Minamihashi, A., Ugai, H., Murata, T., Yokoyama, K.K. 2006. Derivation, maintenance, and induction of the differentiation *in vitro* of equine embryonic stem cells. Methods Mol. Biol. 329: 59-79.

Samper, J.C. 2001. Management and fertility of mares bred with frozen semen Anim. Reprod. Sci. 68: 219-228.

Slade, N.P., Takeda, T., Squires, E.L., Elsden, R.P. and Seidel, G.E. 1985. A new procedure for the cryopreservation of equine embryos. Theriogenology. 24: 45-58.

Smith, R.K., Webbon, P.M. 2005. Harnessing the stem cell for the treatment of tendon injuries: heralding a new dawn? Br. J. Sports Med. 39: 582-4.

Squires, E.L., Seidel, G.E. and McKinnon, A.O. 1989. Transfer of cryopreserved equine embryos to progestin-treated ovariectomized mares. Equine Vet J. (Suppl). 8: 89-91.

Squires, E.L., Brubaker, J.K., McCue, P.M. and Pickett, B.W. 1998. Effect of sperm number and frequency of insemination on fertility of mares inseminated with cooled semen. Theriogenology. 49: 743-749.

Taylor, S.E., Smith, R.K. and Clegg, P.D. 2007. Mesenchymal stem cell therapy in equine musculoskeletal disease: scientific fact or clinical fiction? Equine Vet. J. 39: 172-80.

Tharasanit, T., Colenbrander, B. and Stout, T.A. 2005. Effect of cryopreservation on the cellular integrity of equine embryos. Reproduction. 129: 789-98.

Tharasanit, T., Colenbrander, B., Bevers, M. M. and Stout, T.A. 2006a. Effects of recombinant human follicle stimulating hormone on follicle development and ovulation in the mare. Theriogenology. 65: 1071-1081.

Tharasanit, T., Colleoni, S., Lazzari, G., Colenbrander, B., Galli, C., Stout, T.A. 2006[b]. Effect of cumulus morphology and maturation stage on the cryopreservability of equine oocytes. Reproduction.132: 759-69.

Tremoleda, J.L., Stout, T.A.E, Lagutina, I., Lazzari, G., Bevers, M.M., Colenbrander, B. and Galli, C. 2003. Effects of in vitro production on horse embryo morphology, cytoskeletal characteristics and blastocyst capsule formation Biol Reprod. 69: 1895-1906.

Vanderwall, D.K., Woods, G.L., Roser, J.F., Schlafer, D.H., Sellon, D.C., Tester, D.F., White, K.L. 2006. Equine cloning: applications and outcomes. Reprod Fertil Dev. 18: 91-98.

Vidal, M.A., Kilroy, G.E., Johnson, J.R., Lopez, M.J., Moore, R.M., Gimble, J.M. 2006. Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity. Vet. Surg. 35: 601-610.

Watson, P.F. 2000. The causes of reduced fertility with frozen semen. In: Proceedings of the 14th International Congress on Animal Reproduction. 2-6 July 2000. Stockholm Sweden. 481-492.

Wobus, A.M. and Boheler, K.R. 2005. Embryonic stem cells: prospects for developmental biology and cell therapy. Physiol Rev. 85: 635-78.

Woods, G.L., White, K.L., Vanderwall, D.K., Li, G.P., Aston, K.I., Bunch, T.D., Meerdo, L.N., Pate, B.J. 2003. A mule cloned from fetal cells by nuclear transfer. Science. 301: 1063.

Zhang, J.J., Muzs, L.Z. and Boyle, M.S. 1990. *In vitro* fertilization of horse follicular oocytes matured *in vitro*. Mol. Reprod. Dev. 26: 361-365.