Equine Reproduction Symposium,

January 9-10, 2015

organised by the FRENCH ACADEMY OF AGRICULTURE,

sponsored by FONDS EPERON and by IMV TECHNOLOGIES

Académie d'Agriculture,18 rue de Bellechasse, Paris

FOREWORD

ERIC PALMER, Convener, Chairman of organising committee

Dear friends,

When the French bid to hold the IETS in 2015, the local Chair-person (Pascale Chavatte) asked me to organise an equine reproduction satellite meeting. Unfortunately I was not in a position to refuse anything to Pascale and I took this task as my last contribution to equine theriogenology.

For this I have been helped by an informal committee composed of Pascale Chavatte, Michele Magistrini, Pierre Del Porto and Pierre Julienne. Each of them contributed in their area of expertise.

As I was not in the research field any more, I asked to former participants to ISER meetings and to EQREPRO web forum to indicate what they felt were the hot topics and the authors most active in the fields. I tried to follow their suggestions as much as possible and most contacted authors responded to my mails.

The result of this approach allowed the setting-up of sessions focusing on topics with recent results and/or important for future applied developments. However in the area of male reproduction it seemed difficult to choose limited topics and the session of stallion is more a wide vision of diversity of researches than a focus. A few people may miss their preferred topic from this programme, it is the result of the choice of focused sessions and I bear the responsibility for this choice.

Organising this meeting being out of research institutions was also a challenge to organise without the logistics or the funding of research. However I had the chance of being a member of the French Academy of Agriculture where many members, although being retired from active scientific activity, keep all their enthusiasm for modern development in all aspects of agriculture. I found among my colleagues the support to hold this meeting in this venerable institution. The session about acceptability of ARTs is certainly at the centre of their interest.

I want to thank our two main sponsors who have largely supported this symposium: the Fonds Eperon and IMV Technologies. This meeting would not have been possible without their help.

The French Academy of Agriculture is so close from the internationally well-known Musée d'Orsay that it would have been an offence to culture not to convene you to visit it.

Please enjoy this symposium as an informal friendly meeting.

WELCOME TO THE FRENCH ACADEMY OF AGRICULTURE

The **French Academy of Agriculture**, established in 1761 by the King Louis XV, as the "Société d'Agriculture de la Généralité de Paris (Agricultural Society for the Paris region), became "Académie d'Agriculture de France" (French Academy of Agricuture) by a decree issued on February 23 1915.

It is committed to inform the Government and the public opinion of progresses in all aspects of agricultural sciences, being it at national or international levels, as well as to facilitate exchanges between scientists of various disciplines. Its scope encompasses agriculture, livestock raising, fishing, and sea farming, but also food and industrial products, machinery, environment, rural life…as to their scientific, technical, legal, political and social aspects.

The Academy, whose signature is "Agriculture, Alimentation, Environnement", includes 120 members, 180 corresponding members from France and 60 members and 60 corresponding members coming from 38 countries. The Academy is divided into 10 sections:" plant production systems", "forest and forest-based products", "animal production", "human and social sciences", "interactions environment- living organisms " , "life sciences", "environment and rural territories", food industries", "agro-business and non-food products", "economy and political sciences".

A regular public session meeting is being held every Wednesday afternoon, unless otherwise stated, in the premises of the Academy, 18 rue de Bellechasse, Paris 7ème. In addition, members also participate in the organisation of meetings or symposiums with other institutions or sister academies, such as the Academy of Medicine, the Academy of Sciences, the Academy of Veterinary Sciences etc... Each year the Academy distributes awards, medals, and grants in its spheres of competence.

The Academy's solemn "advices" and other works are published and can be consulted at the secretariate 18 rue de Bellechasse Paris 7ème or by internet on :

www.academie-agriculture.fr

Section 3 : Animal production

Section 3 is interested in all topics related to animal breeding (fishes included) and to uses of animal derived products. Attention is paid to technical and economical developments in every type of production, as well as to scientific trends in fields of nutrition, feed production, reproduction, genome knowledge, emerging pathologies. Breeding systems depend on territorial, environmental and human factors which are shared with other people not directly involved in breeding activities. Some intensive systems are questioned because of their economical failure or their impact on natural resources (water polluted by animal dejections, greenhouse gas emissions, competition between feed and food).Animal selection aims might be modified. New skills should be required. Social acceptance for breeding techniques, animal welfare concern (leading sometimes to decline meat consumption), new sanitary and environmental rules contributed to restricting recommendations at the European level that breeders must take into account. Animal production has to face an adverse environment, at least in countries not concerned by food scarcity. Section 3, composed of members originating from a variety of professional and scientific sectors, aims to diversify dialogue inside and outside the Academy to design a sustainable future and a positive vision for breeding.

THE AAF IETS EQUINE REPRODUCTION SYMPOSIUM
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Objectifs du Fonds Eperon

Conformément à l'accord de partenariat entre l'Etat et l'Institution des Courses de 2004, le Fonds Éperon doit permettre de répondre aux objectifs suivants :

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Time Table

Friday 9th of January

8:30 - 9:00: Registration

9:00 - 9:15 Welcome address (Jeanne Grosclaude and Eric Palmer, France)

9:10-10:30 & 11:00-11:40 The Stallions. (chairman, Ed. Squires)

- 9:10 Sorting Stallion sperm cells for diagnosis and/or for improving fertility (Charles Love, USA)
- 9:30 State of the art of semen freezing (Harald SIEME, Germany)
- 9:50 From INRA96 to INRA Freeze, an analytic conception. (Michèle Magistrini, France)
- 10:10 Social interactions and reproductive efficiency (Dominik Burger, Switzerland)

10:30-11:00: Coffee break

- 11:00 Sonography of the stallion reproductive tract (Heinrich Bollwein, Switzerland)
- 11:20 Sperm quality for ICSI (Cesare Galli, Italy)
- 11:40 General discussion on the stallion

12:00- 13:10: Embryo and genetics (Chairman, Cesare Galli)

- 12:00 Introduction by the chairman
- 12:05 Embryo biopsy and cryopreservation in equine (Florence Guignot, France)
- 12:20 Genetic testing of equine embryos (Youg Ho Choi, USA)
- 12:35 Embryo sexing followed by implantation (Carolina Herrera, Argentina)
- 12:50 Questions & Discussion of the 3 previous papers

13:10 -14:30 : Lunch on your own

14:30 – 16:00 Guided tour of the Musée d'Orsay

16:15-18:00 Social acceptance of equine ARTs (chairman, Pierre Del Porto)

- 16:15 The ethics of horse breeding - are ARTs a particular cause for concern? (Madeleine Campbell, UK)
- 16:35 Breeding up to 300 mares or more by natural service, at what cost? (Twink Allen, UK)
- 16:55 Social acceptance of equine ARTs: situation in South America (Carolina Herrera, Argentina)
- 17:15 Social acceptance of equine ARTs: situation in the USA (Ed. Squires, USA)
- 17:35 acceptability of biotechnologies in the horse industry in Europe (Alline Reis, France)
- 17:50 General discussion on social acceptance of ART in the equine

Time Table (continued)

Saturday 10th of January

9:00 – 10:30 : Programming and epigenetics (chairman, Jean-Paul Renard)

- 9:00 Introduction to DOHAD and its possible link to the metabolic syndrome in the horse (Pascale Chavatte-Palmer, France)
- 9:20 Developmental programming of growth, glucose homeostasis and predisposition to osteochondrosis (Pauline Peugnet, France)
- 9:40 Effect of mare and foal nutrition and on the development orthopaedic diseases (Didier Serteyn, Belgium)
- 10:00 Health of horses issued of ART (ET, Oocyte transfer, ICSI and Cloning) (Katrin Hinrichs, USA)

10:30-11:00 : coffee break

11:00-12:45 Equine stem cells (chairman, Louis-Marie Houdebine)

- 11:00 Characterization of Equine mesenchyme stem cells from umbilical cord, bone marrow and adipose tissue (K de Schauwer, Belgium)
- 11:20 Induced pluripotent stem cells or how to turn horse skin into neurons (Xavier Donadeu UK)
- 11:40 Equine autologous pluripotent stem cells from embryos dericed by somatic cell nuclear transfer (Lawrence Smith, Canada)
- 12:00 Stel cells in equine medicine - thoughts (Stéphane Maddens, France)
- 12:20 Equine MAPC as an allogenic cell therapy product – rEQover ™,(Jef Pinxteren, Belgium)

12:45 – 12:55 : Wrapping up (Eric Palmer)

13:00 Departure to the Gala lunch and trotter races in Vincennes (optional)

(*)Preconference Symposium on Equine Reproduction (Académie d'Agriculture de France, 18 rue de Bellechasse , Paris; close to the "Musée d'Orsay " RER C train station, direct from "Versailles Chateau " RER C , or "Solferino" metro station).

SORTING STALLION SPERM CELLS FOR DIAGNOSIS AND/OR FOR IMPROVING FERTILITY

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The use of gradient centrifugation techniques for sorting stallion sperm has become a common practice in the horse breeding industry. The intent of sorting sperm using this technique is to separate "normal" from "abnormal" sperm, resulting in a purified high quality sperm population. Previous studies have reported an improvement in fertility following the application of the technique (Mari, 2011; Morrell, 2014) yet it is still unclear why this technique may improve fertility. While the appearance of better quality is a pleasing result, gradient centrifugation can result in a low recovery rate due to the retention of significant numbers of normal sperm in the discarded layer. Therefore, the gradient centrifugation process can be inefficient and not specific in its ability to separate normal from abnormal sperm. In addition, it is not clear why a higher quality (i.e. higher percent normal sperm), yet lower total number of normal sperm in the inseminate compared to conventional insemination, would yield higher fertility. It is unlikely that the presence of abnormal sperm have a detrimental effect on normal sperm, but there are other factors that may play a role in improved fertility. Simple centrifugation, without the application of a gradient technique, may provide an improvement in fertility similar to gradient centrifugation due to a reduction in seminal plasma level combined with the insemination of a low semen volume deep in the uterine horn, ipsilateral to the side of impending ovulation. There are many factors that contribute to the efficiency of gradient centrifugation which can be defined as the recovery rate of all the "normal" sperm available in the ejaculate. Initial studies used two layers of gradient media, commonly a 40% top layer over an 80% bottom layer. Due primarily to the increased sperm losses (34 vs. 47%) without an increase in sperm quality, the double-layer was abandoned in favor of the single-layer 80% gradient (Edmond, 2012). One of the challenges of processing a stallion ejaculate is providing adequate gradient surface area to allow all of the normal sperm to have an opportunity to pass through the gradient without being limited by the abnormal sperm that have accumulated at the semen-gradient interface. There are several factors that may affect the efficiency of sperm recovery including the size (15 vs. 50 mL) of the centrifuge tube, the height of the gradient solution in the tube, the number of sperm placed above the gradient, and the centrifugation speed and time of centrifugation. In a previous study comparing 15 and 50 mL conical plastic tubes, sperm quality (% normal and total motility) was not different, but the recovery rate was higher (~42 vs. 33%) in 15 mL compared to 50 mL tubes. The height of the gradient media (i.e., 28, 35, or 41 mm) did not affect sperm quality or recovery rate. The number of sperm placed above the gradient (250-500 x 10^6/ml in a volume of 1, 2, 3, or 4 ml) affected the recovery rate such that the higher sperm numbers (i.e., 4 ml) had lower recovery rates than the other volumes. These results suggest that the total number of sperm loaded should not be above 750 x 10^6 sperm in either of the tube types (i.e., 15 or 50 ml). Since recovery rate was higher in 15 ml plastic tubes, in a subsequent study (Teague, 2012) compared the 15 ml tubes to glass nipple-type tubes which narrow from a diameter similar to the 50 ml tubes to a small nipple in the bottom of the tube. This design facilitates the concentration of sperm in the smaller diameter nipple region so that ejaculates with small sperm numbers can be processed efficiently. The motility was higher in the 15 ml tubes, but the recovery rates were similar between the two tubes. In the same study recovery rates were higher (48 vs. 40%) when the centrifugation speed was slower for a longer time (ie., 200 x g for 30 min vs. 300 x g for 20 min) and recovery rates were also higher (50 vs. 38%) when the sperm number above the gradient was 500 x 10^6 compared to 1.0 x 10^9. There are several brands of gradients available including Equipure (EP), Androcoll (AC), and RediGrad (RG). In a recent study (Sabatini, 2014) compared these three gradients. The EP and RG had higher motility, while the recovery rates were similar. Semen can be centrifuged (i.e., simple centrifugation) prior to density gradient centrifugation or semen can be extended and immediately layered above the gradient. In a recent study (unpublished), there was no difference in sperm quality after 24 hours of cooling regardless of whether it was centrifuged prior to gradient centrifugation. It may be appropriate to precentrifuge semen prior to gradient centrifugation if the ejaculate is a high volume sample because the total volume (i.e., semen+extender) may be too large to efficiently process immediately over a gradient. The pre-centrifugation allows a smaller volume and more concentrated sample to be placed on the gradient, thereby reducing the number of centrifugation

tubes that are needed. The pre-centrifugation technique requires more time since the semen must be initially centrifuged for 20 minutes, transferred to the gradient, then centrifuged for another 30 minutes. In the same study, the amount of seminal plasma added to semen sample post-gradient centrifugation (0, 5, 10, and 20%) was evaluated. After 48 hours of cooled storage the highest sperm motility was in the 0% seminal plasma group and the lowest was in the 20% seminal plasma group, therefore, minimal seminal plasma is needed to maximize the longevity.

There are many factors that may affect the sperm quality and the recovery rate following gradient centrifugation. Based on findings in our laboratory, those factors that improve sperm quality and recovery rate include use of a single-layer instead of a double layer; 15 mL conical tubes; slower centrifugation speed (220 vs. 300 x g) for a longer period of time (30 vs. 20 minutes);. The volume of gradient does not appear to affect sperm quality or recovery rate and therefore use of a smaller gradient volume may reduce the cost of the procedure. The number of sperm layered above the gradient (500 million vs. 1 billion sperm) will decrease the recovery rate probably due to the overload of sperm at the gradient-semen interface.

Other factors that require study include the initial sperm quality of the semen sample. Samples of higher quality will have more sperm that pass through the gradient and therefore will be less likely to clog the interface and prevent normal sperm from passing, however, a semen sample with the same number of sperm but of lesser quality is more likely to result in the retention of larger numbers of normal sperm in the interface due to the retention of high numbers of abnormal sperm that clog the interface.

References

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NOTES :

STATE OF THE ART OF SEMEN FREEZING

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Stallion sperm cryopreservation allows for long distance transport of sperm insemination doses. In addition, it is used for long term storage and maintenance of sperm and gene reserves; e.g. in case of stallions participating in competition, endangered breeds, or prior to castration or removal of a stallion from a stud book in case of illness or death. In most countries, stallions used for semen cryopreservation, are held in supervised quarantine during the entire collection and processing period, while taking international regulations concerning animal breeding and disease control into account. Sperm cryopreservation involves collection of semen, dilution in extenders containing nutrients and protectants, packaging in straws, controlled cooling and freezing, and storage in liquid nitrogen. Frozen samples are thawed prior to use. All these procedures expose sperm to stress, which affects survival and fertility rates. In this presentation, the following factors affecting sperm cryosurvival are discussed:

(i) Inter- and intra-individual variation of semen quality and survival after cryopreservation. Not every stallion fulfills the prerequisites that are needed for successful sperm cryopreservation. Warmblood stallions are considered suitable for participation in semen cryopreservation programs when they produce ejaculates containing more than 200 million sperm per ml, with more than 50% progressively motile and 70% morphologically normal sperm. The causes underlying variation in sperm cryosurvival amongst individuals remain to be elucidated. Membrane composition, fluidity and permeability properties have been suggested to be involved.

(ii) Centrifugation processing for sperm concentration and/or selection and removal of seminal plasma. After collection, semen should be diluted with at least one volume of primary extender of 37°C. Ordinary centrifugation is typically done at 400−600×g for 10 min. Higher speeds for longer duration result in lower sperm losses, but packing of sperm in a dense pellet together with cellular debris can be detrimental. Alternatively, high speed cushioned centrifugation can be employed. Density gradient centrifugation can be used for enrichment of samples with increased numbers of morphologically normal and motile sperm with increased chromatin intactness. Removal of seminal plasma, immature and dead sperm is thought to be beneficial; since these are sources for damaging reactions affecting sperm quality during preservation.

(iii) Composition of the extender that is used, for primary dilution as well as cryopreservation. Home-made extenders can be used (e.g. lactose-EDTA extender, or INRA82) as well as commercial extenders. Extenders typically contain a buffer compound, nutrients, as well as egg yolk and milk. In addition, liposomes composed of defined phospholipids (of non-animal origin) can be used. Freezing extenders also include cryoprotectants. Permeating agents like glycerol, ethylene glycol or dimethyl formamide can be used (at concentrations ~2.5%), as well as membrane impermeable disaccharides such as trehalose, polysaccharides or proteins. Such compounds protect sperm by minimizing exposure to osmotic stress, affecting ice formation, stabilizing biomolecules and cellular structures, and limiting the damaging effects of reactive oxygen species. The type of protectant and/or its concentration may have detrimental effects on fertility success rates.

(v) Cooling and freezing protocols employed. After adding freezing extender, sperm samples need to be slowly cooled from room temperature to 5°C (i.e., at 0.1°C/min). Then, cooled semen can be filled into (0.5 mL) straws at 5°C. Membrane domain reorganizations taking place with cooling at supra-zero temperatures, have been suggested to be less detrimental with slow cooling rates. An optimal cooling rate of 45 to 60ºC/min has been determined for freezing of sperm. The optimal cooling rate is defined as the rate where damage due to intracellular ice formation associated with rapid cooling and osmotic dehydration associated with slow cooling is minimal. Mathematical models exist that can be used to predict the optimal cooling rate. When samples are frozen they can be transferred into liquid nitrogen for long-term storage. Thawing is typically done rapidly, by incubating straws for 30 s at 37ºC.

NOTES :

FROM INRA96® TO INRA FREEZE®, AN ANALYTIC CONCEPTION

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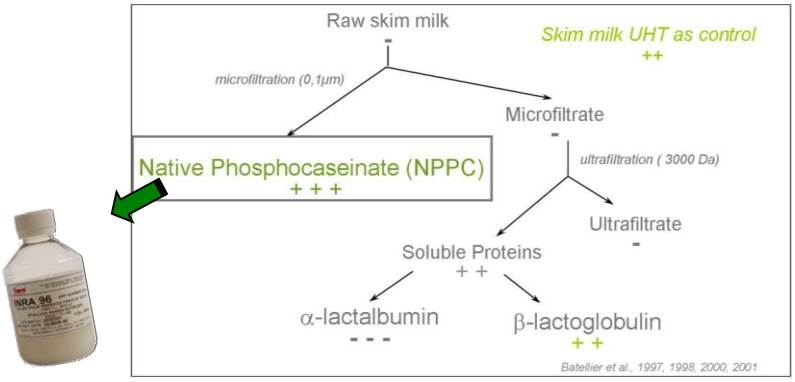
Equine industry needs more and more to improve long-term sperm storage (chilled or frozen) to optimize artificial insemination (AI) and fertility rates and consequently genetic exchanges and biodiversity conservation. In most domestic animal species, sperm extenders are composed of animal products as milk and/or egg yolk (EY). However, these products are potential sources of bacterial contaminations and have a variable composition.

In equine species, milk and egg yolk have been used for years in the composition of extenders. In our laboratory, we decided since 1992 to focus our research on the composition of extenders to improve sperm protection during storage. Our objective was to adapt extenders free of milk and/or egg yolk for both chilled and frozen sperm.

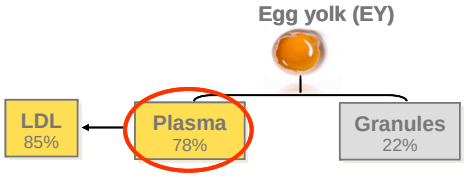
For chilled transported sperm, milk or milk based extenders have been used to dilute and store stallion sperm at 4°C for AI. However, all milk components are not optimal for sperm protection. We tested in our lab milk purified fractions and we demonstrated that one of the milk fractions composed of the native caseins (NPPC) was the most protective (cf. figure below).



Finally we developed and patented an extender named INRA96®, containing the purified fraction of native milk caseins (NPPC), for long-term sperm storage at 4°C or 15°C. INRA96® is a ready to use extender and it can maintain at high rates the fertility potential for up to 24-72 hours. INRA96® has proved itself and lot of breeders use it nowadays in many countries through IMV Technologies commercial network.

Since the first insemination with frozen semen, the low or fluctuating fertility data have limited the use of this technology. To improve the fertility rate of frozen sperm, we focused our research on the composition of the freezing extender. In fact, it seems to be the key factor of the success of cryopreservation. The efficiency of INRA96® extender at 4°C conducted us to test it for freezing. Then our objective was to develop a new freezing extender, easy to use for breeders and able to improve the success of artificial insemination with equine frozen semen.

We first demonstrated that INRA96® extender, supplemented with egg yolk (EY) and glycerol improved significantly the fertility rates of equine frozen sperm compared to our control one (cf. table 1). More sterilized EY-plasma afforded the same protection as EY. EY plasma is the fraction of EY obtained by high speed centrifugations and elimination of EY granules (cf. figure below).



These results lead to the commercialization by IMV Technologies of an extender composed of INRA96, EY plasma and glycerol available sterile, ready to use and called INRA Freeze®.

Our next objective was then to identify the cryoprotective molecule(s) in egg yolk plasma. EY plasma and more precisely Low Density Lipoproteins (LDL), composed mainly of phospholipids, are considered since a long time as cryoprotective agents.

In our analytical approach to develop a new freezing extender, we tested the effect of EY-phospholipids instead of EY or EY-plasma in the extender. Liposomes were chosen as the "vehicle" to transport the phospholipids up to the spermatozoa.

Our results demonstrate that EY-phospholipids can replace EY or EY-plasma as cryoprotective agents in stallion freezing extenders. Liposomes of EY phospholipids are a promising approach to get a chemically defined freezing extender since it is possible to modulate the composition in phospholipids and to sterilize them

Table 1presents the fertility data of the different *in vivo* experiments with frozen sperm conducted on our experimental herd in Nouzilly (Pillet *et al.,* 2008, 2011, 2012).

INRA96® *vs* INRA82 EY+Gly EY+Gly	INRA96® *vs* INRA96® EY+Gly EYplasma+Gly	INRA Freeze® *vs* INRA96® EYPL-Lip + Gly
40 % *vs* 71% (17/42) (30/42)	60 % *vs* 69% (21/35) (24/35)	58% *vs* 54% (15/26) (14/26)

EY : egg yolk ; Gly : glycerol ; EYPL : egg yolk phospholipids; Lip : liposomes

INRA Freeze = INRA96 + EYplasma + glycerol

Our analytical approach of the composition of extenders in the equine species has conducted to adapt 2 new extenders easy and ready to use available by breeders: INRA96® extender for chilled transported sperm, INRA Freeze® extender for frozen sperm

The authors would like to acknowledge the "Equine Reproduction Group" and especially Eric Palmer, Isabelle Couty, Jean-Marie Yvon (INRA, Nouzilly),Marianne Vidament and colleagues from "Les Haras Nationaux", the "Equine facilities" (INRA, UEPAO, Nouzilly), IMV Technologies R&D Department.

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SOCIAL INTERACTIONS AND REPRODUCTIVE EFFICIENCY

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The reproductive efficiency of domesticated horses is often lower than what could be expected from observations in feral herds where stallions typically live with mares in harem bands, with other stallions in bachelor bands, or occasionally in mixed sex transitional bands. Hereby foaling rates of up to 90% are achieved, compared to approximately 75% in well-managed domesticated horse populations. Modern husbandry, breeding procedures and structures of domesticated horses differ greatly from natural conditions. Contrary to almost year-round contact with mares in feral conditions, domesticated stallions are often kept isolated from mares and other horses, with collection for semen carried out using a phantom and artificial vagina or via “in hand” service. This modern breeding industry has resulted in a wide disparity between the sexual behaviour of domesticated horses in comparison with their feral counterparts, where mate choice systems have evolved in natural conditions.

In effect, in most mammal species, mate choice is carried out by females which have to be “choosy” as they usually invest the most concerning reproduction and parental investment and are therefore often the limiting sex. Such seems also to be the case in horses, where pre-copulatory mate choice of females may be based on the good genes model which follows the principal that females will choose males based on certain traits / phenotypes which can be strong indicators of the male’s genotype. This in turn will convey indirect benefits for females such as the optimization of offspring fitness. Selection of a partner may also be based on a complementary mate choice model where individuals should choose heterozygous mates that will minimize inbreeding depression of their offspring. Also male mammals can demonstrate some form of selection via “strategic ejaculation”. Sperm production is costly and therefore adjustments in allocation could depend on post-copulatory selection mechanisms i.e. sperm competition, where males are expected to invest more sperm for a higher quality female. Post-copulatory selection can also occur via “cryptic selection” within the female reproductive tract, perhaps enabling females to avoid inbreeding effects or bias offspring variability.

Such interactions are likely to be influenced by various factors, including the highly polymorphic genes of the major histocompatibility complex (MHC) that have been shown to influence odours and mate preferences in a range of vertebrates. Typically, males and females will avoid MHC-similar mates which may either aim to directly promote MHC heterozygosity or use the diversity on the MHC as a marker to increase overall heterozygosity in offspring, i.e. avoiding inbreeding and/or providing an immunological advantage for the progeny. Such complex selection factors show the importance of the influences of social communication directly impacting reproductive success.

A better understanding of the effects of intra- and intersexual social interactions between stallions and mares on their breeding efficiency provides potentially valuable information, which could be implemented for future optimization of breeding management. We provide here a summary of our present knowledge of female and male reproductive strategies in horses, leading to higher fertility and biodiversity.

NOTES :

SONOGRAPHY OF THE STALLION REPRODUCTIVE TRACT

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Sonography of the reproductive tract is used routinely in breeding soundness evaluation and if a pathologies are suspected in stallions. For a safe examination of the genital tract a proper restraint of the stallions is important. For nervous or intractable animals a twitch or chemical restraint may be necessary. It may be advantageous to scan the genital tract after semen collection, because in these cases stallions will be more relaxed and tolerant compared to examinations carried out before semen collection. Linear array or microconvex transducers with 7.5 to 10 MHz are most suitable. At the beginning of the sonographic examination, the left hand is used to push the right testicle up of the way and extend and stabilize the left testicle to provide firm contact with the transducer. The transducer is held in the right hand and gel is applied to the face. The transducer face then is placed against the lateral surface of the testicle perpendicular to its long axis. Sagittal (long axis) views of the testicular parenchyma can be obtained by moving the transducer over the cranial surface of the testicle directing the beam caudally or by holding the probe on the ventral surface of the testicle with the long axis of the transducer parallel to the long axis of the testicle. Because the epididymis is composed of a single, highly convoluted, fluid-filled duct, it appears to consist of numerous circular, anechoic areas distributed within a soft-tissue background on sonographic images. After examination of the testicles and epididymides, the transducer should be moved up into the inguinal region for examination of the spermatic cord. The cross-section of the spermatic cord has a heterogeneous appearance with distinct anechoic areas surrounded by more hyperechoic regions. This appearance results from numerous cross-sections of the luminae of the testicular artery, the vessels in the pampiniform plexus, and the ductus deferens. By using Doppler mode blood flow can be detected in the testicular artery. Reference values for testicular blood flow are now available for normal stallions, but still only a limited number of stallions with a variety of testicular pathologies have been examined. The entire process of sonography is then repeated with the right testicle. The sonographic appearance of the normal testicular parenchyma is homogeneous, gray, and granula. A small amount (< 5 mm) of anechoic fluid can normally be seen in the vaginal cavity between the visceral and parietal tunicae. The central vein of the testicle is visible as an anechoic line with a diameter of 1 to 4 mm traversing the approximate center of the testicular parenchyma. The volumes of the testicles are determined by measuring their width, height, and length and using the formula of an ellipsoid (testicular volume (mL): 0.5233 x height (cm) x width (cm) x length (cm)). Total testicular volume can be used to predict daily sperm output. If a stallion does not produce enough sperm for calculated testicular volume, then it should be monitored closely and evaluated for possible testicular pathology. The sonographic appearance of orchitis can be highly variable. The testicular parenchyma usually develops a heterogenous or even granular appearance. Echogenicity of testicular tissue may be increased or decreased compared to normal. In cases where focal lesions are imaged, it may be difficult to distinguish orchitis from neoplasia sonographically. Testicular abscesses can often be diagnosed with sonography. A well-defined pocket of purulent fluid is generally visible within the testicular parenchyma. Abscess fluid typically contains large amounts of particulate debris that can sometimes be swirling in real time. In some instances, the fluid may be so echodense that it resembles soft tissue. Acute testicular hematomas appear on sonographic images mottled grayish black to white, depending on the degree of consolidation. If hemorrhage is ongoing, large pockets of relatively hypoechoic, unclotted blood may also be seen swirling in real time. As the hematoma organizes, its sonographic appearance becomes more echogenic. Fibrin tags and adhesions may also form in the affected area. Testicular tumors are uncommon and relatively small in stallions. Although sonography may alert the clinician to the possible existence of a testicular tumor, at this time it is not possible to sonographically differentiate the different tumor types. Definitive diagnosis requires histopathological examination. Testicular degeneration (TD) is a common cause of acquired and often progressive infertility in stallions. It can arise secondarily to a known testicular insult, but more commonly no underlying cause for the degeneration can be identified (idiopathic TD = ITD). The sonographic image of the testicular parenchyma in cases of ITD usually does not differ from that of a normal testicle. ITD could be detected if sonographic measurements of the

testicular volume are performed regularly at least once each year. Testicular cysts are sometimes found in stallions. They do not adversely impact fertility. It can be easy to confuse these cysts with the central vein of the testis. However, Doppler sonography will confirm the diagnosis, as there is no blood flow present within the cyst. Hydrocele can be detected as an increase in volume of the anechoic fluid in the vaginal cavity (depth > 5mm). A hematocele or pyocele can also be visualized sonographically within the scrotum. Unclotted blood appears as heterogenous fluid within the vaginal cavity. Purulent fluid generally has large amounts of particulate debris floating within it. It can be difficult to distinguish it from hematocele. In cases of hematocele and pyocele, fibrin tags may form and may be visible sonographically, as grayish structures floating or waving in the surrounding fluid. Sonography can be a valuable tool for monitoring the progress of affected stallions and is often of prognostic value when used to assess the extent of the pathology. The sonographic appearance of epididymitis varies but, generally, increased frequency and size of hypoechoic regions within the epididymis are seen. These are presumably associated with accumulation of exudate and abscess formation. In cases of spermatic cord torsion in which the cord is compromised, sonographic evaluation of the scrotal contents typically reveals dilated vessels in the spermatic cord/ or testicle and changes in the echogenicity of the cord and the testicular parenchyma. Either an increase or decrease in echogenicity may be seen. Doppler sonography may allow the evaluation of the presence or absence of blood flow to and from the testicle. Examination of the accessory sex glands of the stallions is performed per rectum. The sonographic appearance of sperm-occluded ampullae is highly variable and can be normal. When present, sonographic abnormalities include abnormal amounts of echodense material (inspissated sperm) or echogenic or anechoic fluid within the ampullary lumen. Additionaly, the glandular portion of the ampullae may appear more echodense and may contain hyperechoic spots throughout the parenchyma, probably presenting accumulated sperm. However, a dilated ampullary lumen in and of itself is not enough to make a diagnosis of sperm blockage, because in normal, teased stallions, the lumen of the ampulla can get very large. Seminal vesiculitis is reported uncommonly in stallions. Sonographically, fluid within the affected gland(s) can vary from anechoic to relatively echogenic and often will contain particulate debris or fibrin tags. In cases of chronical seminal vesiculitis, affected glands can become firm and often are almost devoid of fluid. In conclusion, sonography of the genital tract is a valuable tool for examination of breeding soundness in stallions.

NOTES :

SPERM QUALITY FOR ICSI

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Equine assisted reproductive technologies such as Ovum Pick, Intracytoplasmic Sperm Injection (ICSI) and slow freezing of in vitro produced embryos have been developed over the last few years and are becoming an established service offered by a growing number of clinics. The candidates for these technologies are often stallions of low or no field fertility or stallions with limited semen availability because the advantage of ICSI as compared to any other reproductive technology is that only a small quantity of semen, either fresh or frozen, is needed for fertilization of each batch of oocytes collected. Donor mares include both young mares, often at the peak of their sporting activity, or infertile and/or aged females. Since the influence of semen quality on the efficiency of assisted reproduction technologies is well documented in other species, we tried to assess this influence in the horse. For this purpose we compared stallions with different field fertility by doing ICSI of equine oocytes recovered from abattoir ovaries. In the same study we used also pig oocytes for testing the fertilizing ability of the different stallions because these oocytes are easily available in large number allowing to select batches of very high quality as compared to those recovered in limited number from horse ovaries. The objective was to show that both equine and porcine oocytes can be used to test the fertilising ability of stallion semen before using it for OPU-ICSI on valuable donor mares. The frozen semen of 5 stallions with good field fertility (HF) and the semen refrigerated (2) or frozen (3) of 5 stallions with no field fertility (NF) was used in the study with horse oocytes only, while the semen of two stallions (one from each category) was also used with pig oocytes. Horse oocytes were matured in vitro for 24-28 h, pig oocytes for 40-42 h then injected with Percoll separated spermatozoa of HF or NF stallions, selected for motility and immobilized prior to injection using the piezo. A third group of pig oocytes was sham-injected. The horse oocytes were allowed to cleave and develop to blastocyst. After 20-24 h half of the injected pig oocytes were fixed to assess pronuclear formation (both male and female pronucleus developing synchronously), the remaining were cultured for another 24 h to assess cleavage and then fixed to evaluate the presence of nuclei. Seventy-two % of horse oocytes injected with HF stallion cleaved (254/351) and 21% developed to blastocyst while in the NF group 62% cleaved (193/312 and 9% developed to blastocyst. Using pig oocytes injected with the HF stallion we obtained 85% (83/98) fertilization and 75% (53/71) cleavage rate, while with the NF stallion we obtained 25% fertilization and 30% cleavage rate. Sham injected oocytes gave 12% fertilization and 14% cleavage rates.

In conclusion, this study suggests that stallions with low in vivo fertility can be used in vitro for successful fertilization by ICSI but perform less efficiently. Moreover the study shows that in vitro matured pig oocytes can be used as a tool to evaluate the fertilising ability of horse semen both at pronuclear stage formation or by assessing cleavage.

In a second study, performed on OPU donors belonging to our commercial programme, we addressed the effect of the breed on the efficiency of the OPU-ICSI technology in a clinical context. Most of the animals referred to us as OPU donors belong to three breed/category: Arabian, Quarter and Warmblood, therefore only the data from these donors are included in the present abstract and are summarised in Table 1. A total number of 367 OPU-ICSI procedures were performed on 132 donor mares giving rise to 181 transferrable embryos. On average 2.78 OPU/ICSI procedures were performed on each donor mare and the average embryo production was 1.27 per donor and 0.49 per OPU/ICSI. We did not observe large differences between the 3 categories in the number of follicles aspirated and oocytes recovered nor in the percentages of oocytes that reached metaphase

II and were injected. By contrast, cleavage rate and blastocyst rate were significantly lower in Arabian

(44.56% and 2.42%) compared to Quarter (58.11% and 6.44%) and Warmblood donors (62.61% and 7.86%) (Chi square test, p<0.05).

In conclusion these studies demonstrate that also in the horse sperm quality has a significant influence on the efficiency of embryo production by OPU-ICSI. In addition there is a considerable breed effect that overlaps to sperm quality of individual stallions as well as to oocyte quality of individual mares. Finally pig oocytes are suitable for evaluating the fertilising ability of horse semen.

Table 1. Efficiency of OPU-ICSI in different breeds/categories of donor mares and stallions.

Breed/category	n. donors	n. OPU	n. follicles	n. oocytes	% recovery	n. MII injected	% MII	n. cleaved	cleavage rate %	n. embryos	% / oocytes
ARABIAN	56	214	3726	2607	69.97	1526	58.53	680	44.56a	63	2.42a
per OPU			17.41	12.18		7.13		3.18		0.29	
QUARTER	22	43	611	404	66,12	265	65.59	154	58.11b	26	6.44b
per OPU			14.21	9.40		6.16		3.58		0.6	
WARMBLOOD	54	110	1683	1170	69,52	813	69.49	509	62.61b	92	7.86b
per OPU			15.30	10.64		7.39		4.63		0.84	
TOTAL	132	367	6020	4181		2604		1343		181	

NOTES :

EMBRYO BIOPSY AND CRYOPRESERVATION IN EQUINE

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Embryo cryopreservation and transfer is a powerful tool for genetic selection and has extensive economical and clinical application in many species. It is applied for embryos with high genetic value. Moreover, new knowledge about the mammalian genome and significant advances in embryo manipulation allow embryo selection on specific dispositions before transfer: termed preimplantation genetic diagnosis (PGD). Few embryonic cells are required for PGD or just DNA found in blastocoelique fluid. Embryonic cells can be biopsied with a microblade or with a glass pipette attached to a Piezo drill or not. After whole-genome amplification (WGA), several genes or markers can be genotyped. WGA and genotyping take time, and therefore, embryos are preferably cryopreserved before preimplantation genetic selection and transfer in most species.

Unfortunately, in equine species, the embryo cryopreservation remains problematic. Cryopreservation by freezing or vitrification of early equine embryos < 300 µM in diameter has been reported with acceptable pregnancy rates (60-80%), but cryopreservation of expanded blastocysts > 300 µM in diameter resulted in very low pregnancy rates after transfer (0-57%). The presence of an embryonic capsule and the large amount of fluid within the blastocoel could be responsible for cryopreservation failures. In 2011, as previously performed in humans, induced blastocyst collapse allowed successful vitrification in equine embryos >300 µm in diameter. About 70% of the blastocoel fluid was removed by applying suction with a glass pipette. Thereafter, the embryo collapsed and was vitrified. After embryo transfer to the recipient, acceptable pregnancy rates (50 to 70%) were reported.

This new approach is very exciting for equine embryo cryopreservation, especially for expanded blastocysts. Moreover it allows PGD on biopsied cells or on blastocoel fluid DNA with high accuracy (85-100%). Nevertheless, a skilled technician and expensive material are required to perform the biopsy and embryo collapse. The procedure should be simplified before widespread clinical use. An easier process to remove the blastocoel fluid is to put the blastocyst into saccharide solutions: the blastocyst will be quick dehydrated by collapsing itself through osmotic pressure differential.

To conclude, we can say that, at last, equine embryo cryopreservation is an available biotechnology for encapsuled embryos which will facilitate the equine reproduction management. Moreover, actually, in association with PGD, it can allow a widespread development of genetic selection in field and world.

NOTES :

GENETIC TESTING OF EQUINE EMBRYOS

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Hereditary diseases are being increasingly identified in horses and now can be detected on genetic testing. These diseases can be a financial and psychological burden to owners who lose a foal from a lethal mutation, e.g., glycogen branching enzyme deficiency, or raise a horse only to find it unusable, such as in the case of hereditary equine regional dermal asthenia. Most of these diseases are inherited in a recessive manner, because dominant traits can be more easily selected against. For recessive genes, carriers that have one copy of the mutation may not have any symptoms associated with the disorder. Interestingly, in the American Quarter Horse industry, some diseases expressed in a recessive or incompletely dominant manner are actually thought to confer increased competitiveness in the heterozygous state, thus the disease-related alleles are selected for and the prevalence of these alleles is increasing in the horse population. This is associated with an increased risk of a carrier-to-carrier breeding. To ensure that affected (homozygous recessive) foals are not produced, the most logical approach is to perform genetic testing at the pre-implantation embryo stage. Other reasons to perform genetic testing include sex selection, and selection for other measureable traits such as coat color. In humans, embryo testing is done on early cleavage-stage embryos or on blastocysts, and requires production of embryos in vitro. Testing at the early cleavage stage is not a feasible approach in the horse due to the expense and low efficiency of in vitro embryo production. In contrast, the use of blastocysts for biopsy is practical in the field because embryo recovery by uterine flush is a common practice, as used for embryo transfer. The equine embryo is present in the uterus by Day 6 after ovulation, however, flushing embryos at Day 7 or 8 is most commonly done, due to ease of locating the larger embryo and a higher recovery rate. Day 7 or 8 embryos are surrounded by a capsule, a strong acellular glycoprotein coat that could both interfere with the ability to biopsy the embryo, and potentially limit embryo viability if punctured. We conducted a study to evaluate biopsy of both Day 6 early blastocysts and Day 7-8 expanded blastocysts (Choi et al., Reproduction 2010, 140:893). We found that both embryo stages could be biopsied successfully and that the subsequent pregnancy rate after transfer was normal. The cells obtained from the biopsy procedure were evaluated by whole genome amplification followed by PCR; this was accurate in detecting embryonic sex but the accuracy of detecting disease-related alleles (79%) was not high enough to apply clinically, and the allele dropout rate (detection of only one allele at a heterozygous locus) was not tested. Similar results regarding embryo viability after biopsy (pregnancy rate equal to non-biopsied control embryos) have been reported by others (Herrera et al., Theriogenology 2014, 81:758). These authors determined sex on the biopsy samples and reported a 46 to 79% accuracy. To improve efficiency of genetic analysis, we recently evaluated different methods of whole genome amplification in combination with multiplex PCR for analysis of biopsied cells from in vitro-produced and ex vivo-recovered blastocysts, which resulted in > 97% accuracy in interrogation of sex, disease-related, coat-color and identification loci, with < 2 % allele dropout (Choi, Penedo et al., submitted for publication). These findings indicate that equine preimplantation genetic diagnosis can be conducted efficiently without detrimental effects on subsequent pregnancy rates, and may be of value in clinical practice.

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NOTES :

EMBRYO SEXING FOLLOWED BY IMPLANTATION

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Introduction

Preimplantation Genetic Diagnosis (PGD) has already been successfully used to diagnose the sex and certain genetic traits of equine in vivo produced embryos. Different research groups have demonstrated that the pregnancy rates produced by biopsied embryos do not differ from those of intact embryos. Until 2011, this technique had never been applied in a large-scale commercial equine embryo transfer program. Therefore, the aim of our work was to set up embryo gender determination by PGD in a commercial embryo transfer program. Our work was performed between April 2011 and March 2014 in Buenos Aires, Argentina using embryos obtained from artificial insemination of Polo Argentino mares.

Materials and Methods

Experiment 1: The efficiency of PCR to diagnose the sex of the embryo was compared using biopsy pipettes of two different inner diameter and different number of cells in the biopsy sample. Biopsy samples were obtained from in vivo produced embryos by placing the embryos in 50-ul microdroplets of Dulbecco's-PBS supplemented with 10% fetal bovine serum and 50 ug/ml gentamicin under mineral oil, on an inverted microscope equipped with a micromanipulation system. Two different sizes of biopsy pipettes (S: 8-9um or L: 25um) were used to obtain the biopsy samples by aspiration of embryonic cells from the inside of the blastocoele cavity. The number of cells obtained on each biopsy sample was registered. The biopsy samples were frozen at -20°C and shipped to a reference laboratory were the sex of the sample was determined by PCR using specific primers targeting the *AMEL* and *SRY* genes. The results obtained by PCR were compared between biopsy samples obtained using S or L biopsy pipettes and different number of cells in the biopsy sample.

Experiment 2: The sex diagnosed by PCR of biopsied samples was compared to the sex determined by ultrasound at 60 d of gestation and to the sex of the foal.

Biopsy samples from in vivo produced embryos were obtained as described in Experiment 1, except only L biopsy micropipettes were used. All biopsy samples were analyzed at the embryo tranfer center by two consecutive PCRs using specific primers targeting AMEL and SRY. The same cycling protocol and primer concentration were used for both PCRs, except the amplification product from the first PCR was used as a DNA template for the second PCR. After the second PCR the amplification products were analyzed by elecrophoresis in agarose gel, stained with SybrSafe and visualized under blue light. The results obtained by PCR were compared to the sex obtained by ultrasound at 60 d of gestation. Additionally, these results were compared to the sex of the foal.

Results

In Experiment 1 fourty one and thirteen in vivo produced embryos were biopsied using a S or L biopsy micropipette respectively. Eleven out of fourty one biopsy samples obtained using the S biopsy micropipette amplified after PCR (26.8%) while 10/13 amplified when a L biopsy pipette was used (76.9%). Using S or L biopsy pipettes, only biopsy samples containig more than 10 cells amplified after PCR.

In experiment 2, the sex obtained by PCR of 46 biopsied embryos was compared to the results obtained by ultrasound at 60 d of gestation and to the sex of the live foal. Of the 46 foals analyzed, 41 were born female and 5 male. The pregnancies from the 41 females were diagnosed by ultrasound as 40 females and one male and by PCR as 32 females; the other 9 samples did not amplify. The 5 male foals were diagnosed during gestation as 5 males by ultrasound and 4 males and one female by PCR.

Conclusions

Preimplantation genetic diagnosis can be used to diagnose the sex of in vivo produced equine embryos with high efficiency. As demonstrated in Experiment 1, the biopsy sample has to be obtained with a large biopsy micropipette and at least 10 cells have to be removed from the embryo in order to obtain a high diagnostic rate by PCR. In experiment 2, the concurrence of the sex obtained by ultrasound and PCR were compared to the sex of the live foal. Ultrasound allowed the diagnosis of 45 out of 46 pregnancies and only 1 mismatch was obtained when compared to the sex of the live foal. Diagnosis by PCR produced a result in 81% of the samples and 1 mismatch when compared to the corresponding foal. Further work will demonstrate if PGD for embryo gender determination could be improved to produce results with a higher efficiency

NOTES :

THE ETHICS OF HORSE BREEDING - ARE ARTS A PARTICULAR CAUSE FOR CONCERN?

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Introduction

Assisted reproductive techniques (ARTs) inspire particular public interest in veterinary medicine, as in human medicine. In the UK (though not in some other countries) the result of this particular public interest has been that human ARTs are much more tightly regulated than any other field of human medicine. Veterinary ARTs, in contrast, are less strictly regulated, in line with other human and veterinary medical specialities.

This paper aims to answer the question of whether equine ARTs are a particular cause for ethical concern, by considering whether concerns relating to human ARTs apply equally to equine ARTs. I also consider whether there are additional causes for ethical concern which relate to equine ARTs, but not to human ARTs. I shall argue that whilst many of the concerns about human ARTs are not applicable to veterinary medicine, two ethical issues – concern about the effect of ARTs on society, and concern about the effect of ARTs on the health and welfare of future generations – apply equally to human and to veterinary medicine.

However, ARTs also have the potential to improve the welfare of breeding horses. Unfortunately, ethical analysis of whether the potential harms associated with equine ARTs are or are not outweighed by their potential benefits is currently hampered by a lack of basic information about the welfare effects associated with many equine ARTs, ranging from artificial insemination to 'cloning'. By identifying and minimising negative welfare effects, or providing evidence that they are already minimal, we will be able to argue that, although ARTs *are* a cause for special ethical concern, in an ethical analysis the benefits of using them outweigh the harms.

Causes for ethical concern about ARTs

Many of the reasons behind ethical concern about human reproductive medicine are not relevant to veterinary medicine. For example, when IVF was first used in people, there was concern about the psychological impact upon offspring conceived by such techniques, and similar concerns existed around the use of donor sperm and the right of children to identify their father (or not). We can only assume that animals conceived by ARTs do not suffer from such psychological issues. Other arguments, for example that allowing sex selection or permitting pre-implantation genetic diagnosis of disease are the first steps on a slippery slope to allowing 'designer babies'(e.g. [1, 2]), with all the negative eugenic connotations which that entails, again seem irrelevant to animal reproduction. The aim of human-directed animal breeding has always been to deliberately select for desirable characteristics, whether by using ARTs or not.

In relation to human ARTs, there has been significant concern about the need to protect (or respect) the human embryo[3]. Concerns have similarly been raised about embryonic loss / wastage in some equine ARTs. It is illogical to provide embryos with greater protection than we afford the animals which they will become. Those of us who are prepared to kill animals for spurious reasons (for example to eat) cannot therefore make an ethical argument based in moral worth that animal embryos ought to be protected against death (deliberate, or unwanted). However, if we believe that animals generally ought to be protected against unnecessary suffering then we ought also to protect animal embryos against unnecessary suffering. There is currently no evidence that animal embryos are capable of suffering or feeling pain[4] – therefore the high rates of embryonic loss associated with some veterinary ARTs are not a cause for particular ethical concern.

Ethical concerns about the effect of the techniques on health and physical welfare are relevant to veterinary as well as to human ARTs. In equine medicine, there is a paucity of studies on whether animals undergoing ARTs suffer pain/stress. In the absence of such information, it is impossible to know whether ARTs require special ethical consideration because of their welfare effects on the animals undergoing them, and equally impossible to make an ethical argument in favour of ARTs based on their potential to improve welfare (see below).

If ARTs are causing future generations of animals to suffer in the neonatal period, or affecting their life-long health, then we should be giving ARTs particular ethical consideration. Professor Hinrichs will be speaking on the health of horses born from ARTs at the IETS satellite meeting. I shall therefore

confine myself here to commenting that multi-centre long term cohort studies of horses created using such ARTs would inform decision making about use and modification of techniques.

'Cloning' horses has been considered by some to be unethical in the sense that it provides an unfair sporting advantage. There is currently no evidence to support that argument. Anyway, a competition in which all horses were clones of one original elite horse could be considered the ultimately fair test of riders' abilities.

There are legitimate areas of public concern about veterinary ARTs at the interface of veterinary and human medicine, outside the realm in which most of us routinely work. For example, the technical possibility of creating human:animal hybrids/chimeras raises fascinating questions which deserve special consideration because of their direct impact not only on animal welfare but also on human welfare and human moral status[5]. What percentage of a hybrid, for example, would have to be human before one considered it necessary to grant the animal human rights?

Potential for ARTs to improve equine welfare

Set against ethical concerns about equine ARTs are arguments that ARTs are a useful tool in improving equine welfare. For example, use of AI by shipped semen can abolish the need for stallions to 'shuttle' between hemispheres or for mares and foals to travel to stud, thus reducing transport stress, the risk of injury between horses, and exposure to unfamiliar pathogens.

Conclusion

Veterinary ARTs are unique amongst veterinary techniques in their ability to affect both future generations and other species. Furthermore, along with blood donation, they are the only veterinary techniques which are undertaken for reasons other than the benefit of the animal on which they are being performed. For this combination of reasons, ARTs *do* deserve to be given special ethical consideration. However, ARTs also have the ability to improve welfare. If those of us working in equine reproduction wish to be able to ethically justify equine ARTs, we should collaborate to improve the evidence base about both negative and positive welfare effects of ARTs, and to ameliorate negative effects.

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NOTES :

Breeding up to 300 mares or more by natural service, at what cost?

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Nowadays, virtually every breed of horse worldwide other than the Thoroughbred (TB) uses AI to good practical effect and many also accept limited use of ET. The compulsory use of natural mating in the TB clearly supports the vested interests of the relatively small number of stallion owners at the expense and inconvenience of the more numerous, but less pecunious mare owners.

The past 40 years has witnessed dramatic advances in the veterinary management of TB mares which have lifted pregnancy and foaling rates from 65 and 50 percent respectively in the 1960s to >90 and >80 percent respectively today. These include artificial lighting to hasten the onset of the season and prostaglandin, progestagens, GnRH analogues, gonadotrophins and other drugs to induce and control oestrus, follicular growth and ovulation. But perhaps most significant, the introduction of ultrasound scanning by Eric Palmer in 1980 to monitor follicular, embryonic, fetal and placental development. Together these advances have enabled a trebling or more of mare book sizes for popular TB stallions and many horses nowadays cover >140 mares per season, rising to 300 or more per year for those that shuttle between northern and southern hemispheres. Incredibly, the inherent fertility of most TB stallions is high enough to cope with these very large ejaculation loads.

Two examples illustrate the illogicality and unfairness of the present system for mare owners. In 2013, only 8 TB stallions standing in or near Newmarket earned a staggering £40.7M in covering fees, not from the high nomination prices but the fact that they all covered >80 mares in the season; 5 of the 8 covered >130 mares each. In the same season it cost a mare owner with his own private studfarm in the west of England around £3000 in transport, boarding and veterinary fees to send a mare and foal to be covered and spend the first 40-odd days of gestation at the boarding studfarm close to the selected mating stallion in Newmarket in East Anglia, all in addition to the nomination fee.

Looking at ET, two equally outstanding racehorses retired to stud in 2013. FRANKEL, the colt, to cover 159 mares in the UK at a fee of £125,000 per mare and BLACK CAVIAR, the filly in Australia, to conceive her singleton maiden foal with the attendant chances of pregnancy loss or the production of an unraceable foal. A very skewed genetic contribution to the general TB pool and sad that ET could not be employed to produce 3-5 foals per year from BLACK CAVIAR, as is done so successfully with superior polo playing fillies in South America.

Too many foals from too few stallions narrowing the TB gene pool is the principal argument advanced by the TB registration authorities of the world against the introduction of AI. Yet, ironically, it is the stallion owning members of these organisations who have reaped the benefits of the aforementioned veterinary advances of recent years to increase the book sizes of their horses. And, in the Standardbred industry which recently introduced a successful voluntary limit of 140 mares per stallion per year to be inseminated artificially, it has been demonstrated convincingly that overproduction of foals by a single, sought after stallion soon drops the demand for, and price of, his over populous young stock.

Clearly, it is time for mare owners to unite and reconsider the domination of their breeding industry by the stallion owners. Widening the choice of stallions is only one of the many benefits the introduction of AI could bring to them.

NOTES :

SOCIAL ACCEPTANCE OF EQUINE ARTs: SITUATION IN SOUTH AMERICA

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The application of Assisted Reproductive Techniques (ARTs) to the equine industry has seen a great progress during the last decade. The ARTs which are currently being used in the equine around the world are: embryo transfer, intracytoplasmic sperm injection (ICSI), nuclear transfer, oocyte transfer, preimplantation genetic diagnosis (PGD), sexed semen and embryo cryopreservation. Horse breeders from different southamerican countries have demonstrated a recent and increasing interest in applying these techniques to produce foals.

Embryo Transfer

Brazil and Argentina have recently reported an embryo transfer activity of more than 31,135 equine embryos obtained from 44,273 uterine flushes (IETS 2013 Statistics and Data Retrieval Comitee Report), which represent more than half of the world equine embryo transfer production. This technique is also used in Chile, Colombia and Uruguay.

ICSI

Brazil and Argentina are the only southamerican countries that have produced results using this technique. The first three foals produced by ICSI in South America were born in Argentina, in October 2011 at Doña Pilar Embriones, as a result of a collaborative work between Dr. Fernando Riera, Dr. Young-Ho Choi and Dr. Katrin Hinrichs. These foals were produced after ICSI of oocytes obtained from slaughterhouse ovaries. One more team from Argentina has also produced three foals by ICSI in September to November 2012 from oocytes obtained from slaughterhouse ovaries and a Polo Argentino stallion (Herrera et al 2012, abstract). In October 2012, In Vitro Brasil produced the first foal from an embryo produced by ICSI in Brazil. This foal was produced from oocytes recovered from a Quarter Horse dead mare. Since then, they have produced one more live foal and there is an ongoing pregnancy (unpublished data). At least two companies (one in Brazil and one in Argentina) are setting up this technique to be used in equine clinical programs.

Nuclear Transfer

Cloned foals from different breeds have already been produced in South America, in Argentina and Brazil. The first cloned foal in South America was born in Argentina and was produced from a Polo Argentino mare in 2008, but this foal only lived for 16 hours (Miragaya et al 2010, abstract). The first viable foal in South America was also born in Argentina in August 2010 and was the clone of a Criollo stallion (Gambini et al 2012). In Brazil, the first cloned foal was born in 2012 (In Vitro Brasil, unpublished data). In the last four years, more than 20 viable foals have been produced by four different groups, three from Argentina (Clonarg, Crest View Genetics and Kheiron) and one from Brazil (In Vitro Brasil).

Oocyte Transfer

Since 2010, this technique has been applied clinically at Doña Pilar Embriones in Argentina. More than 50 foals have been born by oocyte transfer using oocytes from Polo Argentino mares (Dr. Fernando Riera, personal data). In 2010 another group from Argentina produced one foal by oocyte transfer from an oocyte obtained from a Polo Argentino Mare (Alonso et al 2010).

Preimplantation Genetic Diagnosis

This technology has been mainly been used in South America to diagnose the sex of in vivo produced embryos. Different teams from Argentina, Brazil and Colombia have produced results as part of research projects or within commercial programs.

Sexed Semen

Artificial insemination using sexed semen has been used by one company in Argentina for four consecutive breeding seasons, although the results produced by this group have never been published or revealed to the public.

Conclusions

Assisted Reproductive Techniques in the equine have been widely accepted by breeders and professionals of the equine industry in South America. This is demonstrated by the results produced using all the available techniques, not only as part of research proJects but also in commercial programs.

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SOCIAL ACCEPTANCE OF EQUINE ARTS - USA PERSPECTIVE

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The acceptance of assisted reproductive techniques (ART) in the USA is largely dependent upon the breed. Although most breeds have accepted artificial insemination, except the Thoroughbred industry, the acceptance of the other ART has varied among breeds. All the major breeds experienced a marked decline in foal registrations from 2006 to 2013. For example, in 2006, the AQHA registered 165,000 foals. The drop in foal registrations for the major breeds from 2006 to 2013 was 55, 66, 44, 53, 61, and 60%, respectively, for AQHA, Paints, Thoroughbreds, Arabians, Appaloosa and Pinto breeds. In addition, the stock market crash of 2008 resulted in a major drop in sale prices in all breeds, which was a reflection of too much supply and too little demand. Some owners were quick to state that the increase in supply was a direct result of technologies such as AI, shipped semen and embryo transfer. However, it should be noted that the same decline in price was noted in the Thoroughbred industry, a breed that does not use any of these technologies. The overproduction was from the less desirable mares and stallions and was a result of irresponsible breeding. Nearly 75% of the AQHA mares are bred to stallions that breed less than 24 mares/year, indicating that these stallions are of lesser quality with smaller books. The American Quarter Horse Association (AQHA) is the largest breed in the USA, accounting for nearly 60% of all foals registrations (75,000 in 2013). This paper will primarily focus on the AQHA breed since they have been the most libel in accepting ART and the most information on ART is available.

Within the USA, there are two different approaches that have occurred with regard to acceptance of ART by the horse industry. The Thoroughbreds has accepted no ART, whereas the AQHA and several other breeds have accepted all ART except cloning. Some would say that AQHA is on a ”slippery slope of technology” with no end in sight. In a recent survey by AQHA, the members voiced that one of the top five challenges to the breed was “advanced breeding technology”. The tolerance for ART varies among breeders with some in favor of all ART (except cloning), whereas others would prefer that some technologies such as shipped semen, frozen semen and embryo transfer not be allowed.

The history of acceptance of the various technologies by AQHA is as follows: AI with fresh semen on the premise (1960s); embryo transfer on the premise (1979); cooled shipped semen (1997); Frozen semen (2001); oocyte collection and transfer (2002), multiple embryos (2003); and frozen embryos (2007).

Although most breeders have embraced cooled semen and feel that it has helped them expand their business, others feel that it has hurt the “small breeder” and that it increased the popular stallion syndrome. One of the arguments for frozen semen was to allow shipment of semen internationally. This has in fact been a major part of certain segments of AQHA such as the reining and cutting horse. In fact, international registration has been one area of growth for AQHA. There is, however, a portion of the owners that would like to prevent the use of frozen semen after the stallion dies to “allow the young stallion a chance”. In reality, only a small percentage of semen is used each year after the stallion dies (0.3% in AQHA), and the longer the stallion is dead the less his semen is used.

The unlimited foal registrations from embryo transfer (ET) donor mares in AQHA was a result of a lawsuit to allow more than one foal per mare to be registered. Multiple embryo transfers have been accused of overpopulating the breed and depressing prices. However, the data from AQHA shows that most people using ET only produce 1-2 foals from a given mare/year. In 2013, only 5.4% of the foals registered in AQHA came from ET. It is also felt by some breeders in AQHA and other breeds that the quality of the foal from ET is less than that of a mare producing her own foal. The data particularly in race horses would not support this notion. Surprisingly few embryos are frozen and the demand from the breeder is limited. Other ART that is used sparingly in the AQHA is oocyte freezing, sexed semen, sexed embryos and oocyte collection and transfer.

One technique that has been used more in the past few years is OPU/ICSI. This is being used as a treatment for infertility in mares that have failed in an ET program or for subfertile stallions or stallions that have frozen semen but that have died. This is primarily being used in the cutting horse and racehorse segment of the industry, where the value of the mares and stallions are high. Recently, this technique was made more practical by the ability to ship immature oocytes from small follicles to ICSI

stations. If successful, the in vitro produced embryos were shipped back to the OPU facilities, either as fresh embryos or frozen embryos, and then transferred on the farm non-surgical.
In summary, it is unlikely that the Thoroughbred will accept ART and it is equally unlikely that breeds that have accepted ART will reverse their decisions. It is also likely that breeds will be more cautious about accepting new ART as it become available and that only time will tell whether ART has been good for the horse industry or not.

NOTES :

ACCEPTABILITY OF BIOTECHNOLOGIES IN THE HORSE INDUSTRY IN EUROPE

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Europe is one of the most important suppliers of horses of high economic value at the international level. It is the fruit of a successful horse industry based on the exploitation of the different abilities of horses for work, meat and more recently sports. The development and global expansion of this industry is intrinsically dependent on innovative processes generating and formalizing concepts on genetics and reproductive management for further application in the field. In the domain of reproduction these concepts resulted in different biotechnologies: Artificial Insemination (AI), Embryo Transfer (ET), Intracytoplasmic Sperm Injection (ICSI) and cloning. This participation is aimed to discuss the current acceptability of biotechnologies of reproduction in Europe.

The discussion is structured around the concept of technology life cycle and pathway of development. For the mature or maturing technologies (AI and ET) a global profile of acceptability was drafted. Afterwards, we investigated the saddle breeds markets of three breeding countries: Germany, France and Spain in order to understand if national realities resulted in pathways leading to different standard of exploitation or organisations. This second phase is based on published data (Brade, 2013; MAGRAMA, 2013, Ecus, 2013) and interviews with key actors. Because of the low number of foals born from the more recent technologies (ICSI and cloning), the low technical efficiency and the current attraction of first movers (pioneers), they were classified as immature technologies. They will be discussed only at the European level because of the low level of development and insufficient numbers to build a discussion at the national levels.

Mature technologies (AI and ET):

In the 90's, AI progressively gained popularity in Europe after a long period of public research and a period of market restriction in some countries and studbooks. Currently, all the countries of the European Union count on trained professionals and reproduction centres authorized by the governments to develop activities of semen collection and storage and to perform artificial insemination. Nowadays AI is largely accepted by studbooks and represents a powerful commercial tool allowing Europe to maintain its leading position on the international genetic market through a better valorisation of males. In addition, the sanitary safety is another important advantage of AI for professionals and governments. It is very difficult to obtain statistics of AI in whole Europe because of differences on the national communication systems. However, we observe that the development of AI is not similar in all the European countries. In the example of France, Germany and Spain, we observe three different profiles of adoption of AI. Indeed, in Germany, 90.31% of the broodmares are inseminated and a clear preference for the AI with Fresh / Cooled semen can be observed (AI frozen semen represents only 2.8% of the AI activity). In France only 55.5% of the broodmares are inseminated. Frozen semen represents 52% of the AI activity. 19% of the broodmares are still bred with natural service. The trends observed for France and Germany are relatively stable in the last years. We did not find Spanish AI data. However, the combination of official data and interview with actors allows us to identify a recent development of AI in Spain (more intensive in the 2000's) with a preference for fresh semen AI. However, the natural and on hand services seems to be still dominant in Spain. These differences on adoption of AI in different countries can be explained by national environments including the research environment, technical capacity of local workers, semen quality of the local breeds, culture and market strategy and the logistic systems available.

Embryo Transfer is a maturing technology: available in Europe since the 90's but commercialisation intensified in the beginning of the 2000's. It currently presents an advanced technical knowledge and virtual possibility of utilisation by a large part of breeders. However, ET represents only a marginal activity in Europe. The information collected from the IETS reports, combined with personal communications led us to estimate that the global ET activity in Europe must represent less than 2000 embryos transferred/year. France is the leader of commercial ET in Europe, followed by Germany. Spain officially produces and transfers only a few embryos/year (official ET in 2013 = 22) despite a recent progression on the national implementation of ET. The stimulating factors identified for adoption of ET are: medical indications and valorisation of the female athletes. The barriers are the costs (recipient and donor mares), low technical results and studbook regulations (ex.: authorisations needed – Arabian; exigencies concerning the breed of the recipient mare and limitation on the number of embryos/mare/year – Spanish Pure Bred).

Immature technologies (ICSI and cloning):

These technologies are not present in the official data in Europe. We did not obtain any information about the number of ICSI foals produced per year in European countries. The main barriers for its development are the high costs associated to the low technical efficiency and number of embryos needed to produce one foal. For cloning, based on our previous experience (Reis, 2013) we estimate that the current European demand represents 2 to 5 clones / year, mainly distributed in The Netherlands,

Belgium, Denmark, France and England. We also identified one cloning company based in France and one in Italy. The barriers for the adoption of cloning are the high costs, low technical efficiency, lack of field results (linked to the low numbers and the recent introduction), low public support and lack of prescribers. Despite the low acceptation of cloning in Europe, three European studbooks recognised the interest of this technology to save their very best genetic and authorised the registration of cloned foals and offspring in their books. In addition, FEI recently recognised that cloning does not impact fair-play and authorised the participation of clones and their offspring in official competitions. Interestingly, all the biotechnologies suffered from low acceptation in the beginning of the commercial development.

From these first observations we can affirm that Europe leading position is strongly linked to the development of biotechnologies. The design of a technology (what it is intended for) is not the only factor influencing the adoption of the biotechnologies in the field. Adapted logistic systems, expertise for both female and male management and gametes treatment, social acceptation, support from governments and studbooks are determinant for the success of a biotechnology. The impairing factors include: costs of storage of genetic material and female management, low technical efficiency, lack of adapted information, work organisation. In addition, the recent phenomenon of knowledge concentration in few research centres impairs the transfer of recent technologies to the field and subsequent development. The actors of the European horse industry should open the dialogue in order to improve knowledge about the recent technologies, to establish rules for a rational exploitation instead of ban and to open new ways of development for future innovation. This dialogue would be important to determine the adapted pathways of development of technologies for each country or breed and to keep Europe within the leaders of the reproduction and genetic market.

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NOTES :

Introduction to DOHaD and its Possible Link to the Metabolic Syndrome in the Horse

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About 25 years ago, epidemiological studies described an increased risk of non-communicable metabolic diseases in people born Small for Gestational Age (SGA) and/or with a poor growth rate in infancy[1,2]. It was hypothesized that fetal adaptation to adverse environment in early life lead to what was qualified as a "thrifty phenotype" after birth, which could be particularly challenged in the presence of post-natal plethoric nutrition[3]. It has been suggested that the fetus would indeed develop an "adaptive predictive response", i.e. adapt its metabolism in anticipation of post-natal conditions based on an integrated assessment of maternal conditions[4]. Nowadays, it is generally admitted that early developmental conditions play an important role in the subsequent development of obesity, type 2 diabetes (T2D) and hypertension, as stated in the "Developmental Origins of Health and Disease" (DOHaD) hypothesis (http://www.mrc-leu.soton.ac.uk/dohad/). It has also been shown that any disturbance in the maternal environment leading to abnormal placental function and fetal nutrition may lead to an increased risk of developing metabolic disease as an adult. Epidemiological and experimental reports suggest that epigenetic mechanisms link those early life events to health later in life, with epigenetic marks being considered as long-lasting environmental cues.

In equids, placentation occurs over the entire surface of the uterus. Thus, the nutrient supply to the fetus depends directly on the uterine capacity, which in turn is determined by the mare's size. Based on this observation, Allen *et al* used embryo transfer between breeds of different sizes (ponies and Thoroughbreds) to evidence the developmental origins of glucose homeostasis in equids. Indeed, overgrown ponies (born after transfer of pony embryos into Thoroughbred recipients) showed enhanced insulin secretion in response to exogenous glucose in the early neonatal period (2 days of age)[5,6]. This demonstrated for the first time that an increased nutritional supply to the equine fetus throughout pregnancy has the capacity for inducing insulin sensitivity abnormalities in the newborn. Broodmares, however, are often fed high energy rations so as to maximize fetal growth and this could affect the β cells function. Using obese mares *versus* mares of moderate body condition, Ousey *et al* confirmed that nutritional programming of the foal's sensitivity to glucose depends on the dam's nutritional intake. Indeed, foals born to the "moderate" mares had higher insulin secretion in response to exogenous glucose shortly after birth (3 days of age) compared to the foals born to the obese mares[7]. Subsequently, further studies evidenced some effects of nutritional manipulations at longer term on the foal's post-natal energy homeostasis, using diets with high starch contents[8], high energy levels[9] or the use of concentrated feed supplements[10] in late-pregnancy. Recent work from our lab show, however, that growth and endocrine status do not seem to be affected, at least until weaning[11].

Glucose and insulin sensitivity dysfunctions have been related to a range of pathologies of the adult horse, including laminitis and obesity amongst others. Johnson hypothesized twelve years ago that insulin resistance, laminitis and obesity are part of one same clinical syndrome in horses and ponies and he introduced the concept of the "Equine Metabolic Syndrome" (EMS)[12]. According to the ACVIM consensus statement, the EMS phenotype includes insulin resistance characterized by hyperinsulinemia or abnormal glycemic and insulinemic responses during oral or IV glucose tolerance/insulin sensitivity tests, predisposition to laminitis and local fat accumulation or general obesity. Additional features can be observed such as hypertriglyceridemia, hyperleptinemia, arterial hypertension, alterations of the mare's reproductive cycle and increased concentrations of inflammation markers associated with obesity.

There is growing concern regarding these pathologies which prevalence increases in the equine population. Depending on the study and the breed, laminitis and obesity prevalence can go up to 35%[13] and 45%[14], respectively. Both diseases have heavy consequences for the equine athlete and for the equine industry since they cause partial or total cessation of their careers in equestrian sports. Indeed, laminitis stands behind 15% of lameness cases and can lead to anesthesia, whereas obesity, amongst other effects, is susceptible to alter the broodmares reproductive efficiency.

Knowing the role of the developmental origins in the programming of the metabolic syndrome in humans, predisposition to EMS could also start during development (*in utero* and in the early post-natal period). As a consequence, adequate management of the pregnant and then lactating mare could be determinant in the future predisposition of the offspring to this metabolic syndrome.

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Developmental Programming of Growth, Glucose Homeostasis and Predisposition to Osteochondrosis in Foals

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Background & objective - Fetal adaptations to intra-uterine *stimuli* have immediate and long term consequences on the offspring's health after birth. In equids, this concept of DOHaD (Developmental Origins of Health and Disease) was evidenced using between-breed embryo transfer: the mare's size, *i.e.*, the maternal environment throughout gestation and lactation, was shown to determine the foal's post-natal growth[1,2], as well as the neonate's insulin secretion in response to exogenous glucose[3]. Osteochondrosis (OC), an osteoarticular pathology of the growing horse, has been related to abnormalities in glucose homeostasis[4]. Its antenatal origin is highly suspected[5]. Using between-breed embryo transfer, the present research aimed to evaluate the impact of the maternal environment on fetal and post-natal growth, glucose homeostasis and predisposition to OC of the foal from birth until age 18 months (or 540 days).

Materials & methods - Fetal growth was enhanced or restricted through embryo transfer using pony (P), saddlebred (S) and draft (D) horses. Control pregnancies of P-P (n=21) and S-S (n=28) were obtained by artificial insemination. Enhanced and restricted pregnancies were obtained by transferring P (P-D, n=6) and S embryos (S-D, n=8) into D mares or S embryos into P mares (S-P, n=6), respectively. From birth, control foals were raised by their dams, whereas experimental foals were raised by their recipient mares. All foals were weaned at age 180 days.

- ***Placenta -*** Placentas were collected at delivery. Gross morphology and expression of candidate genes involved in placental growth, vascularization and nutrient transport were investigated.
- ***Foals' growth -*** Body measurements were recorded from birth to age 540 days. Plasma IGF-1, T_4 and T_3 were assayed at age 3, 90, 180, 360 and 540 days.
- ***Foals' glucose homeostasis -*** Fasting glycemia and plasma NEFA concentrations were measured at regular intervals from age 3 to 540 days. Glucose clearance and insulin secretion were assessed with intravenous glucose tolerance tests (IVGTT) at age 3 and 360 days. Insulin sensitivity was measured with hyperinsulinemic euglycemic clamps at age 200 and 540 days.
- ***Foals' osteoarticular status -*** Radiographic examination of the fetlocks, hocks and stifles was performed at age 200 and 540 days.

Results - Two main experimental models were obtained. Fetal overgrowth was induced in ponies (P-D group), whereas intrauterine growth restriction was induced in saddlebreds (S-P group). Interestingly, the S-D group did not differ from the S-S control group.

- **"Enhanced" ponies (P-D group) -** P-D pregnancies were shortened by 6 days and P-D foals were born with a 57% increased birth weight and 16% increased withers' height compared to P-P controls. P-D placentas were heavier and larger as well, without any differential gene expression compared to P-P controls. P-D foals exhibited an overall harmoniously amplified growth of all body measurements: they were still 30% heavier and 9% higher than P-P controls at age 540 days. They also had lower plasma T_3 concentrations than P-P controls from age 3 to 180 days. P-D foals were hypoglycemic version of P-P controls with consistently higher plasma NEFA concentrations from age 30 to 540 days. IVGTT demonstrated increased insulin secretion in response to exogenous glucose in 3-day-old P-D foals compared to P-P controls whereas both pony groups had similar sensitivity to insulin thereafter. Two P-D foals and one P-P foal were found to be OC-positive at age 200 days, but only one P-D foal was still affected at age 540 days. This did not represent a significant increase of the relative risk to develop OC.
- **"Restricted" saddlebreds (S-P group) -** S-P pregnancies were lengthened by 13 to 16 days and S-P foals were born with a 37% reduced birth weight and 12% reduced withers' height compared to the S-S or S-D groups. S-P placentas were lighter and smaller as well, with two genes (the IGF-II- and SNAT2-encoding genes) having their expression down-regulated compared to the S-S or S-D groups. Growth in terms of body weight, shoulder width and hip width was slowed down before weaning and those body measurements demonstrated catch-up growth thereafter in S-P foals. No catch-up growth occurred, however, in terms of wither's height, chest circumference, front leg length and cannon bone width. As a consequence, S-P foals can be said to exhibit slowed-down pre-weaning growth and then disharmonious post-weaning catch-up growth. This was accompanied by higher plasma T_3 concentrations than the S-S or S-D groups. S-P foals were hyperglycemic version of S-S controls from age 30 to 540 days with lower pre-weaning plasma NEFA concentrations. IVGTT demonstrated reduced insulin secretion in response to exogenous glucose in 3-day-old S-P foals compared to S-D foals, whereas increased sensitivity to insulin was evidenced in S-P foals by clamps at age 200 days. All saddlebred

groups had similar sensitivity to insulin at age 540 days. All S-P foals were OC-positive at age 200 days *versus* only six foals in the S-S group and two foals in the S-D group. Thus, the relative risk to develop OC was significantly increased in S-P foals at age 200 days. At age 540 days, however, most OC lesions had disappeared and the relative risk was not significantly increased anymore.

Conclusion - This work confirms the concept of the developmental origins of growth and glucose homeostasis abnormalities in foals from birth to age 18 months[1]. These are important data for embryo transfer practitioners. Indeed, special care should be taken when selecting a recipient mare, in particular with regard to the donor mare features: its size and milk yield, as well as its metabolic status (*e.g.*, pony mares have higher fasting glycemia than saddlebred ones) should match that of the embryo donor mare. Even if foals seem to suffer more from the intra-uterine and early post-natal restriction, the recipient mare should not be too large, especially when stud-book registration includes criteria on withers' height. Moreover, all effects of the maternal environment described above may become apparent with ageing. Experiments in other species also indicate that multigenerational effects may occur[6]. Data relative to the glucose metabolism regulation are also important in light of the increasing prevalence of the equine metabolic syndrome, obesity or laminitis in the equine population[7,8]. In conclusion, *in utero* development can affect the incidence of pathologies in the equine, which underlines the importance of broodmare's management. Indeed, a few studies already highlighted the crucial role of the late-pregnant mare diet's composition on the post-natal metabolic regulation of the foal[5,9,10].

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EFFECT OF MARE AND FOAL NUTRITION ON THE DEVELOPMENTAL ORTHOPAEDIC DISEASES

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Osteochondrosis (OC) is a developmental disease of growing horses. It is defined as a disturbance in the process of endochondral ossification which can lead to the formation of detached fragments (osteochondrosis dissecans, fissures, or subchondral bone cysts at the epiphyseal growth cartilage). OC is a frequent cause of pain, lameness and reduced performance in young athletic horses. The disease appears to be multifactorial in origin, including skeletal growth rates, nutrition, endocrinological factors, exercise, biomechanics, and genetic effects.

Based on a digital gene expression analysis, our group analyzed the transcript profile of leukocytes from horses affected with OC using the high throughput sequencing method digital gene expression analysis. Metabolic pathway analysis showed an obvious dysregulation of several signaling pathways related to cartilage formation and cartilage repair such as Wnt/β-catenin, Indian hedgehog- and TGF-β signaling pathways. Other genes regulated may play a role in high carbohydrate diet, abnormal insulin metabolism or inflammation. Furthermore, the transcript profile of leukocytes demonstrated that OC-related genes are differentially expressed in horses of different ages when compared to their age-matched controls and are differently expressed according the affect joints in young foals (<12 months). High plasma concentrations of sclerostin, an inhibitor of the WNT signaling pathway, in young horses affected by OC was recently observed.

The aim of the present work was to evaluate the effect of mare and foal nutrition on the prevalence and the evolution of OC.

The effect of mare nutrition was studied in a previous work to establish relationship between breeding management and OC. Breeding conditions were recorded, and a radiological examination was performed in 223 foals (1). Feeding practice and housing management were analyzed in a multivariate model to determine risk factors for OC. We observed a significant relationship between OC development and the maternal nutrition during gestation. It appears that mares fed with concentrates during gestation are more likely to produce foals that are subsequently affected by OC compared with other mares ($P<0.05$).

The effect of nutrition after weaning was studied on 204 foals followed from 6 to 18 months of age to investigate the evolution of OC lesions. Foals without OC lesion at 6 months fed with concentrates show a higher trend to develop OC in comparison to foals fed without concentrates ($p=0.06$). Foals with OC lesions at 6 months fed without concentrates had higher probabilities of healing OC lesions and becoming negative at 18 months compared to the foals fed with concentrates ($p < 0.001$).

These results confirm that nutrition conditions influence the incidence of osteochondrosis and the evolution of the disease between 6 to 18 months.

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NOTES :

HEALTH OF HORSES PRODUCED BY ART (EMBRYO TRANSFER, OOCYTE TRANSFER, ICSI AND CLONING)

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Foal production by embryo transfer has been a common clinical procedure since the 1980's, with production of foals via oocyte transfer starting in the 1990's. These assisted reproduction technologies (ARTs) have been joined in the last decade by production of foals via intracytoplasmic sperm injection (ICSI) and nuclear transfer (cloning). Interestingly, there is little information about the effects of these technologies on the physical or mental health of foals, and subsequent adult horses, produced. Effects of embryo transfer are of prime interest, as all of these technologies include transfer of the oocyte or embryo to a recipient mare, and thus effects of embryo transfer are superimposed on any potential effects of the other technologies used.

It has been shown that the size of the recipient mare affects the final adult height and long-bone proportions of the offspring. Recent work from England and France has also established that offspring insulin sensitivity, blood glucose levels, thyroid hormone levels and weight gain are affected by transfer of embryos into "rich" or "poor" environments (uteri of mares larger or smaller than the mare producing the embryo). There is little information on the effect of the recipient mare on behavior of the foals/horses produced; in one brief report, foal "learning capacity" was significantly affected by recipient mare.

There is essentially no information available on health of foals produced by oocyte transfer. This technique involves recovery of oocytes from the dominant preovulatory follicle after gonadotropin stimulation, thus manipulation of the oocyte during meiosis, which has the possibility of disrupting the metaphase spindle and compromising chromosomal integrity; however, this technique has been used clinically since the late 1990s and no adverse effects on offspring are known to have been reported.

Similarly, there is essentially no information available on the health of foals produced by ICSI. There are two major techniques for clinical utilization of ICSI: 1) Recovery of the oocyte from the dominant stimulated follicle, ICSI, and in vitro embryo culture for 1-2 days followed by surgical transfer of cleaved embryos to the oviduct, and 2) Recovery of immature oocytes from small follicles, followed by in vitro oocyte maturation, ICSI, culture to the blastocyst stage, and transcervical transfer to the uterus. The latter technique involves recovery of oocytes from follicles of variable health and maturity, maturation of oocytes in vitro, and extended embryo culture, all of which could more critically affect epigenetic status of the resulting embryos. Both techniques involve in vitro embryo culture to varying degrees, which has been shown in other species to affect health of offspring produced -- to the point of being lethal (large offspring syndrome). In other species, the proportion and type of serum used in embryo culture medium appears to be related to the occurrence of this syndrome. Fortunately, the in vitro manipulations associated with ICSI appear to have no immediately obvious effect on health of the foals produced. Both of the given ICSI techniques have been employed clinically to produce hundreds of foals in the last decade, and again, while no studies have been reported on foal health, no systematic adverse outcomes appear to be observed. On communication, horse owners report they are satisfied with the foals/horses produced (E. Carnevale, personal communication 2014; K. Hinrichs, unpublished data).

One report is available on the health of foals produced by nuclear transfer, from our laboratory. Of 14 live-born cloned foals, six foals were clinically normal. The most common abnormalities detected in the remaining 8 foals included maladjustment, enlarged umbilical remnant, and angular deformity of the forelimbs. Two foals died within 7 days after parturition; in the remaining 6 foals, these conditions resolved with medical or, in the case of enlarged umbilical remnant, surgical management. Large offspring syndrome and gross abnormalities of the fetal membranes were not detected. Personal communication with personnel of other laboratories that are performing equine cloning indicate that similar problems with cloned foals are encountered, and that the proportion of foals with problems, and of foal death, may be higher in some laboratories. Communication with owners of horses produced by NT in our laboratory indicate that the cloned horses are normally fertile, and may perform to the level of the original horse if trained, e.g. in 2013, one clone of a Polo mare, produced using oocytes from slaughterhouse tissue, competed successfully at the Argentine Open, among the top Polo competitions in the world. Since in our laboratory NT is performed using the same oocyte recovery, in vitro maturation,

basic micromanipulation, and embryo culture techniques as for our ICSI-produced embryos, the problems seen in NT foals at birth appear to be related to the cloning procedure itself rather than to the culture system. In our work, we evaluate different methods for donor cell synchronization, oocyte activation, and treatments for aiding chromatin reprogramming, and improvement of these techniques may be related to the birth of a higher proportion of normally-healthy cloned foals in our most recent studies.

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NOTES :

CHARACTERIZATION OF EQUINE MESENCHYMAL STROMAL CELLS FROM NON-INVASIVE SOURCES

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Mesenchymal stromal cells (MSC) have been extensively studied for their promising capabilities in regenerative medicine. In general, most cell-based therapies consist of the use of autologous MSC. Nevertheless, considering their immunosuppressive capacities, MSC seem perfectly suited for cellular therapy in allogeneic settings. An allogeneic source would provide an off-the-shelf, more standardized and readily available product without the inherent lag period associated with isolation and expansion of autologous MSC. Although bone marrow is the best known source for isolating equine MSC, alternative sources such as umbilical cord blood (UCB), umbilical cord matrix (UCM) and peripheral blood (PB) have been reported also.

Equine MSC were isolated from 6 mares (PB) and their foals (UCB & UCM) at parturition. Following parameters were analyzed: (i) success rate of isolation, (ii) proliferation capacity, (iii) tri-lineage differentiation ability, (iv) immunophenotypical protein and (v) immunomodulatory mRNA profiles. Linear regression models were fit to determine the association between the source of MSC (UCB, UCM, PB) and (i) the moment of first observation, (ii) the moment of first passage, (iii) cell proliferation data, (iv) the expression of markers related to cell immunogenicity, and (v) the mRNA profile of immunomodulatory factors.

While equine MSC could be isolated from all the UCB and PB samples, isolation from UCM was only successful in 2 samples. Proliferation data showed that equine MSC from all three sources could be easily expanded, although UCB-derived MSC appeared significantly faster in culture than PB- or UCM-derived MSC. Equine MSC from both UCB and PB could be differentiated towards the osteo-, chondro- and adipogenic lineage, in contrast to UCM-derived MSC where only chondro- and adipogenic differentiation could be confirmed. Regardless of the source, equine MSC expressed the immunomodulatory genes CD40, CD80, HGF and TGF-β. In contrast, no mRNA expression was found for CD86, IDO and TNFα.

Our data strengthen recent findings that inherent differences exist between MSC from different tissues, as suggested for human MSC. Combining all the observations in this present study, we propose UCB as the most promising non-invasive alternative source for MSC and UCM as the least feasible source due to high contamination risks. Moreover, our data indicate that UCB-derived MSC could be suited for allogeneic use, although their immunogenicity potential needs to be addressed in more detail in future studies

These data have been published in:
De Schauwer et al. Stem Cell Research & Therapy 2014, 5:6

NOTES :

INDUCED PLURIPOTENT STEM CELLS OR HOW TO TURN HORSE SKIN INTO NEURONS

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Interest in equine stem cells has boomed over the past decade, and mesenchymal stem/stromal cells (MSCs) are now being used extensively for orthopaedic regenerative medicine. Important limitations associated with the clinical use of MSCs include the very small numbers of stem cells that can be naturally harvested from adult tissues, the difficulties associated with the expansion and maintenance of these cells *in vitro*, and the multipotent rather than pluripotent nature of MSCs, i.e. the fact that, by definition, they can give rise to mesenchymal but no other tissue lineages.

In contrast to multipotent cells, pluripotent stem cells are intrinsically able to self-renew indefinitely, thus can be robustly grown in culture, and can give rise to virtually all cell types in the body, features which confer them with distinct advantages in relation to regenerative medicine applications. However, to this date the robust isolation of pluripotent stem cells from its natural source, the embryo, has only been achieved from a very limited number of species. A historic milestone was reported in 2006 with the successful *in vitro* reprogramming of adult mouse fibroblasts into cells equivalent to embryonic pluripotent stem cells, called induced pluripotent stem cells (iPSCs), by inducing the expression of four transcription factors that are naturally associated with pluripotency (Oct4, Sox2, Klf4 and c-Myc). The same cell reprogramming approach was later used to generate iPSCs from humans and other species including, in 2011, the horse. By definition, iPSCs can be generated from a simple tissue biopsy and can be used to produce virtually all cell types in the body in a patient-specific manner, thus offering unprecedented potential for biomedicine. Many functional cell types have now been derived successfully from human iPSCs, and several disease models based on these cells are being used experimentally. Most remarkably, human clinical trials using iPSCs, for the treatment of age-related macular degeneration, are already underway.

After humans, the horse is probably the species for which iPSC technology holds most promise. To this date, equine iPSCs have been successfully derived from both fetal and adult tissue sources, and from two different cell types, fibroblasts and keratinocytes. In a recent study we showed that equine keratinocytes could be reprogrammed to pluripotency with relatively higher efficiency than fibroblasts. Moreover, keratinocyte-derived iPSCs had remarkably high developmental plasticity compared to fibroblast-derived iPSCs, as indicated by their ability to produce a diverse range of differentiated tissues *in vivo* as well as trophectoderm-like tissues *in vitro*, suggesting that keratinocytes may provide a potentially superior source for reprogramming in the horse.

Generation of equine iPSCs has in most studies involved the use of viral vectors that become naturally integrated in the reprogrammed cell's genome. Producing clinically safe iPSCs from horses will require the use of virus-and integration-free strategies, similar to those more recently used to generate human iPSCs, as well as the establishment of defined, xeno-free culture conditions. Significant progress in that regard has been made recently by our group and others with the successful generation of equine iPSCs that can be robustly grown in the presence of Leukaemia Inhibitory Factor without the need to use serum or feeder cells.

A further challenge will be developing robust and safe protocols for the specific and efficient differentiation of equine iPSCs into clinically relevant cell types. We recently made progress on that front by successfully generating cholinergic motor-neurons *in vitro*, the first functional cell type to be derived from pluripotent stem cells in the horse, providing initial proof-of-concept of the biomedical potential of these cells.

The ability to generate cell types virtually *a la carte* using iPSCs provides enormous potential for understanding developmental and disease processes in the horse, for example, through in vitro modelling of cell-autonomous disorders caused by known, high penetrant mutations such as hyperkalemic periodic paralysis disease or exertional rhabdomyolysis. Moreover, equine iPSCs are most promising at present as a potential clinical alternative to MSCs. Numerous studies have reported

the derivation of mesenchymal precursors from human iPSCs and shown their therapeutic potential in animal models. In this regard, equine iPSCs or their derivatives could provide an off-the-shelf source of readily available cells with well-characterised therapeutic properties for veterinary regenerative medicine. In addition, given existing evidence showing low immunogenicity of these cells after transplantation into horses, iPSCs may open the way to the allogeneic use of cell therapies in the horse, in turn leading to more effective treatments

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EQUINE AUTOLOGOUS PLURIPOTENT STEM CELLS FROM EMBRYOS DERIVED BY SOMATIC CELL NUCLEAR TRANSFER

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Mesenchymal stem cells (MSC) of bone marrow and adipose tissue origin are being used therapeutically in horses for orthopedic and non-orthopedic injuries (De Schauwer *et al.* 2013). Nonetheless, poor long-term proliferative capacity and the limited potential for differentiation of MSCs have instigated the search for equine pluripotent stem cell lines with unlimited differentiation and propagation properties. Equine embryonic stem (ES)-like cells derived from the inner-cell-mass (ICM) of *in vivo* embryos have been shown to express a full range of pluripotency markers and can differentiate into several cell types li (Li *et al.* 2006; Paris & Stout 2010), which pertain exclusively to allogeneic applications. Somatic cell nuclear transfer used initially to derive mouse ES lines has more recently been successfully used to obtain stable ES cell lines in humans (Wakayama *et al.* 2001; Tachibana *et al.* 2013). Our group has attempted to establish protocols for producing autologous ES-like lines to alleviate immune rejection after grafting by using *in vitro*-derived parthenogenic (PA) and nuclear transfer (NT)-derived embryos, (Desmarais *et al.* 2011). We showed that primary parthenogenic (paES) and nuclear transfer (ntES)-derived lines express not only the ICM markers NANOG, OCT4 and SOX2 but also the trophectoderm (TE) stem cell markers CDX2 and EOMES. Moreover, NANOG expression tends to be lost from most ES-like lines after a few passages, suggesting a tendency to differentiate after prolonged *in vitro* culture. Interestingly, immunohitochemical analysis of PA and NT whole embryos showed OCT4 and GATA6 expression in ICM and TE, indicating that the expression of pluripotency genes might be dysregulated in *in vitro*-derived embryos. Our next step was to derive induced pluripotent stem (iPS) cells from fetal fibroblast (FF) using a *piggyBac* transposon-based method to deliver transgenes containing the reprogramming factors cMyc, Klf4, Oct4 and Sox2 expressed under doxycycline control (Nagy *et al.* 2011). Equine iPS lines express hallmark pluripotency markers, display stable karyotype and form teratomas containing all three germ layer derived layers when grafted into immunocompromised mice. The immune potential of iPS lines was further examined *in vitro* and *in vivo* by intradermic grafting into allogeneic hosts (Aguiar *et al.* 2014). Although iPS cells weakly express MHC molecules, skin sections of grafted regions revealed CD4+ and CD8+ mononuclear cells 30 days post-transplantation, indicating that allogeneic iPS cells elicited moderate cellular response. Due to impairments in deriving autologous iPS from adult horses, we derived NT embryos using skin fibroblast from aged horses that were then transferred to recipient mares to obtain FF for autologous iPS cell line derivation (ntFF-iPS). Moreover, to shorten the procedure and avoid the ethical dilemma of fetal miscarriage, we used NT embryos to derive early passage ntES cells that were then employed for induced reprogramming (ntES-iPS). Stable iPS cell lines with improved pluripotency marker expression were rapidly and efficiently obtained by this secondary reprogramming of ntES, thereby opening novel avenues for a rapid and effective derivation of pluripotent stem cells for autologous cell therapies.

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NOTES :

STEM CELLS IN EQUINE MEDICINE - Thoughts

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Mesenchymal stromal cell (MSC) therapy, is not as yet but is becoming a fully accepted practice in equine veterinary medicine. For the moment it may be seen by some as more of a sticking plaster. The lack of definitive proof of efficacy could have led to the approach being abandoned if it was not for a continuous flow of new more verifiable discoveries, be it a novel source of stem cells, a new protein secreted by MSCs or a different indication.

Three reasons however, could have consigned this promising medical tool to a historical dustbin. **1-** Firstly, conventional treatments have largely failed for common injuries and compelled vets and owners to rush into innovative medicine in order to optimize the return on their investment.**2-** Secondly, there has been a lack of statutory regulation, allowing nearly any Tom Dick or Harry to produce "stem cells" in uncontrolled environments and then proceed to sell them as such with unverified claims that exaggerate the customers' expectations. **3-** Thirdly, there is a prohibitive cost of clinical trials to test efficiency and to compare it to other treatments. As a consequence this has limited investment by large societies into this niche market.

Vetbiobank is a 4-year old biotech company based in France at the vet school in Lyon. We provide MSC-based products to equine surgeons for orthopedic treatments (joint inflammation, bone cysts, meniscus damage, desmitis, and tendonitis). Our focus is based on allogeneic neonatal stem cells whose youthfulness confers a biological advantage over adult MSCs, along with reduced immunogenicity.

Discussion around MSC in the veterinary field has been confusing and very dogmatic. Views are often expressed such as "autologous is good, allogeneic is bad". As usual science is not so black and white. For the purpose of this presentation, I will highlight three points currently at the forefront of debate: **1-** Many scientists are interested in understanding how MSCs 'work by studying the MSC "secretome" (the complex array of proteins and other biological substances secreted by MSC). The "old fashioned" notion that cell-differentiation of autologous MSCs is the principle therapeutic action is being replaced by alternative mechanisms since less than <1% of total MSC are actually differentiating *in vivo*. **2-** Autologous doesn't necessarily mean safe if MSCs from bone marrow or fat, are used without setting up the basic legal safety nets employed in the human clinic (i.e. checking the raw material and/or the final product for virus, parasites and bacteria plus evaluating the products *in vivo* efficiency). Bacterial contamination is of particular concern since they may lie dormant in tissue samples obtained from healthy carriers.

3- Furthermore, autologous stem cells expanded in a laboratory are modified both quantitatively and qualitatively and the addition of any *ex vivo* biomaterial in the process changes their status to that of a drug.

Allogeneic, ready to use MSCs are being used more and more for diverse human conditions. Many clinical trials under evaluation are using umbilical cord derived cells. They have three significant advantages which justify their further use and development **1-** During storage all tests can be performed to assess safety and efficiency. **2-** They can be used within 48hrs with or without a short culture period, avoiding a significant waiting period of 2-3 weeks to obtain expanded autologous MSC. **3-** They do not need a surgical procedure to collect the raw biological material from which the MSC will be prepared.

Despite the cost, Vetbiobank is currently conducting in partnership with both academic and private veterinary surgeons a clinical trial evaluating allogeneic MSC injection for fetlock arthrosis. Setting up a randomised, double blind, multi-centric and cross over study has been a challenge in itself. However, we

have ensured the quality of data collected by vigorously homogenizing protocols. We will able to present shortly, a synopsis of our study.

In summary, MSC therapy is an exciting development in animal health management. The scientific data supporting its increasing use are encouraging and clinical development is well established for humans. It should be given every chance.

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Equine MAPC as an allogeneic cell therapy product - rEQover™

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Independent scientific studies illustrate the positive effect of mesenchymal stem cell treatment on the healing capacity of injured tendon tissue. The autologous nature of current cell therapies however, is accompanied by several drawbacks. ReGenesys has successfully developed MultiStem®, an allogeneic, FDA approved adherent stem cell product derived from bone marrow, which is currently being used in Phase II clinical trials for the treatment of ulcerative colitis, ischemic stroke and acute myocardial infarct (AMI) in humans and in Phase I or preclinical studies for a range of other indications. Based on our knowledge about the human MultiStem product, the specific goal of this project is to develop a similar product for horses to treat one of the most common disorders in this species, tendon and ligament injuries.

With the rEQover™ product ReGenesys aims to counteract most of the limitations, autologous mesenchymal stem cell therapies are currently faced with. rEQover™ cells are a subpopulation of equine mesenchymal stem cells based on MAPC (Multipotent Adult Progenitor Cells) isolation and expansion protocols, in analogy with our human MultiStem cells. MAPC are bone-marrow derived, non-hematopoietic, adherent cells that were first described by the Verfaillie group. Currently, rEQover™ cells can be discriminated from MSC by their size, superior proliferation capacity and different expression profile (e.g. IL-8). MAPC are immune-privileged, so they can be used as an allogeneic product. Combined with a capacity to undergo extensive in vitro expansion doublings, these properties enable off-the-shelf use making it immediately available when a horse is diagnosed with a tendon injury. As a result taking a bone marrow aspirate will no longer be necessary and perhaps even more important the time between injury and treatment can be significantly reduced. An indispensable part of the development and manufacturing of an advanced cell therapy product is quality control. In line with, and based on our knowledge regarding the human MultiStem product, we will develop a high level QC pipeline for rEQover™. Screening for growth, marker expression, immunosuppression, angiogenic potential and multipotent differentiation will lead to a comprehensive characterization of all cell batches thereby ensuring identity, quality and safety of the cells. Making use of a hollow fiber expansion system we will be able to enter into relative large scale production resulting in a significant reduction of cost as compared to standing prices for stem cell therapy. Because of its extensive proliferation capacity, about a million fold more rEQover™ cells can be generated from one bone marrow aspirate as compared to MSC, meaning a theoretical 5 million rEQover™ doses.

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