16 COLLOQUE SCIENTIFIQUE

16th SCIENTIFIC MEETING

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Doctor Robert CASSOU

Special Celebration

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Santander 08th and 09th September 2000

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Doctor Robert CASSOU

Pioneer Award 2000

Robert CASSOU

Doctor Robert E. CASSOU is well known to all of us, from his generation, from our generation and also and this is quite noticeable, from the younger generations. All, who have worked one single day on artificial insemination or embryo transfer from whatever species, have come across the word or some materials named by or after CASSOU. So it is no surprise to us, if the Board of the A.E.T.E. has decided to honour Robert E. CASSOU in giving him this pioneer award.

The CASSOU family name per se, comes from the South West part of France, in the middle of the Pyrennees, close to the Spanish boarder, so it does seem quite appropriate that this award should be given to Robert CASSOU as AETE meets here in Santander (Spain), from France, on the other side of the Pyrennees. The genetics of those people at the foot of the mountains are generally strongly marked with strong will (but no stubbornness), perseverance and cleverness. I guess those characteristics fit pretty well to Robert CASSOU's mind and attitude that he had the opportunity to show to us all for over half of a century.

Robert CASSOU's life is extraordinary and in a way is a good example of a success story; a book would be necessary to develop all facets of both his personal and professional life. Be reassured, I will not write here a book neither read it. I will try very briefly to excerpt some of the highlights of Robert CASSOU's life.

Although his family name had its roots in the South West part of France, Robert CASSOU was not born there but in a close suburb of PARIS, a little village that at that time was at full country side, with a landscape of grazing areas, pastures and woods by the name of Clamart at 12 kms South of Paris. This was in 1913. It is true to say that it has quite changed since then, although the "petit bois de Clamart", dear to the heart of one of the most famous contemporary French poet singer Georges Brassens who celebrates it in one of his famous songs, has obviously still some charms. Robert CASSOU's parents were posted there as "instituteurs", professors at the primary school, in the lineage of Jules Ferry's concept of public and free education for all at the end of last century. There is no doubt that this family environment has had a profound influence on Robert CASSOU's all life. In fact, he did start his professional career as a "instituteur" as well, just like his parents, in 1934. He got married that same year and had two sons, Maurice and Bertrand, both of them worked very closely to their father later and did all their respective career behind the authority of their father Robert, in a very complementary manner to his.

There was a first major event in Robert CASSOU's life, in 1942. He was asked to join the "National School of sheep breeding" at Rambouillet, not too far from the parisian suburbs where he was originating from but yet already deep in the animal world - Rambouillet the world heart of the sheep industry with the merinos that were brought in under the reign of Louis XVI, King of France (the same king, later to be beheaded as we all know!) and who had such a big influence worldwide on breeding of this species. There, Robert CASSOU met one of his colleagues by the name of G. LAPLAUD, Professor at Rambouillet also. R. CASSOU found with G. LAPLAUD his master, who convinced him of all the interest there would be for the animal industry, particularly that of ruminants and pigs, of being able of having some kind of control on the animal reproduction and particularly via Artificial Insemination. Then on, Robert CASSOU constantly invented, tested, tried, searched, in brief, put all his intelligence and manual creativity to try to bring the artificial insemination in which he believed then as much as his master, to the farmers in the most efficient way.

There, again, one would need a full book to accurately report all the things, including some which could be qualified of "surprising", that Robert CASSOU got involved in. In fact, one first

characteristics of his approach was **very modern** and was that of what we could call, in our contemporary jargon, of multidisciplinary. He tried to invent things very practical that were felt as a constraint in the whole process of producing semen and delivering it to the farmers which led him to produce close to 100 patents. This includes dummy bulls, holding of bulls with a herdsman on horseback, use of dogs to manage the bulls in lay off, and of course the famous CASSOU straws with its progressive reduction in size up to the most **famous mini-straw** that he developed, all the diluents that have been produced by CASSOU and colleagues, A.I. guns and so many other items. The straw adventure was one of the most remarkable, and it is interesting to look back and admire Robert CASSOU's approach. From what he had learned from the Russian school of IVANOV and MILOVANOV, to how constantly he challenged himself to render his straw concept as efficient as possible. This was adapted to many species including of course the humans for whom he and collaborators created the high safety concept of such mini - containers (CRYO BIO STRAWS – CBS -) particularly adapted to sero-banking or banking of strains of dangerous pathogens. He always participated to the forefront of the research thanks to his good and friendly connections with the scientists in Cambridge (UK) or in Jouy-en-Josas (F). He was then involved in the first insemination of fresh semen in cattle in 1946, in the first inseminations of frozen semen and in the first international transport of semen, in this case from France to Madagascar in the Southern Hemisphere. His research did not only deal with ruminants but also very much with poultry and swine. He later, in 1979 passed a thesis at the University of Caen summing up all his findings during a third of century period of research.

He also always encouraged meetings of colleagues involved in this industry both nationally and internationally and has been since the beginning a strong supporter of the then International congresses of Artificial Insemination (now know as ICAR) and actively participated to those of London, Copenhagen, The Hague, Trento, Paris etc…

By the early 1970's, he was alerted by the new and progressing technologies of transferring embryos from one donor animal to a recipient. He immediately understood all the interest of this second biotechnology of Reproduction and set a convention with the director general of INRA to participate to this new adventure and bring his own capacity in developing this technology. This again mainly dealt with practical issues yet unsatisfactorily resolved and particularly those of the straws again and of the catheters for non-surgical collection or for cervical transfer.

Scientifically and mostly technically, he hence had a major impact of the Reproductive biotechnologies in the world during this last half of the 20th century.

But another major facet of Robert CASSOU was his constant involvement in management and care of people. Parallel to his technical inventions, he first understood all the benefits of this technology could bring to the farmers provided that the service was adequately provided. He founded the first Artificial Insemination Cooperatives of farmers, first in La Loupe (close to Chartres) at the boarder of the Beauce and Normandy regions, which later was moved deeper in the heart of the cattle breeding area in L'AIGLE. He remained several decades at the head of this Cooperative. However he was not imprisoned in one way of thinking, The Cooperatives system was – and the facts have proved that he was right- the most economically effective mean of giving the service of AI to the farmers but this was probably not the most effective way to produce equipment necessary to this industry. He then created a commercial business called Instruments de Medecine Veterinaire (IMV) which happened to be more than a success industrially. Very rapidly, this company developed and was able to sell products for AI and later for ET all around the world. Some of us remember visiting AI centers in all parts of the world, in the old and new world, in developed and developing countries under such or such political regime and yet see IMV boxes of equipment used on the bench to produce millions and millions of doses of semen. More than 120 countries were at a time recorded as clients of IMV. It is a worldwide known company that contributed in a decisive manner to the use of this technology.

Indeed, Robert CASSOU displayed a considerable number of qualities in the scientific, animal breeding and industrial world in the forefront of the technologies involved. Hence, it is more than

appropriate that the Board of Governors of the AETE has recognized his merits and offers him this pioneer award.

I am more than honored on behalf of the AETE members to here deliver this award to Robert CASSOU.

By Professor Michel THIBIER

ALLOCUTION du Docteur Robert CASSOU
A.E.T.E. Pioneer Award 2000

Monsieur le Président, Mesdames, Messieurs, Chers collègues,

Vous m'avez invité à Santander aujourd'hui pour me remettre la distinction suprême que l'Association Européenne de transfert embryonnaire décerne habituellement à un scientifique.
Dans mon cas, c'est plutôt un artisan ayant rendu service à la science que vous voulez honorer. J'en suis très heureux et très fier. Je vous en remercie sincèrement.

Vous soulignez de ce fait une réalité que vos illustres prédécesseurs exprimaient chacun à leur façon.

- «La synthèse du savoir empirique et des acquisitions scientifiques»
  (Jay LUSH - IOWA USA(1937)
- «Il n'y a pas de sciences appliquées mais des applications de la science» (PASTEUR)
- «La mécanique au service de la Biologie» (Etienne LETARD) (7) lorsqu'il venait admirer nos progrès dans le labo de Rambouillet entre 1942 et 1945.

Avec ma très longue expérience d'innovateur, d'inventeur et d'industriel, je vais tâcher de ne pas vous décevoir, et de provoquer de nouvelles vocations.

On a dit et même écrit que j'étais atteint de la boulimie de l'INVENTION. C'est une bonne maladie chronique, contagieuse et même héréditaire dont on ne peut pas se débarrasser et qui vous prolonge de toutes les façons. La gravité de mon cas est telle que je la transmet automatiquement à tous ceux qui m'approchent : mes deux fils et ma femme en furent atteint les premiers et apportèrent dans ma spécialité une collaboration inestimable.

Je serais heureux aujourd'hui de découvrir parmi vous quelques symptômes naissants. Jamais les conditions ne m'ont paru aussi favorables.

Il suffit d'avoir lu quelques unes des publications dont tous ici vous êtes les auteurs, pour mesurer l'immense territoire intellectuel et expérimental dans lequel vous évoluez quotidiennement et qui doit être exploité soit par vous-même soit par l'administration qui vous emploie soit par l'industrie toujours avide de découvrir de nouvelles idées.

Au cours de l'année 1942, je me présentai à l'école des bergers de Rambouillet qui cherchait un enseignant. Je fus reçu par le Directeur lui-même, le déjà célèbre Professeur LAPLAUD (8) dont je n'ignorais pas les premiers résultats en matière d'insémination artificielle des ovins.

Qu'elle ne fût pas ma surprise de le voir lisant mon C.V., s'arrêter sur un vieux certificat de l'usine AVIONS BREGUET me reconnaissant capable d'occuper un poste d'ajusteur outilleur après essais concluants. J'avais 18 ans !

Cet intérêt d'un scientifique pour un collaborateur à aptitudes manuelles caractérisées me sidéra.

Ce fût la chance de ma vie.
Il y en eut d'autres. Il faut être prêt à les saisir. Ce jour-là, même la proposition de me loger avec ma famille dans la cabane du berger ne me rebuta pas.

De nos jours, un inspecteur du travail des temps modernes alerté par le comité d'entreprise au nom de la dignité humaine et de l'épouvantail de la sécurité, s'opposerait à de pareilles pratiques.

Quel dommage !!

La réaction en chaîne ne se serait pas déclenchée.

Toujours des idées pour faire mieux.
Souvent pour faire autrement.
Parfois pour faire autre chose.
CHACUN peut avoir des idées s'il en a l'envie et surtout l'occasion.

«L'occasion d'avoir des idées est offerte par l'expérience pratique :
- par l'observation et la réflexion de ce qui a été observé par soi-même et par les autres
- par l'expérimentation accidentelle ou méthodique
- par les échanges d'informations et la réflexion en groupe notamment à partir des renseignements ramenés par les agents de l'industrie extérieurs à l'entreprise, chez les utilisateurs et clients fidèles ou éventuels».

En résumé, l'envie d'avoir des idées existe chez la plupart des gens mais ne se manifeste pas si l'environnement est pénalisant (tais-toi et fait ce qu'on te dit) si, au contraire, le climat est gratifiant (ce que tu proposes est astucieux : regarde comment le faire essayer), on voit se développer l'envie d'avoir des idées ; cette envie est contagieuse, elle est entretenue par la perspective d'une marque particulière de considération de la part des collègues et des chefs, et éventuellement de quelques primes (24).

Pour moi à Rambouillet, la considération et l'amitié du chef m'étaient tout acquises. Les primes me parvenaient sous forme de gigots ou de petits salé dont me gratifiait en abondance la cuisinière pour nourrir ma famille. C'était inespéré. Nous étions comblés.

Pendant les quatre années qui suivirent auprès de Martial LAPLAUD (8) dans notre modeste labo, encombré de la table à hauteur variable pour la récolte des béliers et de toutes sortes d'instruments variés construits sur place à la demande du patron, à proximité des locaux historiques témoins de l'importation des Mérinos et des Astrakans, nous poursuivimes pendant toute l'occupation allemande les expériences d'adaptation de l'Insémination Artificielle à tous les animaux de la ferme, à l'exception des oiseaux.

Aidés des textes d'IVANOV (1), MILOVANOV (2), WALTON (3), GUNN (4), BONADONNA (5) et au matériel ramené par LETARD (7) de Moscou en 1933, il nous fallut adapter l'équipement aux différentes espèces.

L'aventure de la première récolte de sperme du taureau Gracieux II avec un vagin artificiel en cuivre, trop lourd, trop long, trop étroit, est encore dans toutes les mémoires et fait rire les rares témoins à chaque évocation.

Autre anecdote : celle du bélier Astrakan NEGUS, magnifique animal que Martial LAPLAUD destinait pour la production de fourrures Breischwants et Persianeer par croisement avec les brebis Ile-de-France, et qui se révoltait à toutes les familiarités nouvelles surtout celle du vagin artificiel. Après quelques traitements avec l'électrode bipolaire rectale il changea complètement de comportement, et nous le vîmes arriver longtemps avant le troupeau des femelles, le premier au labo, manifestant violemment son impatience.

Nous nous gardâmes bien dans l'énumération des avantages décrits dans notre brevet d'invention de 1944 de faire allusion en aucune manière aux vertus aphrodisiaques de l'instrument. L'époque ne le permettait pas et je ne l'ai jamais relevé dans les écrits de ceux qui nous suivirent.

J'avoue qu'il fallait disposer d'un mâle chaste et pudique pour s'en apercevoir, ce qui est assez rare par principe.

Déjà, il me fallût des prouesses de persuasion pour que mon vieux patron acceptât de placer son nom à côté du mien dans la demande de dépôt de brevet à l'Institut National de la Propriété Industrielle I.N.P.I. (23).

Je n'allais pas le troubler davantage par des commentaires libidineux. Ce mot de «propriété» choquait profondément le fidèle et dévoué serviteur de l'état. Il se révoltait contre ce qu'il prenait pour une malhonnêteté.

Les temps ont bien changé.

Les deux récentes lois, celle de JUPPE-BAYROU-d'AUBERT datant de 1996 et celle de Claude ALLEGRE de 1999 en sont une encourageante démonstration (14) (15) (16).

La création d'une entreprise ou la participation à sa création ne tient plus du miracle comme de mon temps. Ce n'est plus une utopie. On peut risquer avec de réels soutiens A.N.V.A.R. et autres...

L'Inspecteur Général QUITTET (10) très fier de nos travaux pour lesquels il avait sollicité un léger financement et d'énormes responsabilités, invita ses homologues anglais à assister à Rambouillet à une récolte de sperme de taureau par électroéjaculation, ce qui était considéré comme impossible chez eux. Par souci de réciprocité et par fierté aussi de ce qu'ils pouvaient montrer, ces techniciens nous invitèrent en octobre 1945 à ce fameux voyage au Royaume-Uni.

Quittet s'opposa à ma présence malgré l'insistance de Laplaud. BRACONNIER, plus puissant, exigea ma participation. Après de cocasses cavalcades entre plusieurs ministères, j'arrivai muni de tous les papiers indispensables gare du Nord à la porte du wagon en même temps et à la grande surprise des cinq autres plus favorisés.. QUITTET, LETARD, LAPLAUD, BRUNES, JONDET (13) !

Je venais de découvrir que les problèmes de la nature seraient plus faciles à surmonter que les obstacles de préséance dressés par les hommes entre eux. J'en souffris très longtemps jusqu'au moment où fatigué par trop de tracasseries, je décidai à la surprise générale de donner ma démission de l'Administration.

A notre arrivée à Londres, nous fûmes invités par le Directeur de l'élevage à nous rendre à Cambridge, Reading pour décrire notre méthode et montrer notre matériel.

La visite des centres d'I.A. montés depuis plusieurs années déjà par le Milk Marketing Board et fonctionnant à plein régime nous coupa le souffle.

Quelles surprises nous attendaient.

Nous fûmes reçus par le pionnier WALTON lui-même et par d'admirables vétérinaires anglais passionnés eux aussi par leur travail. Nous devinrent de vrais amis et pendant de nombreuses années encore nous échangeames nos résultats et nos découvertes.

Nous revinmes du Royaume-Uni porteurs de documents, d'instruments et de produits précieux :
- Le vagin artificiel de Cambridge vendu par la firme Holborn,
- Le dilueur de Salisbury (6),
- La méthode recto-cervicale généralisée pour le cathétérisme de l'utérus bovin,
- La façon synoptique de contrôler les résultats.

L'école de Rambouillet et son labo devint le phare de la nouvelle technique qui allait révolutionner tout l'élevage bovin français. On peut dire que sans exception tous les techniciens de l'époque vinrent se former dans nos murs avant d'aller exercer leur savoir dans la plupart des départements où se sont montées une soixantaine de coopérative d' I. A. Les élèves pensionnaires des deux dernières promotions fournirent le plus gros contingent. Sans doute qu'un esprit de corps qui ne se démentit jamais, prit naissance en ce lieu historique.

Mon successeur BERNAGE y créa une industrialisation jamais égalée de la production du gant en latex et plus tard, en plastique jetable, indispensable protégeant jusqu'au dessus du coude le bras de l'inséminateur.

A cette même époque, j'eus la chance de rencontrer en Seine Maritime, à l'occasion d'une typique, traditionnelle et folklorique vente aux enchères de taureaux normands, Maurice ISAMBERT Président de la coopérative beauceronne et percheronne d' I.A. et d'amélioration de la Race Normande pure !!

Il dirigeait la commission chargée d'acheter leur premier taureau (le plus gros comme c'était la mode en ces temps reculés). Bien entendu, Laplaud et moi désirions le même et fûmes très déçus de le voir adjugé à des inconnus. Poussé par une force irrésistible, je m'approchais d'eux et quelques minutes plus tard j'étais embauché moralement sur le RING par celui qui allait devenir, pendant 25 ans dans le privé, mon futur patron Maurice ISAMBERT.

Quand je dis patron, je pense surtout à l'ami, le témoin bienveillant et attentif, passionné de toutes les réalisations en cours et de tous les projets. Sa confiance était totale, son désintéressement aussi.

Comment mesurer le cadeau qu'il fit à l'Industrie française de l' I.A., à l'élevage en général et à moi en particulier en m'autorisant sans contrepartie à déposer le 20 Septembre 1949 et à exploiter par la suite le premier brevet de la fameuse PAILLETTE FRANCAISE qui allait devenir au cours de sa longue vie : en 1965 «LA PAILLETTE AMELIOREE» de 0.5 Ml annonçant le gain de place, l'économie de sperme et l'amélioration de la fécondation,

en 1968 «LA PAILLETTE MINIATURISEE» de 0.25 Ml devenant «UNIVERSELLE» losqu'elle envahit en congélation horizontale plus de 127 pays, soit 45 millions d'I.A. annuelles.

Charles MERIEUX(18) prévoyait déjà l'emploi des paillettes pour :

- le développement des sérothèques,
- la lutte contre le cancer sous les auspices du Pr RIBOLI(17),
- la transfusion sanguine et la lutte contre les erreurs transfusionnelles.

Il fallut 8 ans à ce VISIONNAIRE INSPIRE pour voir son programme se réaliser enfin au delà de toute espérance (près de 5.000.000 de doses stockées en 1999).

C'est le Président ISAMBERT aussi qui m'encouragea le plus naturellement du monde et sans se préoccuper d'avis contraires à donner ma démission de l'Administration pour exploiter personnellement mes idées et mon propre brevet. L'état d'esprit d'après guerre s'y prêtait. Ce serait suicidaire aujourd'hui.

Je l'écoutai.

Bien m'en a pris.

Ma REINTEGRATION aurait changé complètement le cours des choses. Nous aurions été ballottés de méthodes en méthodes ou de toutes à la fois. On se serait vus d'abord anglais puis italiens, allemands, japonais et pourquoi pas danois !! On resta français.

Je n'ose pas croire que l'admirable amélioration génétique des races françaises et leur suprématie dans le monde due à la fois à une technique universelle et à la loi de l'élevage de 1960 auraient pu s'imposer dans une situation concurrentielle sur le plan technique.

Pour la France, une lecture des publications de l'époque est éloquente à cet égard.
On y lit : 2 200 000 vaches inséminées en 1950. 7 000 000 en 1966.

En 1952, Copenhague IIeme congrès international de la reproduction et de l'insémination artificielle. Chris Polge et Rowson (9), au cours d'un exposé magistral, lancent la bombe de la CONGELATION de gamètes.

C'est une révolution technique.
C'est la fin d'une époque et le début d'une autre.

De tous côtés surgissent des propositions prétendant exploiter au mieux cette nouvelle conquête des scientifiques.

Il fallut à l'Aigle un travail acharné et cohérent à la fois des techniciens de la Coopérative d'Insémination et de ceux de l'usine I.M.V. pour résister, fournir des armes et triompher.

La méthode française protégée pour vingt ans par le brevet français de 1949 fut par la suite toujours jalousée. De multiples tentatives risquèrent de l'abattre soit par la copie, soit par le vol de secrets, soit par de mauvais et faux perfectionnements ou même de procédés totalement révolutionnaires tels les pellets, les granules dangereusement séduisants par le prix très bas, la facilité et la rapidité d'exécution, le gain de place et l'attrait de la nouveauté (19).

Les publications de nombreux scientifiques parmi les plus célèbres en faveur des granules étaient prêtes pour leur présentation au congrès de Trento en 1964. C'est de cette catastrophe annoncée par mon fils Bertrand au retour de son voyage en 1963 des U.S.A., qu'est née en 1968 la paillette miniaturisée dont le succès allait être écrasant.

Il fallait se battre et chercher des idées pour frapper fort ! Je triomphai le jour où le Professeur Jacquet (20) me téléphona pour m'annoncer qu'à sa grande surprise l'azote liquide que je lui avais remis 48 heures auparavant avait laissé dans les boites de Pétri une flore abondante des micro-organismes les plus redoutables. La méthode des pellets pourtant portée aux nues ne résista que chez les ignorants et les irresponsables (21) (22).

Tout ce qui précède prouve qu'à cette époque reculée on pouvait encore croire aux miracles et aux secrets ! Tout marchait, il suffisait D'EN VOULOIR.

Bien innocent celui qui y croirait encore aujourd'hui.

A ce propos, je me dois de mettre en garde tout détenteur d'idée géniale qui rêverait d'en garder cachée la paternité avant la venue au monde du bébé. Il en serait vite dépossédé.

Il vaut mieux, dans un premier stade, s'adapter aux méthodes classiques et reconnaître l'intérêt moral, particulièrement en France, des enveloppes Soleau. La rédaction, la gestion et la défense des brevets est un second stade qui exige un certain nombre de qualités personnelles particulières, mais aussi une connaissance approfondie de toute la législation française et étrangère. Il faut une formation que l'on commence à offrir frileusement dans l'enseignement. Je pense que l'expérience d'un professionnel averti et réputé s'impose.

Toute ma vie, j'ai conseillé aux chercheurs que j'ai fréquenté de se protéger et de se rendre à l'I.N.P.I. (23), véritable musée dont l'entrée est libre et dont la visite est passionnante et instructive.

Agé de quatorze ans, j'y accompagnais mon père et mon oncle inventeurs contagieux eux aussi. J'y ai beaucoup appris. Je m'y rends encore. C'est comme un pèlerinage à la source (23).

Plusieurs dates mémorables jalonnent l'épopée de l'entreprise I.M.V. qui fonctionnait à l'époque en symbiose extrêmement fertile avec la coopérative d' I.A. réimplantée à l'Aigle avec la même équipe que celle de La Loupe composée de techniciens formés par mes soins et passionnés par leur mission.

La convention cadre d'I.M.V. avec l'I.N.R.A. renouvelée chaque année depuis plus de quarante ans est un exemple parfait des services que peut rendre, dans un climat très amical, l'association fertile des chercheurs de haut niveau et le génie des gens du terrain.

J'ai plaisir à en citer quelques-uns qu'ils viennent de France, d'Europe et même du reste du monde. Tous devinrent, dès la première heure, de précieux et fidèles amis de la maison ; Heyman, Renard, Nibart, Ozil, Courot, Thibault (11), Goffaux, Parez, Jondet, Thibier, de Reviers, Rasbech, Stewart, Hunter, Massip, Dyrendahl, Kufferschmied, Graham, Radjamannan, Mac Pherson, Almquist et tant d'autres. Je n'en oublierai aucun. S'ils ne sont pas cités, qu'ils veuillent bien m'en excuser (voir bibliographie).

Cette convention a prouvé son efficacité dans la plupart des innovations qui ont jalonné l'histoire de l' I.A. et de E.T. et de l'amélioration génétique.

Avec le même manager qu' à la coopérative, la Firme I.M.V. a toujours pu se permettre de disposer de plus de 200 000 vaches pour les essais et contrôles systématiques. Elle est toujours considérée en tous lieux comme la fille naturelle de la coopérative, ça tranquillise toute la clientèle et c'est précieux pour le moral.

- 1955 Formation pratique de la première technicienne Melle CSYBLIX du C.E.C.O.S. au Kremlin Bicêtre Professeur DAVID promoteur de l'I.A. humaine.

- 1960 et 1961 Expéditions à Madagascar de sperme en paillettes congelées à l'azote liquide avec du matériel américain, pour la fécondation réussie d'un troupeau de vaches normandes inscrites au Herd Book.

- 1963 Miniaturisation de la paillette grosse qui passe à 0.25MI. On obtient en cours de congélation un meilleur gradiant thermique indispensable à une bonne conservation de la cellule. La paillette fine, plus économique en sperme, plus performante en fécondation, adoptée d'emblée par toutes les unités d'amélioration génétique a précipité l'abandon des pellets malgré l'arsenal mis en place dans toutes les communications de l'époque.

- 1968 Grande première mondiale : naissance de la génisse Victoire dix ans après la mort de son père avec du sperme vieux de treize ans. (Filiation confirmée par contrôle des groupes sanguins).

- 1969 La paillette et la technique française de l'insémination avec son schéma d'amélioration génétique bovin deviennent le standard universel.

- 1978 Développement et lancement d'automates chez les utilisateurs permettant le remplissage, le bouchage, l'identification, la congélation dans l'azote liquide des «paillettes» par Maurice CASSOU, Directeur du bureau d'études.

- 1980 Madrid IXeme congrès international Evolution de la paillette fine dans le monde : R et B CASSOU (12). Nous avons eu la fierté d'annoncer qu'en routine work, 444.000 AI pratiquées au cours d'une période de 36 mois par les inséminateurs de l'Aigle avaient permis d'obtenir un avantage de 1,50% de non-retours en mini-paillettes, pour le plus grand bien des éleveurs et d'IMV fabricant du matériel

- 1980 Création de la filiale américaine par Bertrand CASSOU, Directeur commercial.

- 1981 Création et ouverture par IMV d'une école à la Ferme expérimentale de la Sapaie devenue le Centre international de formation aux méthodes de reproduction artificielle des bovins, ovins, équins et autres espèces pour l'insémination et le transfert embryonnaire.

C'est Testart de l'INRA qui déclencha en 1971 «l'emballement» pour l'E.T.. Il nous rendit souvent visite à l'Aigle à cette époque pour échanger nos moyens dont il devinait déjà la fertile complémentarité. Une période de près de dix ans fût nécessaire pour améliorer tous les paramètres.

C'est à l'équipe OZYL, NIBART, HEYMAN de l'INRA et de l'UNCEIA que nous devons les progrès continus poussant les ateliers ultra modernes de plasturgie d'I.M.V. à réaliser le tour de force de l'extrusion de la sonde à trois voies, permettant d'abandonner la laparoscopie au profit de la méthode cervicale qui tient lieu de référence pour la France.

Le Docteur Xavier LEGENDRE Professeur au muséum engagé par IMV eut pour mission, à cette époque, d'assurer la mise au point de cette nouvelle technique et la formation dans les conditions réelles de la pratique des moniteurs chargés par la suite d'instruire les 400 stagiaires qui se succédèrent sans interruption pendant les quatre années qui suivirent.

Le Docteur Gustavo DECUADRO HANSEN successeur de Xavier LEGENDRE dans les mêmes fonctions I.A. et T.E. nous fait savoir qu'en France le nombre d'embryons bovins collectés dépasse la barre des 65.000 et que le nombre d'embryons transféré se situe autour de 34.850 en 1999.

Par ailleurs, les unités de sélection française importent plus de 1000 embryons chaque année.

I.M.V. a vendu au total 418.000 gaines pour E.T. en 1999 dont 180000 pour l'Amérique qui reçoit ainsi de France à ce titre 90% de ses besoins.

- 1982 à 1989 Déclinaison du savoir-faire bovin dans les autres espèces animales (aviculture, ovins, caprins, lapins, équins...)

- 1992 Mise au point de la paillette CRYOBIOSYSTEM (CBS et première étude épidémiologique avec le centre international de cancérologie de Lyon portant sur 350 000 européens. (Docteur Elio RIBOLI O.M.S. recherche sur le cancer Lyon. Fondation Charles MERIEUX).

- 1993 Lancement de la gamme de produits (cochette et sonde Goldenpig) très innovatrice dans le domaine porcin sous la. responsabilité de Bertrand CASSOU son initiateur. L'entreprise possédait à cette époque 157 brevets d'invention. Elle fabriquait 450 produits. Elle réalisait 150 millions de francs de chiffre d'affaire, avec un personnel bureaux, ateliers et filiales de 150 personnes.

- 1994 Reprise d'I.M.V. par Jean-Gérard SAINT RAMON, docteur en pharmacie, homme universel ayant fait ses preuves de manager au plus haut niveau chez Bayer.
  - ✓ Ouverture d'une salle blanche et d'un laboratoire pour la définition et la fabrication de milieux biologiques (en particulier conservation des gamètes).
  - ✓ Lancement du produit de conservation de semences bovines BIOCIPHOS, innovation biologique majeure.

- 1996 Lancement de gammes de milieux de conservation de semences porcines mises au point avec une université vétérinaire américaine.
  - ✓ BICEF, filiale d'I.M.V. est rebaptisée CRYOBIOSYSTEM.

- 1997 Mise au point
  - ✓ Milieux de conservation des semences avicoles très innovants permettant un renforcement de la pratique de l'insémination artificielle.
  - ✓ Gamme de produits pour l'insémination artificielle humaine.
  - ✓ Nouveaux types de paillettes permettant d'aborder de nouvelles applications (cryoconservation des vaccins à cellules vivantes, conservation des patrimoines génétiques des espèces en voie de disparition).
  - ✓ Lancement du DEC, détecteur électronique de chaleurs chez le bovin, sous les auspices de Michel Lombard
  - ✓ Introduction d'IMV Technologies au second marché de la Bourse de Paris

- 1998 Transformation de l'office commercial indien en filiale sous forme de «private limited».
  - ✓ Introduction au second marché de la Bourse de Paris.

- 1999. OUVERTURE DE LA BRANCHE AQUACOLE

IMV fabrique les cinq milieux spécifiques mis au point au cours de longues années de recherche et expérimentation par Gérard MAISSE (INRA). 80% des SALMONIDES, BARS, TURBOTS élevés en france dans les fermes aquacoles lui sont redevables de cet immense progrès.

Les sociétés de toutes tailles qui font systématiquement appel aux idées du personnel sous les formes les plus diverses ont généralement de bonnes performances. C'est le secret d'I.M.V. depuis les origines.

On observe alors à tous les niveaux un phénomène de cooptation et d'élimination qui rejette les «mous» ou les incompétents prétentieux, puis un phénomène d'entraînement des éléments apparemment moins doués qui deviennent de précieux auxiliaires des innovateurs. On voit réaliser des choses assez extraordinaires par des gens assez ordinaires, avec des moyens réduits, tandis qu'un management trop centralisateur, malgré un personnel de haute qualité et des budgets considérables, n'obtient que des résultats médiocres avec un climat démotivant ...

La rémunération des seuls inventeurs pourrait casser l'esprit d'équipe : favorisant «la course individuelle au brevet» elle peut nuire au succès final de l'innovation.

L'invention n'est qu'un maillon de la chaîne de l'innovation. Le succès de l'innovation est le résultat d'un effort collectif impliquant un échange intensif d'idées et d'expériences entre individus variés : marketing, chercheurs, metteurs au point, techniciens, dessinateurs, vendeurs, etc...

Finalement l'analyse montre que l'appel aux idées des collaborateurs est à la fois la cause et l'effet d'un management efficace et rigoureux ; il s'agit d'un style de management où le vrai professionnel à tous les niveaux trouve sa satisfaction dans la qualité de son travail, à la fois exigée et reconnue et ou la qualité de la vie rejoint la qualité de l'action. Elle nécessite un effort considérable d'information, de formation et de sélection pour lutter contre le laxisme stupide et ruineux, mobiliser l'intelligence et... LUTTER (24) !

Robert CASSOU

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National Statistical Data of Bovine Embryo Transfer Activity in Europe (1999)

OVERALL BOVINE EMBRYO TRANSFER ACTIVITY IN EUROPE* IN 1999

(Data collected from 19 countries)

TOTAL NUMBER OF TRANSFERABLE EMBRYOS	**170 451**
➢ *In vivo* produced	
- flushed donors	**26 429**
- transferable embryos	**145 305**
- mean per flushed donor	**= 5.49**
➢ *In vitro* produced	**25 146**
Proportion of IVF produced	**14.7 %**
TOTAL NUMBER OF TRANSFERRED EMBRYOS	**143 168**
➢ *In vivo* produced	**129 779**
➢ *In vitro* produced	**13 389**
➢ Proportion of IVF transferred	**9.13 %**
➢ Proportion of frozen embryos transferred	**58.0 %**
TOTAL NUMBER OF FROZEN STORED EMBRYOS	**63 403**

(Y. HEYMAN, AETE Santander, 2000)

EMBRYO TRANSFER ACTIVITY IN OTHER SPECIES
EUROPE year 1999

SPECIES	EMBRYO PRODUCTION	EMBRYO TRANSFERS	COUNTRIES
SHEEP	6744	6330	Czech rep Denmark Greece France Romania UK
SWINE	3995	534	Czech Rep Romania
GOAT	303	176	Czech Rep Denmark Romania
HORSE	222	194	Austria Czech Rep Denmark France Italy Netherlands UK

(Y. HEYMAN, AETE Santander, 2000)

INVITED LECTURES

IN VITRO TECHNOLOGIES RELATED TO PIG EMBRYO TRANSFER

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Abstract

Embryo transfer in swine (ETS) has been used for commercial and breeding application only to a limited extent. However this technique is an essential prerequisite for the application of new reproductive techniques in pigs. This paper will give an overview on steps of pig embryo transfer including selection and stimulation of donor sows, recovery of embryos, embryo handling and the transfer of recovered embryos into recipients. Furthermore the current status and further application of ET related *in vitro* technologies in pig production are described.

1. INTRODUCTION

Global needs for foods and animals require the development of strategies beyond traditional breeding ensuring offspring of value characteristics, of high productivity but maintaining genetic diversity. Oocytes and embryos are escaped from traditional animal breeding methods. However, porcine embryos are an excellent carrier to introduce a novel or altered genetic inheritance into individuals and herds. Embryo transfer and associated reproductive techniques enable the manipulation of germ cells and can in conjunction with conventional breeding improve the rate of genetic progress in domestic animal. ETS is a complex of measures including (i) selection and stimulation of donor sows, (ii) recovery of embryos, (iii) embryo handling, i.e. morphological assessment, intermediate storage, cultivation and transport, and (iv) the transfer of recovered embryos into recipients.

In the pig, so far, the application of reproductive techniques is limited. The present paper will focus on the current status of embryo transfer in swine (ETS), on associated techniques of *in vitro* production of embryos, of sexed and splitted embryos, on cryopreservation of embryos, gentransfer and on its practical application in the pig industry.

Compared to ruminants pig embryo transfer for commercial and breeding applications has been used only to a limited extend. This is due to the high fecundity and to the mainly surgical embryo collection and transfer techniques. First ETS was performed 1951 by Kvasnicki [47] where one out of four recipient sows was pregnant after transfer of 9 embryos, recovered from the oviduct, and 4 piglets were born. Starting from the sixties ETS has been developed and practised [23, 31, 86]. At present embryo transfer in the pig is available as a basic tool of biotechnological procedures.

2. SELECTION OF DONOR SOWS AND SUPEROVULATION

Prepuberal, puberal cycling gilts and sows can successfully be selected as donors. Prepuberal gilts are preferable used because of higher superovulatory response and easy handling. Gilts and sows, especially at the end of their reproductive life, are chosen for the propagation of value breeds.

In pigs, PMSG is exclusively used to induce superovulation (SO). SO is stimulated in prepuberal gilts by application of 1,000-1,500 IU PMSG followed by an injection of 500 IU hCG 72 h after PMSG. In cycling gilts SO is induced after estrus synchronisation (feeding of Regumate® for 15 days) by administration of 1,500 IU PMSG and of 500 IU hCG 78 h later. Multiparous sows are injected with 1,000-1,250 IU PMSG 24 h after weaning and 500 IU hCG 58 h after PMSG. The use of GnRH is an alternative to hCG in triggering ovulation. Gilts and sows are fixed-time inseminated 24 h and 38 h after hCG injection. Results of superovulation and embryo recovery in 146 gilts and sows are presented in Table 1.

Table 1. Superovulatory response (Mean±SD) in prepuberal and cycling gilts, and multiparous sows [7, 9]

Parameters	prepuberal gilts	cycling gilts	multiparous sows
No. of animals	83	139	24
No. of ovulation	40.1 ± 12.8[a]	29.4 ± 12.1[b]	24.3 ± 7.2[b]
No. of recovered embroys	32.5 ± 13.6[a]	23.4 ± 12.8[b]	19.3 ± 7.4[b]
No. of transferable embryos	27.2 ± 12.9	20.5 ± 12.8	11.8 ± 8.2
Recovery rate %	81.1 ± 22.1	78.7 ± 24.1	79.0 ± 20.0

[a,b] Means with different superscripts within a row differ significantly ($p<0.05$)

Gilts and sows show a high variation of ovarian response to superovulatory stimulation. The individual superovulatory reaction, however, is impossible to predict. Multiparous sows (litter 3-11) demonstrating lasting fecundity (litter size 11.7 ± 2.5) show a less superovulatory response compared to prepuberal gilts.

3. EMBRYO COLLECTION TECHNIQUES

No successful non-surgical embryo collection has been reported in pigs so far, except the studies of Kobayashi *et al*. [44] and Hazeleger *et al*. [32] following surgical resection of uterine horns. The major reason of this restriction is the anatomy of the porcine genital tract. Therefore embryo collection can be performed *in vitro* by flushing the genital tract after slaughter, but this technique has the limit to use donor sows only once. Embryo recovery in swine is usually accomplished surgically under general anaesthesia, with the genital tract presented through a midventral incision in the caudal abdominal region involving retrograde flushing of the oviducts and/or uterine horns [6, 20, 31, 84]. The disadvantage of surgical procedures can be diminished by minimal-invasive endoscopic techniques to recover embryos [1, 15, 77].

Embryos can be recovered at different stages of development. Embryos at the one-cell up to the four-cell stage are collected from the oviduct on day 1 to 3 after ovulation, whereas embryos from the four-cell stage up to the hatched blastocyst are flushed from the uterine horn on days 4 to 7 [6].

4. EMBRYO HANDLING

Embryos are morphologically examined *in vitro* under a stereo microscope after they were isolated from the flushing and transferred into a culture media. Embryos are evaluated according to their morphological characteristics and the expected stage of development referring the day of recovery, and they are classified into transferable or non-transferable embryos. Embryos at the one-cell stage (zygotes) are present in the oviduct on day 1-2. The presence of two polar bodies and of accessory sperm cells is a criterion of fertilisation of one-cell eggs. On days 2-4 embryos are at the two- and four-cell stage within the oviduct. Embryos at the four-cell up to the eight-cell stages can be recovered from the uterine horn between days 4 and 5. Normal developed embryos are in the stage of early to late morula between day 5 and 6, and of early to expanded and hatched blastocysts on days 6-7, respectively. Embryos with a difference of one cell cycle can be accepted as transferable, whereas embryos in difference of more than two cell cycles have to be discharged [9].

Following morphological evaluation embryos are stored for a short time (<4 h) in a culture medium until they are transferred into recipients. Storage medium has to ensure the viability of embryos during this time. Therefore it is necessary to stabilise temperature, pH, osmolarity and metabolic requirements. Media which not require supplementation of CO_2 during short time culture are useful, like phosphat-buffered saline (PBS) with 3%BSA or HEPES buffered modified Tyrode solution**.**

5. ET ASSOCIATED *IN VITRO* TECHNIQUES

5.1. *In vitro* production of embryos (IVM/IVF/IVC)

5.1.1. Source of oocytes

In the pig 10-20 mature oocytes are ovulated, so that only about 200 oocytes are used for fertilisation during life span. However, the porcine ovaries contain a relatively large number of primordial follicles (approximately 420,000) [27]. This pool of oocytes is a challenge to use it for *in vitro* production.

The knowledge of requirements of growing oocytes is still limited [18, 49, 64]. Oocytes derived from preantral follicles grow *in vitro* up to their final size, acquire meiotic competence and can be penetrated by spermatozoa, but the success rate is still very low. Only 4.8 % of oocytes from preantral follicles progress to metaphase II, and 0.4 % were penetrated by spermatozoa [36].

The main source of oocytes for *in vitro* maturation is collected from antral follicles of slaughtered prepuberal pigs. Sorted oocytes with uniform ooplasma and surrounded with compact cumulus may undergo nuclear maturation up to 90% if they were incubated in maturation medium containing porcine follicular fluid and gonadotropins [62]. Usually TCM 199, NCSU 23 or Waymouth Medium were used as basic maturation media.

However, *in vitro* maturation influences nuclear and cytoplasmatic maturation of porcine oocytes, and subsequent pronuclear formation and first cleavage [48]. Difficulties of *in vitro* maturation are overcome if oocytes are derived from mature prevulatory follicles by means of endoscopic ovum pick up [14]. Cumulus oocyte complexes recovered from PMSG primed pubertal gilts 22-34 h after hCG showed a high incident of cumulus cell expansion (86.7 - 98.3%) and oocyte nuclear maturation (82.4 - 100 %) [89].

5.1.2. In vitro fertilisation

Pig oocytes matured *in vivo* and *in vitro* can be fertilised *in vitro* using fresh boar semen or frozen-thawed epididymal spermatozoa [54 57, 73, 97]. However, differences were obtained between *in vivo* and *in vitro* matured oocytes according to sperm penetration rate (69.8 vs. 35.0 %) and completion of second meiotic division (41.7 vs. 20.8 %). Furthermore the *in vitro* matured oocytes displayed asynchronous pronucleus development, lower cleavage rate and delayed cleavage [48]. The low incident of male pronuclear formation after *in vitro* fertilisation is due to suboptimal hormonal conditions, high concentration of NaCl and oxidative stress. However, this problem has been currently solved by modifications of maturation media adding thiols and organic osmolytes [24].

Polyspermy is a lasting problem of IVF. Polyspermic penetration of porcine oocytes range between 13 and 90% [62]. Usually a relatively high number of spermatozoa is added to fertilization media to maintain capacitation. However a high number of spermatozoa per oocyte is associated with high incident of polyspermic penetration. One possibility to reduce the occurrence of polyspermic penetration is to minimize the number of spermatozoa per oocyte [73]. However a low rate of polyspermic is accompanied by reduced penetration rate [62]. Preincubation with porcine oviductal cells or follicular fluid did not considerably reduce polyspermic penetration.

5.1.3. Development of embryos in vitro

Embryos, developed *in vivo* up to the 8-cell stage, advance under *in vitro* conditions in a high degree to blastocyst stage. However, the *in vitro* development from 1- or 2-cell through the 4-cell up to the blastocyst stage is critical. Several media and culture conditions have been created that allow embryo development *in vitro* [65]. Up to now NCSU23 containing taurin and hypotaurin promote best the development from the 1-cell to blastocyst stage. In NCSU23 90% of *in vivo* derived and *in vivo* fertilized 2-cell embryos overcome the 4-cell block and 85% developed to the blastocyst stage compared to 70% and 46% in modified Whittens medium, and 46% and 32% in modified KRB, respectively. However, embryos derived *in vivo* but fertilized *in vitro* progress compared to *in vivo* fertilized embryos a lower rate of overcoming the block stage (57 vs. 92%), and of blastocyst formation (43 vs. 75%) [74]. The cleavage rate of oocytes achieved after *in vitro* maturation and fertilisation was 73%, and the blastocyst formation rate after 6 day culture was 42%, respectively [Rath, personal information]. Although embryos cultured in NCSU23 develop well up to the blastocyst stage they have a lower cell number (28 vs. 59) and embryos are a full cleavage division

behind *in vivo* controls [51]. NCSU23 benefits the embryonic protein metabolism. However, the lower incorporation of methionin into *in vitro* developed compared to *in vivo* derived blastocysts demonstrate that cultivation over a longer period has yet to be substantially improved [93].

5.2. *In vitro* production of sexed embryos

Prediction of sex and production of respective offspring could be a noteworthy application in the pig industry. Sex determination of porcine embryos has been done by chromosomal analysis of cells from blastocysts [41] or by PCR [67]. However these methods are not qualified for practical application to produce sexed embryos. Gender preselection by means of high-speed sorting of X-and Y-chromosome-bearing sperm in conjunction with *in vitro* fertilisation enables the production of sexed embryos and offspring. Both, *in vivo* and *in vitro* derived oocytes were fertilised with sexed semen. The cleavage rate was 56.2% and 30.4 to 43.1%, respectively. Altogether 10 and 34 piglets were born, where 100% and 97% were female [75, 76].

5.3. Production of identical multiplets

Embryo bisection, proliferation of single blastomeres, nuclear transfer and parthenogenesis are possible methods to generate identical multiplets. In the pig these methods are rare applied. Piglets were obtained after transfer of bisected [58, 79, 81]. Pregnancy rates up to 82% are possible [79], however, the survival rate of demi-embryos is reduced and range between 17% and 34% [10, 58, 79] compared to 52% after transfer of complete control embryos [10]. The outcome of identical twins achieved by this method is low (2.3%) [79].

Single blastomeres derived from 4- and 8-cell embryos developed *in vitro*, and the blastocyst formation rate ranged between 4-13 % [55] and 34-50 % [61, 83], but piglets were not produced.
Up to now only one piglet was born after transfer of 88 reconstituted embryos which were produced after cloning by fusing single 4-cell blastomeres with enucleated, activated meiotic metaphase II oocytes [68].

5.4. Gene transfer

First transgenic pigs were born 1985 after microinjection of human growth hormone into pronuclei of zygotes [29]. Microinjection of several hundred copies of foreign DNA is still the only promising method to produce transgenic pigs. In the pig, microinjection requires the visualisation of pronuclei by centrifugation. Centrifugation and microinjection influence blastocyst formation. The cleavage and the blastocyst formation rates after 4-days *in vivo* culture in porcine oviducts were 60 % and 38% compared to 74% and 56% of non-injected zygotes, respectively [11]. Besides growth-related genes other gene constructs have been injected into porcine zygotes for different aims (for review see [61, 70]). The efficiency of pronuclear microinjection, however, is still low. About 8% of injected ova result in offspring and 0.7% in transgenic piglets.

Future alternative methods of foreign gene integration into pigs could be sperm-mediated and stem cell-mediated gene transfer. Although after incubation 12-17 % of sperm cell were associated with plasmid DNA molecules, none of the piglets born after insemination with DNA-treated sperm showed sign of exogenous DNA incorporation [26]. Destruction of developing male germ cells by busulfan and injection of DNA-liposome complexes transformed spermatocytes. Foreign DNA was expressed in 15-25 % of male germ cells [43].

5.5. Cryopreservation

Preservation of embryos over a period of 24-72 h maintaining its viability benefits the collection of embryos for world wide trade. Furthermore, sometimes is a need of holding embryos to collect the optimal number or to find a suitable recipient for transfer. Cryopreservation of porcine embryos would be benefit these requirements. However, routine methods of cryopreservation of porcine embryos are not available. Pig embryos are very sensitive to hypothermic conditions. Although there are reports on birth of live piglets after cryopreservation [35, 40, 45, 56], porcine embryos are limited in their ability to withstand freezing and cryosurvival. This can be overcome using cytoskeletal stabillizer. Embryos cryopreserved by conventional freezing and vitrification under

the influence of cytoskeletal stabilization have resulted in pregnancies (60%) and live offspring after transfer (litter size 5 to 7.3) [21, 22].

6. RECIPIENTS

Selection of recipients has a mayor impact on embryo transfer results. Although prepuberal gilts can be used as recipient, cycling gilts and sows are privileged. This is due to their strong endocrine and uterine condition. Recipients are synchronised to donor sows and treated in the same manner except for lower PMSG application (750-1,000 IU) to avoid superovulation. The stimulation of recipients with a lower dose of PMSG creates an endocrine situation which is asynchronous to the donor [13]. An asynchronous transfer (recipients ovulate one day later) benefits embryo survival [66]. The ovarian response of recipients has as well an influence on embryo survival. Recipients with ≥ 6 compared to ≤ 5 ovulation per ovary demonstrated a higher rate of pregnancy (77.8% vs. 55.6%) [8].

Donor gilts have been also used as recipients, reducing the number of animals required for gene transfer programs [3, 71]. However, hormonal secretions after superovulation in donor gilts do not benefit embryo survival and pregnancy [13, 71].

7. EMBRYO TRANSFER

Surgical procedures to transfer embryos into recipients have been available for several decades. The genital tract is exposed under general anaesthesia. The embryos, depended on the stage of development, are transferred either into the oviduct (one- to four-cell embryos) or into the tip of the uterine horn (four-cell embryo to blastocyst). Embryos are transferred in a small amount of medium by means of transfer pipettes or catheters.

Endoscopic procedures were developed recently [1, 78, 88]. This minimal-invasive technique has some advantages compared to surgical procedure but requires endoscopic equipment and experienced handling.

Several attempts were done to transfer embryos non-surgically using AI-spirettes or special designed instruments [25, 33, 50]. Despite several limitations (i.e. use of pluriparous sows only, deposition into the uterine body, stage of embryo development) progress in non-surgical embryo application is promising for expanded application of ETS.

The transfer of 16-20 embryos seems to be optimal to achieve high pregnancy rates [8, 17, 84]. *In vitro* conditions and manipulation of embryos, i.e microinjection of foreign genes, nuclear transfer, bisection, cryoconservation, limit embryo survival [11, 28, 53]. Therefore, to maximise pregnancy rates a higher number of embryos, that is between 30 and 35, would appear to be optimal for transfer [71, 75, 87, 91].

On average, the pregnancy rate is about 60% and the litter size is 6.5 piglets, with a range of pregnancy from 17% with 2.4 piglets to 100% and litter size of 10.8 piglets [5]. Under optimal conditions, using high quality embryos, pregnancy rates and litter sizes can be obtained similar to results after AI irrespective of the embryo transfer technique, i.e. surgical, endoscopic, non-surgical. Application of *in vitro* techniques lowers the results of embryo transfer in a wide range. However acceptable results can be achieved observing optimal conditions in all steps. References on results of embryo transfer and related *in vitro* techniques are shown in Table 2.

8. CURRENT STATUS AND APPLICATION

Although embryo transfer techniques have been developed and tested in several fields of swine production, its practical application is still rarely. ETS has been used to introduce new genetic material into closed herds [17, 19, 37] and for extracting healthy stock from diseased source [52, 72, 85]. ET in pigs has been used, but rather to a limited extend, for the export of embryos [39, 60, 94], for the exploitation of superior sows near end of useful reproductive life [7, 42, 52] and for the propagation of endangered swine breeds [78].

Embryo transfer, however, is an essential prerequisite for the application of new reproductive techniques in pigs. Research and initial application in the field of gene manipulation to improve growth and disease resistance, to generate foreign proteins in blood and milk, and to create tissues and organs for xenotransplantation need embryo transfer [4, 12, 16, 29, 61, 82]. Other reproductive techniques to produce offspring like ovum pick up, and *in vitro* maturation and fertilization [14, 54, 57, 74, 95, 98], generation of sexed embryos [75, 76], cryoconservation of embryos [22, 35, 40] and cloning [10, 58, 68, 81] require also successful handling of embryo transfer technique. However, these reproductive techniques did not find practical application in swine production, yet.

Progress in several steps of porcine embryo transfer like optimized IVC systems, cryopreservation, minimal-invasive recovery and non-surgical embryo application advance further application of this sophisticated biotechnic. One benefit of ETS will be the international exchange of porcine embryos of value breeds instead of live animals. Nevertheless the prospective application of ETS will not become the same significance compared to embryo transfer in other livestock species.

Table 2. Results of embryo transfer following surgical and non-surgical embryo application, and different embryo manipulation

Application	No. of transfers (n)	Pregnancy rate (%)	Litter size (mean)	References
surgical transfer	27	70	5.7	Dziuk et al., [23]
	77	73	6.2	Schlieper [84]
	46	68	6.7	Kruff [46]
	206	53	7.0	Holtz [38]
	39	80	8.1	Cameron et al., [17]
	112	63	7.7	Brüssow [8]
mean		68	6.9	
(range)	(27 - 206)	(53 - 80)	(5.7 - 8.1)	
non-surgical transfer	58	9	5.2	Reichenbach et al. [80] Hazeleger and Kemp [33]
	21	33	6.7	
	46	22	4.3	Galvin et al. [25]
	16	31	6.2	Li et al. [50]
	25	64	3.1	Yonemura et al. [96] Hazeleger and Kemp [34]
	27	59	10.9	
mean		31	6.1	
(range)	(16 -46)	(9 - 64)	(3.1 - 10.9)	
transfer after IVF/IVC	8	38	9	Mattioli et al. [54]
	1	-	3	Yoshida et al. [98]
	3	33	2	Rath, [73]
	6	0	0	Rath et al. [74]
	26	35	6.3	Rath et al. [76]
mean		27	4.1	
(range)	(1 - 26)	(0 - 38)	(0 - 12)	
transfer after gentransfer	10	33	5.0	Brem et al. [2]
	49	59	7.8	Pursel et al. [69] Springmann et al. [87]
	98	37	3.9	
	14	29	4.3	Vize et al., [90]
	39	44	5.9	Brüssow et al. [12]
	17	41	7.1	Williams et al. [92]
	36	81	7.4	Nottle et al. [63]
mean		46	5.9	
(range)	(10 - 98)	(29 - 81)	(3.9 - 7.8)	
transfer after bisection	2	-	4	Rorie et al. [81]
	12	42	5.2	Nagashima et al. [59]
	7	14	4	Brüssow and Schwiderski, [10]
	27	82	6.0	Reichelt and Niemann [79]
mean		45	5.6	
(range)	(2 - 27)	(14 - 78)	(4 - 6.0)	

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EXPRESSION AND DETECTION OF OESTRUS IN CATTLE

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Reproductive efficiency is a major factor affecting the production and economic efficiency of dairy and beef herds. For herds using AI heat detection rate and calving rate are the two major determinants of compactness of calving, of the proportion of cows that fail to conceive in a defined 13-week breeding season (Tables 1 and 2) and ultimately of the calving to-calving interval. Heat detection is a time consuming repetitive chore that must be carried out up to 5-times a day each day for as long as AI is used. Heat detection rate, usually measured as submission rate, is hugely variable from herd-to-herd and between 30 and 70% of cows that exhibit heat are usually detected in heat by the stockman. The objective of this paper is to review physiological, management and environmental factors that affect the expression of oestrus and the technologies that have potential to improve heat detection rates.

Table 1. The effect of different heat detection and conception rates on the % of the dairy herd that is pregnant at 45 days after onset of breeding season.

		Conception Rate %			
		60	**50**	**40**	**30**
Heat Detection Rate %	**90**	96	91	83	71
	70	89	82	73	61
	50	76	68	59	48
	40	67	59	50	40

Table 2. The effect of different heat detection and conception rates on the % of the dairy herd that is non-pregnant after a 90 day breeding season.

		Conception Rate %			
		60	**50**	**40**	**30**
Heat Detection Rate %	**90**	4	9	17	29
	70	11	18	27	39
	50	24	32	41	52
	40	33	41	50	60

Endocrine control of oestrus

During the course of the oestrous cycle, waves of follicular growth occur at regular intervals, with two to four distinct cohorts of follicles emerging together during each cycle. Each follicle wave has an inherent lifespan of 7-10 days as it progresses through emergence, selection dominance and atresia or ovulation. The emergence of each new wave is stimulated by a transient (1-2 day) increase in FSH (Adams *et al*., 1992; Sunderland *et al.*, 1994; Stagg *et al*, 1998) with selection occurring during declining FSH concentration, and the dominant follicle then maintaining FSH at nadir concentrations until it either ovulates or succumbs to atresia, depending on the pattern of FSH secretion. The first or subsequent dominant follicle of the cycle is capable of producing sufficient oestrogen to induce oestrus and subsequently ovulate, if the corpus luteum is lysed by exogenously administered $PGF_{2\alpha}$ (Stevenson *et al*., 1998). Oestrogen, specifically oestradiol-17β, is the primary signal to the hypothalamus that induces oestrus, but only in the absence of progesterone (Vailes *et al*., 1992). When progesterone reaches a threshold concentration early in the luteal phase, it can inhibit the occurrence of oestrus (Vailes *et al*., 1992).

It appears that stressors which elevate blood concentration of cortisol are capable of delaying or blocking the preovulatory LH surge and affecting the expression of oestrus without altering pro-oestrous concentrations of blood oestradiol 17β (see review by Stevenson *et al*., 2000). Occurrence of oestrus seems to be an all-or-none phenomenon with the intensity of oestrous expression apparently not related to either dose or blood concentrations of oestradiol-17β (Coe and Allrich, 1989).

Duration of oestrus

The duration of the oestrous cycle averages 21 days in cows within a normal range of 18-26 days (Woody *et al*., 1965, Mulvehill, 1977) and 20 days in heifers (Gordon, 1976). Published estimates of the duration of oestrus vary considerably. This variation is probably due in part to different criteria used to define oestrus (Table 3).

Table 3. Summary of the average duration of oestrus in cattle

Study	Method of observation	Animal Category	Average duration (hours)
Trimberger, 1948	Observation	Dairy cows	17.8
O'Farrell, 1980	Observation	Dairy cows	8.8
Dransfield et al., 1998	HeatWatch	Dairy cows	7.1
Xu et al., 1997	HeatWatch	Dairy cows	8.6
Mawhinney & Roche 1980	Observation	Beef cows	12.8
Trimberger 1948	Observation	Heifers	15.3
Mawhinney & Roche 1980	Observation	Heifers	12.8
Diskin 2000(unpublished)	HeatWatch	Heifers	14.5

Breaks or quiescent interludes in standing activity have also been observed (O'Farrell, 1980; Allrich, 1993; Stevenson *et al*, 1996). O'Farrell observed that breaks in standing activity occurred in 30 % of dairy cows at pasture while Stevenson *et al*., (1996), using HeatWatch, recorded breaks with an average duration of 2.6 hours in 67% in beef heifers.

Pattern of heat onset: The onset of heat activity follows a distinct pattern, with greatest activity in the early morning and late evening. Data from this laboratory for dairy cows at pasture is presented in Table 4.

Table 4. Percentage of dairy cows first observed in standing heat at specific hours

Hours	**07:00**	**10:00**	**13:00**	**16:00**	**22:00**
Percent detected	40	5	7	18	30

From this data we conclude that careful checking for heat in the early morning and late evening minimises the night interval and results in the detection of at least 70% of cows in heat. Three further checks during the day, at about 4-5 hour intervals, are required to detect 90% of the cows in heat.

Factors affecting the expression of heat.
Numerous factors affect the expression of heat, the more important of which are briefly discussed.

Housing arrangement: For satisfactory expression of heat cows must have adequate space to allow cow-to-cow interaction. If the stocking density is too high the expression of the signs of heat are reduced, consequently making detection more difficult. Checking cows in holding pens or collecting yards is not to be recommended.

Milk yield: There appears to be a weak antagonistic relationship between milk yield and expression of heat with higher yielding cows showing slightly weaker signs of heat than lower yielding herd mates (see review by Stevenson *et al*, 2000).

Floor surface: Cows dislike being mounted while standing on concrete and have a preference for softer underfoot surfaces such as grass, dirt or straw bedded yards. Mounting activity was reduced by almost one half when cows are kept on concrete as opposed to softer underfoot conditions while the duration of oestrous activity was reduced by about 25% (Britt *et al.*, 1986). Cows distinctly dislike being mounted by herd mates if the floor surface is either slippery or very coarse.

Feet and leg problems: Cows with sore feet or legs or that have poor structural conformation exhibit less mounting activity and have fewer "stands". Furthermore, such cows may well stand to be mounted when not in heat because it is too painful to escape from the mounting cow. Consequently, lame cows have significantly longer calving to service and calving to conception intervals.

Status of herd mates: The number of cows in heat simultaneously has a major impact on overall heat activity in the herd and on the average number of mounts per cow (Table 5). The number of mounts per cow increases with the number of cows that are in heat simultaneously up to about 3-4 cows in heat. Thus, in smaller herds and as more cows become pregnant the likelihood of more than one cow being heat on any given day becomes less, consequently, making heat detection more difficult.

Table 5. Effect of number of cows in heat on mounting activity

Number of cowsin heat simultaneously	Average mounts per cow in heat
1	11.2
2	36.6
3	52.6
4+	49.8

Source: Hurnick *et al*., 1975.

To be detected in standing heat a cow she must engage the attention of a herd mate willing to mount her. Generally cows that are themselves in heat, coming into heat or were recently in heat are most likely to mount a cow that is in heat. Cows that are at the mid-stages of their cycles (Day 5 to about day 16) are least likely to mount a cow that is in heat and consequently could be termed "poor heat detectors". Similarly, cows that are pregnant show less interest in mounting other cows that are in heat. Consequently, as more cows in a herd become pregnant it become increasingly difficult to identify the few remaining open cyclic and repeating cows.

About 10% of the reasons for failure to detect heats can be attributed to cow problems and 90% to "management" problems. The latter would include too few observations per day for checking for heat activity, too little time spent observing the cows or observing the cows at the wrong times or in the wrong place such as at feeding time or in the collecting yard at milking time. Another major reason for failure to detect heat is that those involved in heat detection do not understand the signs of heat. To optimise heat detection a number of the signs of heat, both primary and secondary, must be clearly understood.

Primary Signs of Heat

Standing to be mounted by herd mate or bull is the most definite and accurate sign that a cow is in heat. During the period of standing heat cows stand to be mounted by other cows or move forward slightly with the weight of the mounting cow. Cows that move away quickly when a mount is attempted are in not true heat.

Secondary signs of heat

Because standing heat may not always be observed stockmen must frequently use other signs of heat in arriving at a decision as to whether or not to inseminate a cow. These secondary signs of heat and may indicate that a cow is coming in heat, in which case closer attention should be given to her over

the following 48 hours, or they may be indicative of a recent heat in which case she should be given close attention 17-20 day later.

1. **Discharge of clear mucus:** This originates in the cervix and uterus and is a good indication of imminent heat. The passing of long clear elastic strings of mucus is indicative of an imminent heat while a thicker cloudier and more viscous mucus is indicative of a recent heat.
2. **Mounting other cows.** Cattle that mount other animals may be in or approaching heat. Generally, cows that are at the mid-cycle stage of their oestrous cycles or that are in-calf perform mounting activity much less frequently.
3. **Restlessness:** Signs of restlessness such as increased walking, trailing of other cows and bellowing are characteristic of individual cows that are either approaching or are in heat.
4. **Swelling and reddening of vulva:** Hormonal changes associated with heat cause an increased blood supply to the reproductive organs which in turn causes swelling and reddening of the vulva.
5. **Hair loss and dirt marks:** As a result of frequent mounting by herd mates, the hair on the tail-head is usually removed and the skin on either side of the tail-head is often scarred and dirty. This is indicative that the cow was recently on heat.
6. **Blood stains on the tail or vulval area (metoestrous bleeding):** These are indicative of a recent heat. Such animals should be watched closely for heat 17-20 days later.
7. **Decreased feed intake and milk yield.** Cows in or approaching heat spend less time feeding. In some studies a drop in milk yield has also been observed. However, because numerous other factors affect milk yield this should not be taken as a reliable sign of heat.

Improving heat detection efficiency

The single most important factor affecting heat detection efficiency is that those responsible for checking for heat should fully understand the signs of heat and be fully committed to heat detection for as long as it is planned to use AI.

Records: Individual animal records are an essential part of good breeding management. All animals must be clearly and permanently identified by one of several methods, such as plastic ear tags, neckbands or freeze branding. Whichever system is preferred, it is essential that the animal number be clearly legible from a reasonable distance. Breeding records should include (i) animal number (ii) calving date, and other information relevant to the calving (iii) pre-breeding heat dates (iv) first and repeat service dates and sire used on each date and inseminator code (v) date and result of pregnancy diagnosis and, (vi) date of expected calving. Good records are not only part of good farm management practice but are the first essential step in all infertility investigations.

Monitoring submission rate: This is calculated as the proportion of cows calved at the beginning of the breeding season, that are intended for re-breeding and that are submitted for insemination. A submission rate of at least 80% should be achieved in the first 21 days of the breeding period. Submission rate, which is easily calculated, is an excellent measure of heat detection rate and should be calculated at the end of the first 21 day period of the breeding season. A submission rate of less than 80% indicates a problem with heat detection and diagnosis of this problem at such an early stage allows corrective action be taken before much of the breeding period has elapsed.

Pre-breeding heat detection: For seasonally calving herds heat detection should ideally commence 3 weeks before the planned onset of the breeding season. All cows calved 3 weeks before the planned onset of the breeding season should be tail painted and checked 3-times daily until the onset of the breeding season. Cows not observed in heat during this 3-week pre-breeding period and, that are more than 42 days calved, should be either selected for examination by a veterinary surgeon or identified for special observation during the first 3 weeks of the breeding season. If such cows are not observed in heat during this second 3-week period they should definitely be examined and preferably scanned by a veterinary surgeon to identify the cause of the "apparent" non-cyclicity. The objective is to identify and treat potential problem cows early in the breeding period and thus enhance their chances of being successfully bred.

Synchronisation to reduce heat detection: While methods of controlled or synchronised breeding were developed with the objective of achieving compact calving, they can play an important role in reducing the burden of heat detection. If efficient heat detection is not possible an alternative strategy would be to synchronise all cows calved at least 35 days at the start of the breeding period. Synchronisation allows fixed-time AI without reference to heat. Most of the repeat heats fall over a confined period and can be easily detected. Where calving is compact, about 70% of cows and all the replacement heifers are available at the start of the breeding season. A synchronised and one repeat insemination should provide adequate herd replacements. Synchronisation procedures for both dairy (Diskin *et al*., 2000) and beef cows (Roche *et al*., 1999) have recently been reviewed. The former study clearly shows that oestrus or ovulation control programmes have significant benefits particularly in herds where existing levels of heat detection are low but have lesser benefits in herds where heat detection rates are already high.

Technological aids to improve heat detection

The low to moderate heat detection efficiencies achieved on most farms reflect the difficulty of detecting heat in cows. While undoubtedly this low detection efficiency is mainly management-related, cow factors also contribute. The latter include the variable signs of heat, the propensity for many cows to show heat from late evening to early morning and the short duration of standing heat all of which add to the difficulty of achieving high heat detection rates. Consequently, it has been and is the goal of many animal science programmes to develop more objective systems to overcome some of the problems of heat detection. An ideal system for detecting oestrus should have the following characteristics (Senger, 1994): 1) continuous surveillance of the cow; 2) accurate and automatic identification of the cow in oestrus; 3) operation for the productive lifetime of the cow; 4) minimal labour requirements; and 5) high accuracy and efficiency (95%) for identifying the appropriate physiological events that correlate with oestrus or ovulation or both. A number of both inexpensive to expensive aids and technologies are available to meet some but not all of these criteria. In any case, use of various technologies to identify symptoms associated with oestrus, ovulation, or both will require judgment of herd management to verify whether or not the cow seems to be in oestrus based on common husbandry experience.

Tail-painting: Research from a number of laboratories have shown that applying paint or chalk to the tailhead of cows is effective in indicating standing activity (Foote, 1975; Macmillan & Curnow 1977; O;Farrell. 1980). When such "tail painted" cows are mounted from the rear some or all of the chalk or paint is rubbed off indicating that the painted cows possibly stood in oestrus while mounted by a herd mate. When a cow stands in heat this strip of paint is either partially or totally removed by the mounting animal. Combined with early morning and late evening observations, checks for paint loss at milking times should result in a heat detection rate of close to 90%. Efficiencies of oestrous detection using tail paint vary from 44 to 96% (Macmillan & Curnow, 1977; Pennington & Callaghan, 1986; Sawyer *et al*., 1986)

Vasectomised bulls with chin-ball marking harness: Active vasectomised teaser or detector bulls are useful in identifying cows either coming into or on heat. Vasectomy should be carried out 40-60 days prior to introduction to the herd. Many herds are now finding that teaser bulls are particularly useful after the first 3 weeks of the breeding season when fewer cows are in heat each day and when the level of heat-related activity in the herd is reduced as more cows become pregnant. However, considerable variation in libido exists among bulls and they require the same management as full bulls without conferring any of the advantages. As an alternative to vasectomised bulls cows or heifers treated with testosterone or oestradiol can be useful in detecting cows in oestrus. A full description of hormonal treatments regimens for such females is given by Stevenson (2000).

Pressure activated heat mount detectors
These devices including these marketed as Kamars, Bovine Beacon and Mate Master are affixed to the tail head of the cow and change colour when pressure is applied by the weight of the mounting animal. Reported efficiencies of heat detection using such heat mount detectors vary from 56 to 94% while the accuracy of heat detection is reported to vary from 36 to 80% (see review by Stevenson, 2000). The relatively low accuracy of heat detection combined with difficulties in keeping the devices affixed to the tail head limit the potential of this approach.

Pedometers: Oestrus in cattle is accompanied by increased physical activity. Cows that are in heat do 2-4 times more walking than a non-oestrous cow. Pedometers can be attached to the leg of the cow to measure the amount of her activity over a unit time span. Earlier pedometric-aided heat detection systems operated with a reported heat detection efficiency of 60-100% and with an accuracy in the range of 22 to 100%. The low level of accuracy was related to a high proportion of false positives and to technical problems that led to either breakage, malfunction, or loss of the pedometers (see reviews by Lehrer *et al*., 1992; Senger, 1994; Stevenson, 2000). New improved pedometric technology has now led to improved information storage systems, improved analytical capabilities to allow comparison of current with previous physical activity, incorporation of internal power supply to operate the electronics, the development of self-contained devices to interrogate the pedometers in milking parlour and relay or store information in a personal computer. Some systems have an inbuilt alert system such as a bleeper or flashing light which alerts the farmer when a cow is deemed to be in heat. A number of pedometric systems are commercially available in the US. While scientific information on their operating efficiencies is not yet available these systems would appear to have significant commercial potential.

Radio telemetric devices: The primary sign of heat is standing to be mounted. A number of research laboratories have attempted to develop pressure sensitive devices that measure such standing activity. Such a system (HeatWatch; DDX INC, Colorado, USA) is currently commercially available in the US and in a number of other countries. This system involves the location of a pressure sensitive battery-powered transmitter on the cows tail head which, when activated by the mounting cow emits a radio signal which is picked up by either a receiver or repeater (see Figure 1) and relayed to a buffer and ultimately to a personal computer where the information is digitized and stored. The time, date and duration of each mount along with the identity of each cows is recorded. From this information the time of heat onset is calculated. The HeatWatch software generates management and individual cow reports that can be viewed or printed. HeatWatch classifies a standing heat as a cow having 3 standing events in a four-hour period. A cow with fewer standing events is recorded as a "suspect heat" and such a cow should be checked for secondary signs of heat prior to deciding to inseminate her. Periodically during the day the farmer checks the computer for a listing of cows in heat. The limited data available suggests that HeatWatch operates with both an efficiency and accuracy of almost 100% in detecting cows in heat Stevenson *et al*, 1996; Walker *et al*., 1996; Nebel *et al*., 1995; Xu *et al*., 1997). The limited data from this laboratory suggests that HeatWatch is an extremely powerful research tool, operates well under both indoor and pasture conditions.

As a lower cost alternative to the complete HeatWatch system DDX have recently launched a system **(MountCount)** that counts the number of mounts received by a cow in heat. The MountCount is a manual version of the current HeatWatch transmitter. The battery-powered unit is affixed to the tail head of the cow. It contains a pressure switch that is activated when the cow is mounted. When a certain threshold of mounts is reached a light is activated on the device the alerts the farmer that the cow is in heat and should be inseminated. Different flashing light patterns alert the farmer when the cow is in suspect heat, standing heat and when she is considered ideal for breeding.

Yet a further DDX heat detection product also cheaper that the HeatWatch sytem is HeatWatch Express. This does not need a computer or software to process and display the data. Again like the other DDX systems a small batter-powered radio transmitter is affixed to the tailhead of the cow. The mount data is relayed to a radio receiver and then to a buffer in the farm office from which the data can be printed. The cost of this product is about $2000 or about £1700.

IMV Technologies in France have also developed a pressure sensitive mount count detector. This system known as **DEC** is commercially available in Ireland. The device is programmed in such a way that when a certain number of valid mounts have been recorded a light, incorporated into the DEC, starts to flash. The number of flashes is in proportion to the time elapsed since the first valid mount was recorded. The number of flashes in a 10-second period indicates the time of heat onset and most appropriate time to inseminate the cow.

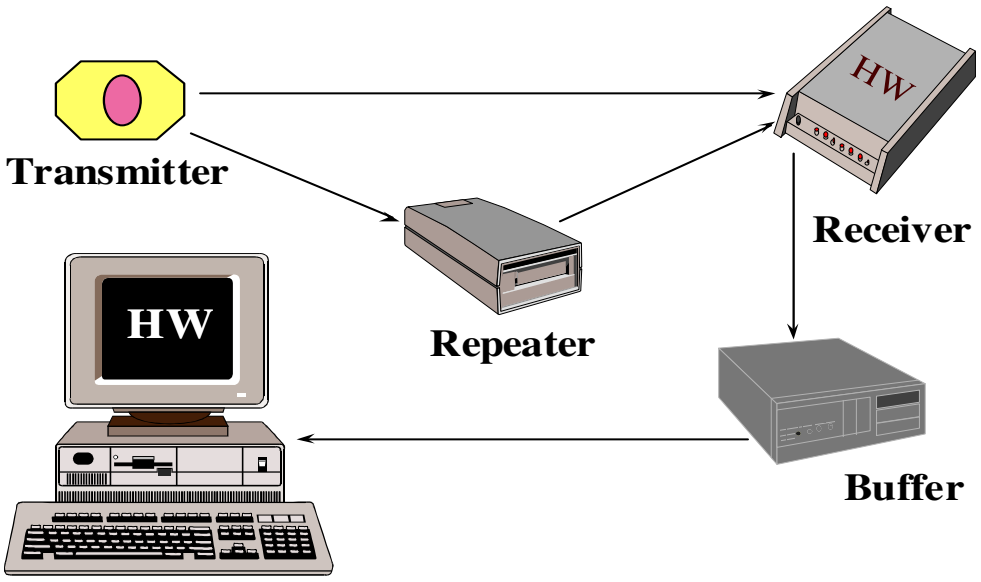

Figure 1. Components of the HeatWatch 24 h oestrus detection system. (Courtesy DDx, Inc.)

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REPRODUCTIVE BIOTECHNOLOGIES FOR ENDANGERED MAMMALIAN SPECIES

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Abstract *- Assisted reproductive techniques (gamete cryopreservation, artificial insemination, embryo transfer, and in vitro fertilization) allow to propagate small fragmented populations of wild endangered species or domestic breeds. This is the best way for producing several offspring from selected genitors in order to avoid inbreeding depression. However, few mammalian species are well studied for their reproductive biology whereas huge differences have been observed between these species. Furthermore, materials, methods and experimental designs have to be adapted for each case and each limiting factor (wildness, poor quantity of biological material, disparate locations). Genome resource banking is currently arising and the most applied reproductive biotechnology remains the artificial insemination. Assisted reproductive techniques currently developed in domestic species (intra-cytoplasmic sperm injection, nuclear transfer) may offer new opportunities for propagation of endangered species.*

endangered species /artificial insemination/cryopreservation/in vitro fertilization/embryo

Résumé - Les techniques de reproduction assistée (cryoconservation des gamètes, insémination artificielle, transfert embryonnaire, fécondation *in vitro*) permettent d'accroître des populations, parfois dispersées géographiquement, d'espèces sauvages ou domestiques en voie de disparition. Ces méthodes sont le meilleur moyen pour produire plusieurs descendants à partir de géniteurs sélectionnés de façon à éviter la consanguinité. La biologie de la reproduction est connue pour peu d'espèces de Mammifères alors que de grandes différences ont pourtant été mises en évidence entre ces espèces. En outre, les matériels, les méthodes, ainsi que les schémas expérimentaux doivent être adaptés à chaque cas et pour chaque facteur limitant (animaux sauvages, peu de matériel biologique, populations dispersées). Des banques génétiques sont actuellement en voie de constitution mais la technique la plus utilisée reste l'insémination artificielle. Cependant, des biotechnologies de la reproduction en cours de mises au point chez les animaux domestiques (injection intra-cytoplasmique de spermatozoïde, transfert nucléaire) pourraient offrir de nouvelles possibilités pour l'accroissement de populations menacées.

espèces menacées/insémination artificielle/congélation/fécondation in vitro/embryon

1. INTRODUCTION

The pressure of selection has resulted in thousands of different mammalian species, each with their own genetic make-up and each adapted to its own environment. Extinction of species is part of the natural process of evolution and is irreversible, but is now occurring at a much higher rate than speciation because of human activities, such as habitat destruction, over-hunting, or competition with introduced herbivores. For some domestic species, extinction has been rather due to intensive selection of few breeds imposed by management techniques and market demands. The aim of animal conservation is to maintain biodiversity because removal of a single species can affect the functioning of entire ecosystems [25].

A species is endangered when its survival in the wild is unlikely if causal factors of extinction continue to operate. Threatened populations may be extinct in the wild and composed by less than 50 mature individuals raised in captivity. For domestic breeds, populations are considered as endangered when it remains less than 1000 females or less than 20 fertile males [25]. Thereafter, factors that may reduce the population size of a small breeding group of animals may be the variations in litter sizes, a skewed sex ratio in offspring, preferential mating between individuals, random fluctuations in birth and death rates, overlap of generations [3]. Isolated populations have little or no genetic exchange between them and the main problem is the mating of closely related animals that increases homozygosity and inbreeding depression. The lack of genetic diversity leads to a bad adaptive capacity and risks of transmission of inherited diseases, congenital defects and fertility problems [74, 57].

Virtually, habitat preservation is the best way to conserve biodiversity [72], but small population propagation is part of a multidisciplinary research including genetic and ecological characterizations and needs further strategies. In situ conservation enables to maintain live population of animals in their adaptive environment (usual strategy for endangered domestic breeds). But these efforts are sometimes insufficient to propagate small populations and maintain adequate genetic diversity. Thus, ex situ conservation aims to establish a viable population in captivity for eventual reintroduction and to cryopreserve animal genetic resources (gametes, embryos, DNA, serum). However, reproduction process may be impaired in captivity by small space, health and husbandry problems, non-adapted diet, modified sexual behavior or infertility [40]. Therefore, field conservation and captive breeding need the help of assisted reproductive techniques (ART) including gamete cryopreservation, artificial insemination (AI), embryo transfer and in vitro fertilization (IVF). ART allow to obtain more offsprings from selected genitors to ensure genetic diversity and may reduce the interval between generations. As we will analyze in the first part of this review, utilization of reproductive biotechnologies for endangered mammalian species is not easy because of the broad biological variability between species and the sparse knowledge about it. In a second part, the current status of the ART in endangered domestic breeds and non-domestic species will be reviewed.

2. LIMITING FACTORS AND STRATEGIES WHEN USING REPRODUCTIVE BIOTECHNOLOGIES FOR ENDANGERED SPECIES

2.1. Great variability in reproductive physiology, anatomy and behavior

Success in producing new individuals with the help of ART requires as a first step a greater knowledge in basic aspects of reproductive biology. Among more than 4000 mammalian species, fewer than 100 have been studied to any descriptive detail of their reproductive biology, many of them are livestock and laboratory animals [40, 72]. Unfortunately there are few studies about wild species which differ enormously in physiology, anatomy and behavior.

Different reproductive strategies are used by eutherian mammals for the control of ovulation and pregnancy [3, 2, 17]: spontaneous ovulation (ruminants), ovulation induced by coitus (felids), luteal life span not prolonged by mating (canids), embryonic diapause (mustelids, roe deer, bears, seals), extra corpora lutea during pregnancy (equids, deer). Furthermore, marsupials differ from eutherian species in several aspects of their reproduction [44]. Variations also exist in the reproductive regulatory processes within the same genus. In deer species, breeding seasons are not similar and not controlled in the same way; breeding is aseasonal in axis deer (Axis axis), rusa deer (Cervus timorensis), and sambar (Cervus unicolor) whereas red deer (Cervus elaphus) and sika deer (Cervus nippon) are seasonal breeders [31]. Melatonin treatment is an efficient strategy to control the circannual cycle of reproductive activity in red deer [1] but not for all seasonal species. Length of the estrus cycle may vary from 18 days in red deer to 27 days in white-tailed deer (Odocoileus virginianus), and gestation period lasts from 180 days in hydropotes (Hydropotes inermis) to 285 days in Pere David's deer (Elaphurus davidianus) [31]. However, hormone profiles (ovarian activity) of different close species do not differ enormously as shown in felids [7]. In addition to the divergent physiology, oocytes, spermatozoa, embryos or cells from different species usually require different nutritive media for in vitro culture, these media have yet to be defined for most endangered species.

In order to assess the moment of the estrus cycle, vaginal cytology is not well adapted to wild species, but modern approaches to non invasive endocrine monitoring play an important role in optimizing the success of breeding programs [61]. Fecal steroid metabolites analysis have been used to estimate the pregnancy rate of free ranging herd [63], or to assess the reproductive status of males and females for various species as sika deer, wild black rhinoceros (Diceros bicornis minor) and clouded leopard (Neofelis nebulosa) [75, 22, 8]. Ovarian cycle may be also characterized by steroids and peptides analysis in urine [55]. In Asian and African elephant (Elephas maximus, Loxodonta africana), ultrasonography is also a good tool for characterization of the female reproductive status, for monitoring of the ovarian function or assessing the male reproductive tract [27].

For AI, it is important to know precisely the appropriate site to deposit the sperm (vagina, cervix or uterus) and the appropriate time during estrus. Because actual time of ovulation may be difficult to assess, the best strategy is to control the ovarian functions in order to detect and manipulate more easily the sexually receptive period of females. Unfortunately, commercially available gonadotropin preparations are not efficient in all species, ovarian responsiveness to synchronization treatments may be variable [59, 68].

For every species, there are also technical limitations linked to various anatomies. In small size animals, as in common marmoset monkey (*Callithrix jacchus*), sperm recovery by vaginal washing after copulation is the best collection technique [49]. When animals are too small for ultrasonography or blood collection, they may also benefit of ovarian monitoring or gestation diagnosis by fecal steroid metabolites as shown in the pygmy loris (*Nyctecebus pygmaeus*) [34]. In contrast, manual collection of elephant semen seems to be more efficient with the help of ultrasonography [27]. Genital tracts have also anatomical species specific characteristics [59], especially in marsupials that have two separate uteri, each connected to lateral vaginae by twin cervices [44]. Transcervical embryo recovery or AI may be achieved in most large mammals, but some species have impenetrable cervix like giraffe (*Giraffa giraffa*) and okapi (*Okapia johnstoni*) [42], thus laparoscopic methods offer a good alternative for these kinds of animals.

In wild species, sexual and social behaviors play also a key role for application of reproductive biotechnologies. In deer species, only the dominant stags may be collected because only these individuals produce good quality sperm. In a group of animals, only one female may be sexually active. To reduce the vulnerability to predators, some species (e.g. *Oryx dammah*) exhibit a small window of receptivity to mating [42]; thus monitoring of the ovarian activity by fecal steroid metabolites is once again a good strategy in this case. Captivity may also induce physiological or behavioral troubles, propagation may be impaired because of sexual incompatibility between paired individuals (aggressiveness), and sexual activity may be also modified in solitary animals [40]. In order to reduce handling stress that lead to pathologies or traumatism, manipulation of deer species are performed in darkness. Semen collections of aggressive males are feasible with the help of internal artificial vagina or vaginal condom. Thus reduced handling and non-invasive method (administration of drugs and hormones with projectile dart, non-surgical method for AI or embryo recovery and transfer) are suitable for endangered wild species.

2.2. Few individuals are available and sometimes in widely disparate locations

In captivity, 200 to 250 individuals in disparate locations often compose populations of endangered wild species. For breeding programs, number of founder animals should be as large as possible to maximize genetic diversity. Animals with difficulties in collecting, cryopreserving or transferring their gametes, or individuals dispersed geographically could not be discarded from ART program [3]. Technical adaptations such as portable incubator or mobile laboratory may solve the problem of time elapsed between the gamete recovery in the field and its treatment (cryopreservation, culture) [10].

The poor availability of biological material is a major limiting factor for the study of reproductive physiology and the set up of adapted ART in endangered species. Alternative methods are necessary to characterize some parameters and to select the best donors. We therefore used heterologous in vitro fertilization (IVF) with zona-free in vitro matured bovine oocytes in order to assess the fertilizing ability

and the developmental potential of cryopreserved semen from different stags. In vitro capacitation of the Oryx sperm (Oryx dammah) [58] and fertilizing ability of different spermatozoa of the genus Bos [46] have been assessed by heterologous IVF with bovine oocytes. Oocyte penetration assay is also used for testing sperm in canids [26].

Background data are often sparse and opportunities for research may be limited. Another way to solve the problem could be the use of a closely related non endangered species as a model for the study of physiological parameters or set up of techniques. For example, a third of the world deer species are rare or endangered but the reproductive physiology is supposed to be close to the common species studied for farming [3]. There are other models like domestic bovines for wild oxen [62], domestic cat for endangered felids [54], domestic dog for fox [17], common marmoset monkey for endangered callithrix species [43], South American camelids for endangered camelids [6]). Domestic ferret (Mustela putorius furo) and domestic rabbit (Oryctolagus cuniculus) have been also studied for developing non-surgical method of embryo collection and transfer in small species [35].

Since the number of individuals is often poor, the number of recipient mother for embryo transfer is also a limiting factor for a breeding program. Interspecies embryo transfer is therefore a key technique in the conservation of endangered species by choosing appropriate related surrogate species with similarity between body size, estrus cycle and gestation pattern. Embryo transfer of gaur (*Bos gaurus*) embryos in Holstein cows has been the first successful interspecific embryo transfer [64]. Other examples concerned wild horse embryos transferred into domestic horses [65], Indian desert cat embryos (*Felis silvestris*) into domestic cat (*Felis catus*) [54], mouflon embryos (*Ovis orientalis*) into domestic sheep (*Ovis aries*) [19]. Successful interspecific-bispecific transfers (Spanish ibex embryos (*Capra pyrenaica*) + goat embryos) into domestic goat have been also reported [18]. Even if treatment with interferon could reduce embryonic loss due to asynchrony between the embryo and the recipient mother [15], the immunological barrier remains a major restriction for interspecific pregnancies. In deer species, common related subspecies are easier to find. In our endangered deer breeding program [45], Japanese sika deer hinds (*Cervus nippon nippon*) will serve as surrogate mothers for Vietnamese sika deer (*Cervus nippon pseudaxis*) or Formosan sika deer embryos (*Cervus nippon taiouanus*). When there is no related surrogate species (e.g. panda, *Ailuropoda melanoleuca*), interspecific embryo transfer could be feasible by creation of chimeras (trophectoderm of the recipient mother with inner cell mass of the endangered species) as previously shown in ovine chimeras [9]. However, interspecies embryo transfer will require further studies about the sexual preference of offspring after interspecific birth.

Physiological, anatomical, and behavioral knowledges are not limiting factors when using reproductive biotechnologies for endangered domestic breeds. Indeed, more common related breeds are often studied. Individuals are not in disparate locations, and appropriate surrogate mothers are easy to find for intraspecific embryo transfer. The main problem is to propagate a small population avoiding the genetic drift.

2.3. Regulations and institutional support

In addition to practical or technical limiting factors, institutional and economical constraints are also to take into account. Even if the 'Convention on International Trade in Endangered Species' (CITES) play a key role in animal conservation, rules bring sometimes new problems to conservationists by limiting the acquisition of animals that are needed to maintain traditional captive breeding programs. Furthermore, embryo importation and cryobanking strategies for wildlife species are currently not well defined [60]. Institutional supports are important (e.g. Conservation Breeding Specialist Groups, Species Survival Plan, Taxon Advisory Group, World Watch List of FAO for domestic animal diversity, European global databank for farm animal genetic resource). However, species to save may be chosen according to different interests (political, cultural, economical) sometimes outside the control of biologists. The maintenance of large captive populations of wild animals in parallel to a reintroduced stock represents considerable problems in terms of costs. However this provides a back up for successive releases if there are problems in the reintroduced population. The most attractive strategy in respect of costs and low inbreeding is that involving cryopreservation of semen plus a breeding herd [41]. Long term financial support is also necessary as shown in the European Union (EEC 2078/92) which supports

breeders of endangered domestic breeds, but the questions are: which intervention strategies will have the desired effects for a precise situation, what is the costs/benefits ratio? Even if embryo transfer or AI are not the most efficient methods to quickly propagate small populations, it may be sometimes more suitable than sophisticated techniques (good AI program versus poorly efficient IVF program).

3. CURRENT STATUS OF REPRODUCTIVE BIOTECHNOLOGIES FOR ENDANGERED MAMMALIAN SPECIES

Kraemer [38] used for the first time ART in a wild species (embryo transfer in baboon *Papio sp.*). In Europe, ART have been used for endangered domestic breeds for more than 10 years.

3.1. Genome resource banking

Genome resource banking (GRB) refers to the collection, processing, storage and use of gametes, embryos and other biological material. GRB are in combination with ART, for interface with in situ and ex situ conservation [29]. It is currently more developed for rare domestic breeds (bovine, ovine, caprine, porcine), but the concept of using GRB to facilitate the management and the conservation of endangered species is being promoted extensively [72]. If properly used, GRB have the potential to decelerate the loss of gene diversity in captive populations through reintroducing original genetic material, without removing genetically valuable individuals from the wild. Like for set up of ART, factors that need to be considered in developing GRB are: the conservation justification, knowledge of life history and natural reproduction, knowledge of assisted reproduction, demographic distribution of donors and recipients, accessibility of donors for banking, type and amount of biomaterials to store. A small space for storage is needed but liquid nitrogen supply must be efficient. Furthermore, cryobanks have to be held in two different sites in order to avoid the risk of total destruction.

A bovine breed could be saved with 1000 sperm doses collected on 25 different males or 300 embryos (non-sexed) from 90 donors. Cryopreservation of embryos is currently not routinely possible in porcine but there is a semen bank for endangered breeds in Europe [39]. Another example is given by the embryo bank of White Caceres cattle breed [4]. In wild species, a GRB program (semen) has been initiated for the Siberian tiger (*Panthera tigris altaica*) [29], and the Wildlife Breeding Resource Center has established the first GRB in Africa.

3.2. Sperm collection and cryopreservation, artificial insemination

Semen collection may be achieved by artificial vagina, electroejaculation or flushing of the epididymis. These methods have been used successfully in deer species [3]. Additionally, post-coital sperm recovery has been also described in marmoset monkey or in rhinoceros (*Dicerorhinus sumatrensis*, *Diceros bicornis*) [49, 50]. Epididymal sperm has been successfully cryopreserved in chinchilla (*Chinchilla laniger*) [52] or in red deer [21], this method is suitable for cryopreservation of spermatozoa after death of the male or after the rut period.

Cryopreservation techniques are well controlled in domestic ruminants, but less in equids and porcine. For wild species, standard domestic animal extenders (TRIS-buffer + egg yolk) have been tried. We are currently cryopreserving ejaculated and epididymal sperm from different deer species by using a protocol developed for the ram semen [11]. However, physico-chemical requirements differ between species as shown by glycerol concentration tolerances: 5% in bovine, no more than 4% in deer, 3% in pigs, 1.75% in mice, 6% in Chinchilla, and large differences are also observed in marsupials [30]. But for white rhinoceros (*Ceratotherium simum*) sperm cryopreservation, glycerol seems to be not acceptable [73].

AI allows the controlled propagation of genetic material from selected males, it is the most extensively applied ART, examples include wild bovids, cervids, canids and wild felids [28, 48, 32, 54]. Intrauterine laparoscopically insemination is necessary when catheter insertion through the cervix is not possible or is ineffective as demonstrated in felids [67]. In the USA, implementation of an AI program for the black footed ferret (*Mustela nigripes*) allowed a significant propagation and the reintroduction of this threatened animal [72].

3.3. Induction of ovulation, superovulation, embryo collection and transfer

In sable antelope (*Hippotragus niger*) and in other wild ruminants, ovulation may be induced artificially by PGF2α injection or by removal of progesterone-releasing implants. Fecal steroid monitoring has been performed for assessing effectiveness of treatment and adapting the doses [68]. For felids, the induction of ovulation is possible at any stage of the reproductive cycle by using eCG and hCG injections but immunological reactions may impair the stimulation [67].

The advantage of superovulation is to propagate female genetic material. It has been already performed in various species as African antelopes, giraffe, deer, wild cattle, wood bison (*Bison bison*) and camelids [42, 51]. Unfortunately, as in domestic animals, exogenous gonadotropins may lead to abnormal oocyte or follicle development, immunization, and variable ovarian response.

For large mammals (bovids, cervids, equids), transcervical embryo collection or transfer are used [59, 42, 69]. However, laparoscopic embryo transfer is performed in various species (silver fox *Vulpes vulpes*, bear *Ursus americanus*, swine breeds) when non-surgical methods are not possible [32, 5, 56]. In wild species, scarse knowledges about kinetics of embryo development and foeto-maternal recognition may lead to asynchrony between the transferred embryo and the recipient mother. In a recent study in the red deer, treatment of recipient mother with interferon reduced significantly embryonic loss after asynchronous transfer [15]. For endangered wild species, cryopreservation of embryos remains to be developed. We are currently trying to develop vitrification procedure adapted to in vitro produced deer embryos.

3.4. In vitro production of embryos

It is the most efficient technique for propagation of small populations but it is also the most expensive method. There are different steps: gamete recovery, *in vitro* maturation (IVM) of the oocytes, sperm in vitro capacitation, *in vitro* fertilization (IVF), and in vitro development (IVD) of the resulting embryos. Immature oocyte recovery (by transvaginal or laparoscopic Ovum Pick-Up (OPU) on living females) enables to avoid the problem of timing of ovulation and allow to collect dead or sick females (e.g. with obstruction of genital tract), prepubertal or pregnant animals. In contrast to AI, handling is reduced and more embryos may be produced with the same dose semen.

IVM or IVF have been tried in various species as mink whale (*Balaenoptera acutorostrata*) [20], African elephant (*Loxodonta africana*) [36], gorilla (*Gorilla gorilla*) [53], and zebra (*Equus burchelli*, *Equus zebra*) [47]. In our deer preservation program, we developed a method for repeated immature oocyte recovery on live sika deer hinds by OPU through laparoscopy. We defined standard conditions adapted from domestic ruminants for IVM/IVF and IVD in the red deer and the sika deer before the application to related endangered subspecies [45, 15]. Maturation rates were about 75-80%, fertilization rates were 60% (with ejaculated or epididymal sperm), finally 20% of the fertilized oocytes reached the blastocyst stage after 6 days of culture in SOF medium. We noted that supplementation of media with biological fluids from the same species (follicular fluid, serum) was not necessary.

3.5. Other biotechnologies and future applications

Assisted hatching, embryo bisection, sperm or embryo sexing are not routinely used in domestic species, there are currently no references in endangered species. However, intra-cytoplasmic sperm injection (ICSI) is an alternative to IVF and may be a useful technique for endangered species when not motile sperms are retrieved from cadavers [37]. This ART is now performed in felids [72, 54], equids [23] and in the Rhesus monkey (*Macaca mulata*) [66], this last example has not only a laboratory interest but could be useful for endangered primates.

Restoration of species by transfer of somatic nuclei into enucleated recipient oocytes has been already considered. Calves of an endangered breed of cattle (Enderby Island) adapted to extreme climatic conditions were born after nuclear transfer of granulosa cells into enucleated oocytes from domestic cows and transfer of the resulting embryos in domestic cow recipients [70]. Additionally, a pregnancy was observed after the transfer of embryos reconstructed from cells of argali *Ovis ammon* and enucleated oocytes of domestic sheep [71]. In the giant panda, blastocysts have been also obtained after nuclear transfer of panda cells into rabbit enucleated oocytes [12]. Furthermore, it is clearly demonstrated that bovine oocyte cytoplasm could also serve as recipient for somatic cells from different mammalian species [16]. Cloning might even serve a useful purpose with species that have

never bred in captivity such as the giant armadillo (*Priodontes giganteus*), or the saola (*Pseudoryx nghetinhensis*).

Germlines preservation (male and female) followed by transplant in SCID mouse could be also an interesting alternative when unexpected death of valuable individuals (in complement with oocyte recovery, epididymal flushing and somatic cell collection). Moreover, techniques to rescue gonads (preantral follicles culture) have been already tried in non-domestic felids [33]. Finally, antral follicles development in xenografted cryopreserved elephant ovarian tissue [24] or spermatogonial sperm cell transplantation after thawing in mice followed by restoration of spermatogenesis [13] could be future reproductive biotechnologies for endangered species.

4. CONCLUSION

Application of reproductive biotechnologies for the preservation of endangered mammalian species is limited by several factors. Production of embryos and offspring depends on the existing knowledge on the reproductive physiology of each particular species and little is known about the physiology of most wild animals. Captivity and poorly available biological material (often in disparate locations) increase obstacles for research progress. Thus, ART progress for endangered species depends on multidisciplinary research. ART for endangered species are adapted from technologies developed in domestic species although all problems are not solved in these species (e.g. variable ovarian response to hormonal stimulation). Additionally, wild species are more sensitive to stress as compared with domestic ones and require to reduce the handling of individuals. Furthermore, the methods and materials used have to be adapted to allow the work in the field conditions. Implementation of ART program for endangered wild species are more rare than for endangered domestic breeds. However, all over the world there are endangered species or domestic breeds which may be candidates for conservation programs. An optimal genetic management system would consist of a captive population and a cryopreserved genetic resource bank in constant dynamic interaction. But ART are not the only solution for animal conservation, people education and habitat preservation are essential, and it is important to consider that a species requires a conservation action even if it is not threatened.

ACKNOWLEDGEMENT

The author thank the staff of the Espace Animalier de la Haute Touche. P. Comizzoli is supported by a grant (N° 97 298 035) from the Conseil Régional de la Région Centre.

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CURRENT PROGRESS ON ASSISTED REPRODUCTION IN DOGS AND CATS

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INTRODUCTION

The improvement of reproductive performances is the ultimate goal of assisted reproductive technologies (ART) and the main impulse to these studies in carnivores started when the scientific community began to be interested in the conservation of biodiversity. In fact, carnivores have been considered not only as pets, but also as comparative models for studies aimed at non-domestic canine and feline species.

In the last 200 years more than 50 animal species disappeared and more than 200 are considered endangered by the Convention of International Trade in Endangered Species [4]. There are 37 feline species and all of them, besides the domestic cat, are endangered, as well as some wild canine species.

Placing animals in captivity, even under intense management, does not always resolve the loss and variety of species or ensure that a viable population will be established. Certain species thrive in captivity and others experience subfertility or infertility [40].

Assisted reproductive technologies as artificial insemination (AI), in vitro fertilization (IVF) and embryo production, embryo transfer (ET) and gamete cryopreservation, are important biotechnologies for basic research, and for achieving improvements in reproductive performance. Among these, IVF and embryo production potentially are the most powerful reproductive tools currently available for studying fertilization and preserving genetic material of rare species.

Current progress in assisted reproduction techniques concerns mainly in vitro embryo production and the scope of this paper is to provide a review of this aspect of ART in canids and felids.

Successful in vitro techniques depend on the possibility to mimic in vivo conditions. For this reason knowledge on reproductive physiology is necessary to define an efficient entirely in vitro system to produce embryos.

IN VITRO EMBRYO PRODUCTION IN DOGS

In canine species the female gamete has unique characteristics compared to oocytes of many other domestic mammals. The main differences are represented either by the follicular environment or the oocyte meiotic stage at ovulation. In fact, in most mammalian species estrogen dominates the preovulatory follicular environment and the meiotic division is resumed shortly before ovulation as a consequence of the preovulatory LH-surge. Ovulated oocytes are in metaphase II of the meiotic division and are ready for fertilization.

In dogs, foxes and other Canids, ovarian follicles luteinize prior to ovulation, exposing oocytes to high concentrations of progesterone. After the LH-surge, the oocytes are ovulated spontaneously as primary oocytes, at the beginning of the first meiotic division. Subsequent stages of the meiotic maturation are resumed in the oviduct and take 2-3 days to complete [14].

The presence of primary oocytes in the oviducts increases the chance of the oocyte meeting spermatozoa before or during maturation and it has been shown in the fox that primary oocyte can be fertilized and a male pronucleus can be formed irrespective of the stage of oocyte maturation [7].
The canine embryos require long time for the passage of the oviduct and enter the uterus as a morula of 16 cells or more [14]. Thus, the oviduct supports long term survival of oocytes that in this tract complete maturation, undergo fertilization, and develop up to the morula stage. Moreover the canine oocyte is very rich in lipids and the cumulus cell mass around the oocyte is tight and multilayered and remains attached to the gamete longer after fertilization [37].
These interesting dissimilarities of reproductive physiology of the dog compared to other species and the lack of precise information on the oviductal environment, make the defined in vitro system routinely applied for oocytes of other species, unsuitable for canine oocytes.
In the last few years, research has been directed to the identification of adequate cultural conditions for canine oocytes, but still relatively limited success in terms of embryo development has been achieved.

In vitro maturation

Collection of follicular oocytes from ex situ ovaries obtained post mortem or at ovariectomy could provide a large source of potential embryos. A prerequisite for fertilization and embryo development is oocyte full nuclear (metaphase II stage) and cytoplasmic maturation.

In vitro oocyte maturation is a complex process in which the attempt is made to mimic the dynamic changes occurring in the preovulatory ovarian follicle and in the oviduct. A successful maturation system supports all components of the cumulus-oocyte complex, including the cumulus cells, nuclear material and cytoplasm.

In vitro maturation of canid oocytes is, at present, characterized by low and greatly variable success rates. It has been shown that canine oocytes resume meiosis in vitro, although at a much lower rate than oocytes of other species. In fact, full nuclear maturation is achieved in about 20% of cultured oocytes [6].

Low maturation rate could be due either to low meiotic competence of the oocytes or to suboptimal culture conditions.

In vitro studies allowed a better understanding and the definition of some parameters indicative of maturation competence. Morphological appearance of the cumulus-oocyte complex and the diameter of the oocyte, have been already identified as factors influencing *in vitro* maturation rates. In fact, oocytes collected from ovarian follicles with dark and homogeneous cytoplasm, completely surrounded by two or more layers of compacted cumulus cells and with a diameter of more than 110 μm, have better chances to mature *in vitro* [12].

Moreover, a technique for culturing oocytes in intact follicles dissected from the ovaries has been employed [3]. The results show that dog oocytes cultured within advanced preantral and early antral follicles *in vitro* are competent to resume meiosis to the metaphase II stage.

The immature stage of oocytes at ovulation and the persistence of cumulus cells during the transport and maturation period within the oviduct suggest that the investigation of the relationship between cumulus cells and oocyte could have contributed to clarifying the reasons behind the low efficiency of *in vitro* maturation of canine oocytes. It is well known, in fact, that in mammalian ovaries communications through gap junctions between the somatic compartment of the follicle and the oocyte are involved in the regulation of its meiotic differentiation and maturation leading to the acquisition of meiotic and developmental competence [30]. In fox oocytes gap junctions are present within the ovary and all junctional contacts between cumulus cell projections and the oocyte are disrupted 2-3 days after the LH-peak when the metaphase I stage is reached [15]. Viable and differentiated cumulus cells are needed to control the resumption of meiosis in vitro of fox oocytes [19]. These authors concluded that the role of cumulus cells in these species seems to be even more essential than in other animals.

In the bitch, it has been shown that the stage of the oestrous cycle influences the functional status of communications between cumulus cells and oocyte [23]. The results of this study indicated that dog cumulus-oocyte complexes isolated from the ovary during anoestrous are unable to complete meiosis and communications between the germinal and the somatic compartment through gap

junctions are absent, thus suggesting a relation between presence of communications and meiotic competence. This was consistent with the observation that open communications between cumulus cells and oocyte, were present in cumulus-oocyte complexes isolated during late proestrous, which were also capable to complete meiosis at a higher rate.

This finding seems particularly relevant to the understanding of the mechanisms which limit the efficiency of IVM in these species.

A similar effect of the phase of the oestrous cycle on meiotic competence of bitch oocytes has been previously described by Yamada et al. [44]. These authors reported that 32% of preovulatory oocytes collected from superovulated bitches reached metaphase II after 72 hours of culture, while oocytes from anoestrous bitches showed no tendency to resume meiosis even after a culture period of up to 144 hours.

Research is in progress for studying culture requirements for dog oocytes. Culture media such as Tissue Culture Medium 199, modified Krebs Ringer Bicarbonate or Hams F-10 with or without serum or hormonal supplementation [5, 27, 28, 44, 45] have been used for IVM, but the requirements of canine oocytes in culture have not yet been elucidated. Recently, Hewitt and England [13] investigated the effect of using an environment which is different to that normally used in studies of mammalian oocyte maturation in vitro and more similar in composition to oviductal fluid. These authors demonstrated that synthetic oviduct fluid (SOF, 39) with high concentrations of proteins (Bovine Serum Albumin) and in the presence of oviductal cells improved maturation rates, but only after a prolonged maturation time (96h).

All these studies confirm that the morphological appearance and diameter of the oocytes, the developmental stage of the follicles, and the stage of the cycle, coupled with culture conditions are important factors to take into account for the successful IVM of dog oocytes.

In vitro fertilization and embryo development

From what has been mentioned above concerning the difficulties encountered in canine oocyte IVM, it follows that results of in vitro fertilization and subsequent embryo development are still limited in this species.

In 1992 Yamada *et al*. [44] reported 2% of inseminated oocytes that reached the 8-cell stage and recently Otoi and coworkers [29] obtained one blastocyst out of 217 inseminated oocytes.

New microinjection techniques such as intracytoplasmic sperm injection (ICSI) have been reported in the dog [8] and male pronucleus formation was observed in 7.8% of oocytes, but no further cleavage occurred.

IN VITRO EMBRYO PRODUCTION IN CATS

Reproductive physiology of the domestic cat is characterized by induced ovulation. Ovulation requires the release of LH from copulation, even if recent studies have demonstrated that ovulation can also occurs in group-housed females in the absence of mating [20].

The ovulated cat oocyte is in metaphase II of meiosis and it is very dark in appearance because of a high intracellular concentration of lipid [11]. Moreover the cat zona pellucida is bilayered and the inner zona layer appears to function as a partial barrier to sperm penetration affecting the number and kind of sperm entering the oocyte [1].

In the last decade considerable progress has been made in in vitro embryo production in domestic cats and important goals have been achieved applying these techniques to non-domestic feline species [31].

In vitro maturation

Selection of oocytes and cultural conditions, as has been already stressed for dog oocytes, are the most important factors that affect *in vitro* maturation results.

Morphological criteria used to select cat oocytes do not differ from those used for canine oocytes and immature oocytes with dark and homogeneous cytoplasm, surrounded by compacted cumulus cells are destined to maturation *in vitro*.

The important role played by cumulus cells during maturation has been confirmed in cat oocytes by different authors [34, 43] that obtained the highest percentages of maturation and fertilization in vitro by selecting oocytes with intact cumulus cells.

Cultural conditions have also been investigated and gonadotropins were found to have a positive effect, but bovine serum [17, 24, 42] and estrual cat serum [10] had a negative effect on maturation compared to bovine serum albumin. Moreover it has been shown that antioxidant components, such as cysteine, significantly improve maturation rates that reach 70% [22].

In vitro fertilization and embryo development

Although cultural conditions for *in vitro* development of cat embryos are not yet completely defined, results are encouraging even though they are still lower than those obtained in other mammalian species such as bovine.

Cleavage rates *in vitro* range from 30 and 50% [18, 21, 38], and 20-30% of inseminated oocytes reach the blastocyst stage [34, 43]. Recently, *in vitro* development of embryos produced from IVM/IVF oocytes was improved (40% to >50% blastocysts) by adding cysteine during IVM and culturing in a reduced oxygen atmosphere [35].

Embryo transfer of in vitro derived embryos

The ultimate goal of the *in vitro* procedure is the transfer of produced embryos and the subsequent pregnancy in the recipient animal and in 1997 kittens were born after transfer of IVM/IVF derived embryos [34].

Recently, new techniques for producing embryos *in vitro* have been applied in cats. Microinjection of sperm cells in the cytoplasm of the *in vitro* matured oocytes (ICSI) resulted in cleavage rates ranging from 40-60% [2, 36] and in developmental rates to the blastocyst stage of around 15% [9, 32].

Up to now, kittens were born after transfer of the embryos obtained by ICSI only when *in vivo* matured oocytes had been used [33].

Cryopreservation of oocytes

To maximize the practical effectiveness of *in vitro* protocols in the preservation of genetic material of feline species, improved techniques for long-term storage of gametes and embryos would be very useful.

Sperm cells and *in vivo*- or in vitro-derived cat embryos have been successfully frozen [31], but cryopreservation of oocytes is still considered experimental because adequate rates of survival, fertilization and embryo development of frozen oocytes have been reached mainly with murine oocytes.

The cryopreservation of oocytes would greatly increase their availability to a range of reproductive technologies.

Wolfe and Wildt [41] observed that cat oocytes collected from antral follicles can be stored at +4°C for 24 h without loosing their ability to mature and develop *in vitro* after fertilization and small preantral follicles seem to be more sensitive to prolonged cold storage than larger preantral follicles. Recently, Jewgenow et al. [16] reported that preantral follicles from the domestic cat ovaries survive cryopreservation and remain structurally intact and physiologically active after thawing.

Moreover, it has been shown, by evaluating their subsequent development following IVM, that immature cat oocytes are able to survive after cryopreservation and results indicated that dimethylsulphoxide (DMSO) and ethylene glycol (EG) were both suitable cryoprotectants for slow freezing of immature cat oocytes. The ultrarapid procedure never resulted in resumption of meiosis in vitro, despite post-thaw intact morphology of the oocytes [26]. This demonstrates that morphology of oocytes after freezing and thawing is not predictive for ability to resume meiosis and it underlines the importance of freezing damage to the cytoplasmic system, which is not revealed by a

morphological evaluation, and which affects maturation.

Recently, it has been demonstrated [25] that cryopreserved cat oocytes can be fertilized successfully and that their development in vitro after fertilization is enhanced when mature oocytes (metaphase II stage) are frozen with a slow procedure and EG. In fact, the cleavage rate obtained after IVF of mature oocytes cryopreserved with EG was greater that the rate obtained by freezing oocytes at the immature stage (38.7% vs. 6.8% $P<0.001$). Moreover only those embryos derived from metaphase II oocytes developed beyond the 8 cell stage (11.3%), thus demonstrating a higher developmental competence than that of germinal vesicle stage (immature).

In order to facilitate the design of cryopreservation protocols which optimize the *in vitro* embryo development after thawing, further studies on intrinsic characteristics of cat oocytes are needed. In fact the permeability of the membrane or the presence of the intracellular lipid droplets can be responsible for uneven intracellular ice formation which could affect the freezing-thawing process.

SUMMARY

The objective of the development of assisted reproduction techniques in dogs and cats is their application to non-domestic canine and feline species, most of which are considered threatened or endangered.

In the last decade, progress has been made in assisted reproduction techniques in carnivores and a entirely in vitro system for embryo production is an important tool for conservation of wildlife.

It has been shown that canine oocytes can resume meiosis *in vitro* and that oocytes can be fertilized and developed *in vitro*, although at a much lower rate than most other domestic animal oocytes. The reason lies in the dissimilarities of reproductive physiology of the dog compared to other species and the lack of precise information concerning the oviductal environment, in which oocyte maturation, fertilization and early embryonic development take place.

Successful *in vitro* embryo production in the domestic cat has been attained with oocytes matured in vitro and kittens were born after transfer of IVM/IVF derived embryos. On the basis of these results the *in vitro* fertilization of oocytes has also been applied in several non-domestic feline species.

The effectiveness of such protocols in the preservation of genetic material of rare species can be improved by developing better techniques for long-term storage of gametes. In dogs and cats sperm cells have been successfully frozen and the cryopreservation of oocytes would greatly increase their availability for a range of reproductive technologies.

It has been demonstrated that cryopreserved cat oocytes can be fertilized successfully and that their development *in vitro* after fertilization is enhanced when mature oocytes are frozen.

Thus refined techniques of oocyte maturation and fertilization *in vitro* coupled with oocyte cryopreservation could allow for an easy establishment of genetic combinations when male and female gametes in the desired combination are not simultaneously available, and the propagation of endangered carnivores would be facilitated.

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SHORT COMMUNICATIONS

EVALUATION OF THREE FSH STIMULATION TREATMENTS AND A NEW METHOD FOR OVUM PICK UP (OPU) IN SHEEP

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Laparoscopic ovum pick up (OPU) in sheep has been recently developed and its performance evaluated. However, repeated treatments for ovary stimulation have not been studied enough. The aims of this work were to evaluate a new approach to perform follicular punction and to compare different strategies for ovarian stimulation with FSH. FGA sponges (40 mg) were inserted (day 0) to 12 Rasa Aragonesa adult ewes weighting 57.6 ± 6.3 kg. FSH treatments started at day 11 (total dose: 4.4 mg NADDK-oFSH-7 in 5 ml; OVAGEN). Ewes were allocated to be treated as follows: 1- Pump of permanent infusion (European patent nº 9902484, from GOTAGOT SL) of FSH to assure administering the total dose in about 30 hs (P group); 2- Application of whole FSH treatment in a single dose, half im and half sc (1I group); 3- Three decreasing im injections (2, 2 and 1 ml) at 12 hs intervals (3I group). OPU was carried out between 48 and 51 hs after the beginning of the FSH treatment and sponge was withdrawn at this moment. FSH and OPU were repeated 5 times, one week apart. Ewes were anesthetized with a mix of xylazine, ketamine and midazolam. Follicular visualization was made by means of a laparoscope connected to a video camera and the follicular fluid was aspirated through an Aspic currently used for intrauterine insemination (IMV) connected to a vacuum pump (-25 mm Hg; V-MAR 5100; Cook Ltd.). Three incisions were made to introduce the laparoscope, the catheter for Aspic and the atraumatic forceps. Follicular punction was made by observation of follicles on the monitor. Number of follicles greater than 2 mm and time of operation were individually recorded while oocyte quantity and quality, fertilization rate and embryo production were recorded grouping ewes within treatments. Oocyte maturation, fertilization and embryo development methods were previously described by Cognie *et al*. 1998 (J. Reprod. Fert. 112: 379-386). Follicle population was highly different between animals ($P<0.01$). Number of follicles punctionned per ewe and OPU session was higher in group 3I in comparison with group 1I (16.2 ± 5.9 and 10.8 ± 3.8 $P<0.05$) both being not significantly different from P group (15.2 ± 4.5). Oocytes recovered per ewe and session were 4.6 ± 1.6; 3.7 ± 1.5 and 6.2 ± 3.8 in groups P, 1I and 3I respectively ($P>0.05$). Quality of recovered oocytes (evaluated as the percentage of very good and good oocytes) was similar in all groups (84.8, 85 and 90% of recovered oocytes for P, 1I and 3I respectively; $P>0.05$) with no more than 7% of denuded oocytes. Fertilization rate (72.2; 61.5 and 54.5%) and blastocyts production in relation to the number of oocytes in culture evaluated in two OPU sessions (22.5; 24.1 and 12.2% for P, 1I and 3I respectively) showed no differences between treatments. Time expended on follicle aspiration was 20.5 ± 6.7min/ewe, without differences between treatments, OPU session or interaction and with a good repeatability within ewes. No adhesions in ovary or oviduct were observed and abdominal wall was in very good condition at the end of the experiment. In conclusion, the OPU system evaluated here was useful for the aims of our work and only FSH treatment in a single dose should be rejected. With this system it would be possible to produce about 2 embryos per ewe and session.

We are grateful to MEC for supporting the first author sabbatical year and to INIA for supporting experimental work (Proj. No SC-97-020-C2).

Notes

FIXED TIME INSEMINATION IN DAIRY CATTLE AS A TOOL FOR AN EFFECIENT REPRODUCTION MANAGEMENT

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Reliable oestrous detection in cattle is time consuming and expensive. Therefore the identification of a precise method of inducing and synchronising oestrous and ovulation in cattle would be an important advance in cattle reproductive management.
Many studies confirm that the method of luteolysis by application of $PGF_{2\alpha}$ is practicable. But in spite of a relatively uniform process of luteolysis ovulation ranged with large variations.
For that reason three experiments were conducted to establish a synchronisation regime with artificial insemination at a predetermined time in dairy cattle. In experiment 1, altogether 43 dairy heifers were synchronised at days 5, 8, 11 and 14 administering $PGF_{2\alpha}$ to record LH-peak. The release of LH ranged on average from 57.0 ± 8.6 h to 76.3 ± 10.0 h depending significantly on day of induced luteolysis. The portion of animals with LH-peak increased, when $PGF_{2\alpha}$ is injected later in the oestrous cycle. The release of LH was followed by ovulations. The mean intervals from LH-peak to ovulation were not considered significant between experimental groups.
In experiment 2 only heifers of day 8, 11 and 14 were synchronised. Animals got an injection of GnRH 65 h after induction of luteolysis. Uniform intervals from $PGF_{2\alpha}$ application to LH-peak and to ovulation were obtained in animals given $PGF_{2\alpha}$ on day 11 and 14. There were significant differences to animals starting luteolysis on day 8. But there were no significant differences between means in interval from LH-peak to ovulation. In experiment 3 a fixed time insemination scheme was tested on the base of recovered data from experiment 1 and 2. Synchronised heifers (unit A and B) and high yielding cows with poor reproductive performances (unit C) received GnRH 65 hours after $PGF_{2\alpha}$ Artificial insemination was carried out 13 h after GnRH-application. Results are shown in Table 3.

Table 3: Oestrous- and pregnancy rates in fixed time inseminated animals (FAI) or cattle inseminated after oestrous detection (DAI) in different units

		Unit A		Unit B		Unit C	
		FAI	DAI	FAI	DAI	FAI	DAI
Number of synchronised animals	(n)	108	107	26	140	18	47
Number of animals with oestrous and AI	(n)	105	106	25	133	15	40
Oestrous rate	(%)	97.2	99.1	96.2	95.0	83.3	85.1
Pregnant animals	(n)	83	74	19	84	8	18
Pregnancy rates	(%)	79.0	69.8	76.0	63.2	53.3	45.0

Pregnancy rates show that fixed time artificial insemination, independent of onset of oestrous can result in high pregnancy rates.

Notes

DETECTION AND QUANTIFICATION OF ENDOGENOUS TRANSCRIPTION IN BOVINE EMBRYOS AS EARLY AS THE PRONUCLEAR STAGE

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During a long time, due to the poor sensitivity of the methods used, onset of zygotic transcription has been identified to major activation phase in numerous species. Transgene integration in zygotic genome has then demonstrated that zygotic transcription was possible earlier, as soon as the pronuclear stage in the bovine (Gagné *et al*., 1993, Theriogenology, 39, 223 for example). However these results do not inform about endogenous genes transcription. In the mouse, BrUTP incorporation has proven to be sensitive enough to detect endogenous transcription as early as the S-phase of the 1-cell stage (Aoki *et al*., 1997, Dev. Biol, 181, 296-307). We were then interested in examining the onset of zygotic transcription in bovine embryos and in following transcriptional activity during embryo development.

Bovine embryos were obtained after in vitro maturation and fertilization of oocytes obtained from slaughterhouse ovaries. At different stages of development, they were processed for BrUTP incorporation, following a method adapted from Aoki *et al*. (1997). The incorporated BrUTP was detected by immunostaining (anti-BrdU antibody -Boehringer, 1:100- revealed by an anti-mouse FITC-labelled antibody from Sigma). Chromatin is stained with 10 µg/ml propidium iodide before mounting. Labeling quantification was then performed under confocal microscopy (Zeiss LSM). Immunocytochemistry was also performed with the Pol3/3 antibody, directed against the largest subunit of RNA polymerase II (Bellier *et al*., 1996, EMBO J., 16, 6250-6262).

Pol3/3 labeling was present in pronuclei and the very sensitive technique of BrUTP incorporation allows the detection of endogenous transcripts formation as early as the pronuclear stage (24-26 h post IVF). Labeling also appears in polar bodies. Transcriptional activity increases very slowly until the 8-cell stage. Between the 8-cell stage and the morula stage, it abruptly increases from about 3 fold (table 1). Pol3/3 nuclear labeling intensifies parallely.

Table 1 : Quantification of BrUTP incorporation of bovine embryos. Results are expressed as the labeling nuclear intensity minus cytoplasmic labeling, both measured in each cell examined.

	Day after IVF	Mean	SEM	Number of nuclei
1 cell	D1	7.35	1.04	20
2 cell	D1	8.77	0.85	13
4 cell	D1	14.50	2.07	9
8 cell	D2 + D3	11.77	1.93	13
Morula	D4	41.58	4.34	31
Morula	D5	61.27	5.96	11
Blastocyst	D6	45.56	3.27	32
Blastocyst	D7	40.89	3.65	19

A hughe increase of transcriptional activity appeared to be concommitant with the transition from maternal to zygotic control of embryonic development (i.e. 8-16 cell stage in the bovine). A more precise follow-up after in vitro fertilization would allow a better determination of the exact time of transcription onset in this species.

Notes

ARTIFICIAL REPRODUCTION IN THE SPANIH BREED TORO DE LIDIA FIVE YEARS OF EXPERIMENT

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The herd book of the TORO DE LIDIA named UNION DE LOS CRIADORES DE TOROS DE LIDIA was established in 1906, but the breeding of cattle for fighting against human started in the late 18th century. This is the only breed of cattle selected on a psychological behaviour.

For the past five years, we have been working with OCCITANIA, the only 1st category herd of toro de lidia registered outside the Iberian Peninsula. This herd is based near Arles in the South East of France. The aim of the owners is to produce every year about 40 bulls (3 to 4 years old) for fighting in the arena. The herd is limited to only 70 cows, so we needed to optimise the reproduction of these wild cows.

When we started this programme, only half of the cows were pregnant after running with the bull for six months. We therefore decided to organise the breeding into two periods : 1st period from 01/06 to 01/11 and 2nd period from 01/01 to 01/03. All the cows are checked for pregnancy two months after the end of the period, the empty ones are treated and put back to the bull or are inseminated during the next period. With this protocol, we obtained 17 calves more in the first year. We have kept this programme running and we are now trying to decrease the interval between calving as much as possible.

Artificial insemination

In order to run our ET programme, we have to produce straws of frozen semen from the different bulls. To collect the semen, the bull is caught in a crush and we use an electro-ejaculator because these animals are too wild for normal collection We also sometimes collect semen from excellent bulls which have died after fighting in arena. In the first three years, our results are 37 positive pregnancy diagnosis out of 76 inseminated. That's nearly 50%, but we are working only on synchronised cycles with a progesterone device and prostaglandin because it's impossible to catch the cows one by one to AI on natural heat.

Embryo transfer

For the first two years, we flushed 7 cows and did 15 flushes. We obtained 44 embryos with 34 good (2,27 per flush), we transferred 13 fresh which gave us 8 pregnancies and did 20 frozen transfers which gave 8 calves.

Since 1998 we have increased the number of flushes to give us more embryos to transfer and so more calves. A programme now is about 12 donors superovulated and about 15 recipients from the Aubrac breed, and we run such a programme every two months.

Day 0	CID'R (ND) on for donors and recipients
Day 8	2 000 UI PMSG for donors
Day 10	Remove CID'R (ND) and 2ml Estrumate (ND) donors and recipients
Day 11	AI donors in the evening
Day 12	AI morning and evening + Neutra PMSG (ND) morning
Day 19	Flush and transfer

Nb° of donors flushed	Nb°of flushes	Embryos collected	Good embryos	Transfers	Positive progesterone test	Frozen embryos
17	77	218	121	100	55	21
	mean/flush	2.83	1.57		55%	

These results show that the number of embryos collected per flush is lower because during this second period, we were unsuccessful in flushing some cows with excellent genetics but who were very bad for embryo production.

We are also now running an embryo splitting experiment. We are doing this for two reasons. Firstly, we want to prove that there is no difference in the behaviour in the corrida between calves born to a domestic recipient and natural calves (this experiment was carried out in Dr Victorino MARTIN's herd, which is the most famous in Spain for several years, with Dr CHANTEFORT and GELDHOF's help for splitting). The second reason is to increase the number of calves obtained from the small number of embryos we flush. In May 2000 we splitted 10 embryos and transferred the two half in 10 recipients.

Notes

QUANTIFICATION OF TRANSCRIPTIONAL ACTIVITY DURING EARLY DEVELOPMENT AND AFTER NUCLEAR TRANSFER IN RABBIT

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When a transcriptionally active cell is transferred into a transcriptionally inactive oocyte, what happens to transcriptional activity in the reconstituted embryo ? We specially adress the question with a very sensitive method allowing detection of endogenous gene transcription (and not a transgene randomly integrated in the genome) : the aim of this study to determine the time of the onset of zygotic transcription in normal rabbit embryos and to quantify levels of transcriptional activity during normal development and after nuclear transfer.

MATERIAL AND METHODS : Rabbit embryos and oocytes have been collected from superovulated and mated New-Zealand does at slaughterhouse. Embryos have been harvested at 14 h or 19 h post coïtum (h pc) and then in vitro cultured. Nuclear transfer has been performed with frozen-thawed morula donors into in vitro aged oocytes (Heyman et al., 1990 *CR Acad Sci Paris* 311, 321-326). BrUTP incorporation was performed according a method adapted from Aoki et al. (1997 *Dev. Biol*, 181, 296-307). The incorporated BrUTP was detected by immunostaining (anti-BrdU antibody - Boehringer, 1:100- revealed by an anti-mouse FITC-labelled antibody from Sigma). Chromatin was stained with 10 □g/ml propidium iodide before mounting. Labeling quantification was then performed under confocal microscopy (Zeiss).

RESULTS (Table 1) : Fertilization occurs in rabbit around 12 hpc. Inactive at 15 hpc, transcription begins at 17 hpc in both pronuclei. The quantity of zygotic nascent transcripts in the nucleus increases until the 4-cell stage and decreases markedly around the time of major activation (8-16 cell). After nuclear transfer, transcription never stopped. At equivalent time elapsed after fertilization or fusion, the quantity of nuclear transcripts is firstly significantly higher in reconstituted embryos (4 and 6 hpf ; due to the swelling of the transferred nucleus). Then the transcriptional activity becomes equivalent at 8 hpf and tends to be lower at the 2-cell stage (16 hpf). It would be interesting to observe what happens with more differenciated donor cells or with donor cells placed in quiescent G0 phase.

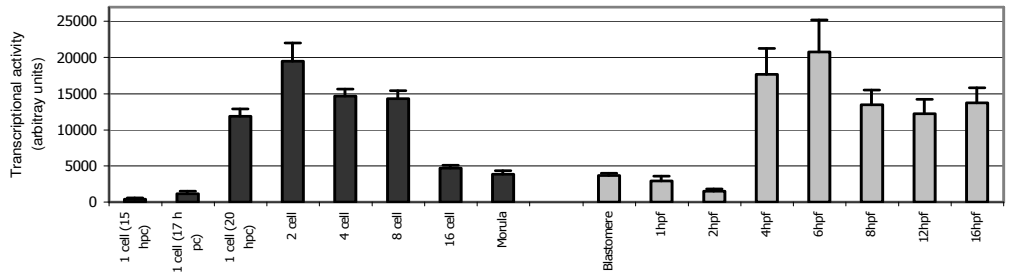

Figure 1: Quantification of BrUTP incorporation during early development and in reconstituted embryos. Transcriptional activity is expressed as intensity of nuclear labeling by area unit x nuclear area measured at its larger diameter. Hpf: hours post-fusion ; Black: normal development ; Grey: nuclear transfer.

Notes

DIFFERENTIAL *IN VITRO* DEVELOPMENT OF GYNOGENETIC AND ANDROGENETIC RABBIT EMBRYOS

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In mammals, the two parental genomes are required for full development to term. In the mouse where major zygotic activity begins at the two-cell stage, gynogenotes (only maternal genome) develop better *in-vitro* to the blastocyst stage than androgenotes (only paternal genome). The aim of this study was to determine whether the differential ability to form blastocysts is also observed in rabbits where the genome becomes active only after the third cleavage, at the eight-cell stage.

Two-pronuclei rabbit embryos recovered 17 hours *post-coïtum* were used. The male or female pronucleus was removed by micromanipulation in M199 with hepes supplemented with 10% FCS in the presence of 7µg ml^{-1} cytochalasine B and 1µg ml^{-1} demecolcine. *In vitro* development to the blastocyst stage was compared (table 1) with normal control embryos, first for haploid uniparental embryos (n ploidy), then for diploid uniparental embryos (2n ploidy) following electrofusion of the blastomeres at the two-cell stage (BTX stimulator, 3 pulses of 3.2Kv.cm^{-1} for 20µsec each in mannitol 0.3M).

Table 1 . *In vitro* development of haploid / diploid gynogenetic and androgenetic rabbit embryos

	Ploidy	Total number	Cleavage	Morulae at 65h pc	Blastocysts at 89h pc
Gynogenotes	n	296	253 (85.5%)	217 (73.3%)	88 (29.7%) a
	2n	191	187 (97.9%)	109 (57.1%)	92 (48.2%) b
Androgenotes	n	334	321 (96.1%)	274 (82.2%)	24 (7.2%) c
	2n	190	182 (95.8%)	148 (77.9%)	30 (15.8%) d
Control		56	56 (100.0%)	56 (100.0%)	52 (92.8%) e

Values with different letters were significantly different ($P<0.001$)

At the morula stage, the mean number of cells was 22 ± 3 and 48 ± 3 for haploid gynogenotes and androgenotes, respectively, compared with 36 ± 3 for control embryos. At the blastocyst stage, it was 46 ± 2, 64 ± 5 and 79 ± 4, respectively.

These results show that gynogenotes, either in the haploid or diploid state develop significantly better than androgenotes to the blastocyst stage. We also found that haploid two-cell stage androgenotes cleaved more rapidly than gynogenotes and had a higher number of cells at the time of evaluation for morula stage although most of the resulting blastocysts had compromised further development up to the hatched blastocyst stage.

The differential ability of uniparental embryos to form a blastocyst is not dependent on the first cell cycle occuring but depends on embryo divisions only after the two-cell stage. This suggests that the lower ability of androgenotes to form a blastocyst is dependent on some defective gene regulation occurring at or after the morula stage.

Notes

THE OVULATION RATE OBTAINED AFTER A SUPEROVULATION TREATMENT ASSOCIATING GNRH ANTAGONIST AND PFSH IS HIGHLY REPEATABLE

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A pretreatment with GnRH antagonist can improve the number of ovulations and reduce the variability of response to pFSH between ewes by suppressing large follicles (Cognié, 1999; Therio 51: 105-116).

The objective was to study the repeatability of superovulation and embryo yield using such a pretreatment.

17 adult ewes (7 Prealpes and 10 Ile-de-France) were superovulated during the breeding season (July = T1) and the treatment was repeated 2 months later (September = T2). Pretreatment consisted of subcutaneous injections (0.5 mg/ewe Antarelix-Europeptides) throughout the first 10 days of the FGA sponge treatment. Purified pFSH (36 mg Armair-Beckers) was given twice daily in 8 decreasing injections (7-5-4-2 mg/day) between d 11 and d 14 (d 14 = removal of sponge). Laparoscopic intra-uterine insemination was practiced 20 hrs after an ovulating injection (iv) of 3 mg pLH. Oulation rate was determined and embryos were recovered surgically 7 days after the onset of estrus.

No significant difference was observed between breeds and results were pooled to compare the superovulatory response for each period.

Table: Comparison between 2 repetitions of superovulation treatment for w production

	T1	T2
No of ovulations (mean ± sd)	21.1 ± 6.8	20.4 ± 9.3
Recovery rate	62^{a} (211/342)	29.5^{b} (102/346)
Cleavage rate	98^{c} (206/211)	88^{d} (90/102)
% of transferable embryos	93^{e} (192/206)	69^{f} (62/90)
Transferable embryos/treated donor (mean ± sd)	11.3^{g} ± 6.0	3.6^{h} ± 5.6

a vs b; c vs d; e vs f; g vs h;: P<0.01

Superovulation rates were high during the first and the second treatments and individual ovulation rates obtained at T1 and T2 were highly correlated (Pearson test: r = +0.75; Spearman test: r = +0.76). However, recovery rate and embryo yield decreased significantly at T2. It was shown in a subsequent study that the repetition of Antarelix treatment is not involved in the drops of embryo yield. This could be attributed to alterations of genital tract during the first embryo recovery.

Notes

USE OF IMAGE ANALYSIS TO INVESTIGATE SPERM-OVIDUCT INTERACTIONS IN 4 DIFFERENT MEDIA IN THE BOVINE

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Bovine spermatozoa attached to oviduct epithelium vesicles in vitro maintain their viability and motility much longer than free-swimming spermatozoa incubated in the absence of oviduct vesicles. For more detailed investigations of this sperm-oviduct interaction, we have used a method to quantify sperm-oviduct interactions by combining fluorescent staining and image analysis. The aim of this study was to evaluate the influence of different media on sperm-oviduct interactions after 30 minutes, 24 hours and 48 hours by making a quantification of 1) the area of oviduct vesicles and 2) the number of spermatozoa bound to the oviduct vesicles.

Frozen-thawed and fresh spermatozoa from the same bull were washed and incubated together with oviduct vesicles at a concentration of 1×10^6 spermatozoa/ml medium. The different media HEPES-TALP, IVF-TALP, TCM-199 and TCM-199 + 10% ECS were incubated at 39°C in 5% CO_2 except for HEPES-TALP (in air). After staining the oviduct vesicles and spermatozoa with the fluorescent mitochondrial marker JC-1 (Molecular Probes) in combination with the nuclear stain Propidium Iodide, the area of 5 oviduct vesicles per point of time and per medium was measured with the 'Image Database' programme from Leica and the number of spermatozoa bound to the oviduct vesicles was calculated. This experiment was repeated 3 times.

Table 1: The effect of different media on the binding capacity of spermatozoa to oviduct epithelium after 30 min and 1 or 2 days of culture. (Geometric mean (GM) of spermatozoa number/ 0.1 mm² oviduct epithelium)

	Fresh spermatozoa				Frozen-thawed spermatozoa			
Media	30 min	24 h	48 h	GM	30 min	24 h	48 h	GM
HEPES-TALP	79	36	23	40^{a}	59	29	6	22^{a}
IVF-TALP	90	52	1	19^{a}	150	8	0	8^{ab}
TCM-199 + 10% ECS	16	5	0	3^{b}	15	4	0	3^{b}
TCM-199	9	4	0	2^{b}	11	4	0	2^{b}

^{ab}GM values in the same column with different superscripts differ significantly. $P < 0.05$ (Repeated measures-ANOVA with $\log_e$ transformation of dependent variable- SAS V8 Proc Mixed).

The overall number of fresh spermatozoa bound to oviduct vesicles incubated in HEPES-TALP and IVF-TALP was significantly higher ($P<0.001$-Table 1) than in the other media. Fresh spermatozoa remained bound to the oviduct vesicles in HEPES-TALP in substantial numbers even after 48 hours of incubation. For frozen-thawed spermatozoa, differences in sperm-oviduct binding were observed between HEPES-TALP and TCM-199 with or without serum ($P = 0.008$). The decrease in the number of fresh and frozen-thawed bound spermatozoa was larger over time in IVF-TALP ($P < 0.001$).

In conclusion, HEPES-TALP was the most useful medium for the evaluation of the binding of fresh and frozen-thawed spermatozoa to oviduct vesicles. Currently this evaluation technique is being used for its repeatability by using semen of different bulls.

This research was supported by the Ministery of Agriculture of the Belgian government, grant no. S5867 and by the Research Fund of Ghent University, grant no. 011B8698

Notes

DEVELOPMENT OF *IN VITRO*-MATURED AND FERTILIZED BOVINE EMBRYOS CULTURED IN SOF MEDIUM OR CO-CULTURED WITH VERO CELLS.

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This study was conducted to evaluate the ability of two culture systems (SOF medium vs co-culture with VERO cells in B2 INRA-Menezo) to support *in vitro* development of bovine embryos.

Cumulus-oocyte complexes (COCs) were matured in TCM-199, 10% foetal calf serum (FCS), FSHp (20 µg/ml) and estradiol-17β (1 µg/ml) for 24 h. Matured COCs were incubated together with Percoll-separated sperm in Fert-TALP and 10 µg/ml heparin for 18 h (Day 0). Presumptive zygotes were vortexed and separated in two groups which were cultured up to Day 7 as follows:

- Group mSOF: 50 µl drops of mSOF (Takahaski and First, 1992; Theriogenology 37: 963-978) + 10% FCS added 42 h post IVF. The ratio volume/embryo was 1-2 µl/embryo.
- Group VERO: 50 µl drops of B2 INRA medium + 10% FCS in coculture with VERO cells. The ratio volume/embryo was 1-2 µl/embryo.

Culture conditions were 39ºC, 5% CO_2 in air and high humidity. After 72 h of culture, 1-cell embryos were separated anf fixed in ethanol and acetic acid (3:1 v/v) to evaluate the fertilization rate. Data were analysed by LSD´s test and results are shown in Table 1.

Table 1. Development of *in vitro* fertilized bovine embryos cultured in SOF medium and co-cultured with VERO cells.

			Day 3		Day 6	Day 7
Group	n	% IVF	%Cleavage	% 5-8 cells	%Blastocysts	%Blastocysts
mSOF	1464	75.6 ± 2.3	93.7 ± 5.2	35.4 ± 5.7	12.4 ± 1.7^{a}	22.0 ± 2.2^{x}
VERO	1406	79.1 ± 3.5	93.6 ± 4.3	43.1 ± 4.7	5.4 ± 1.1^{b}	17.1 ± 1.7^{y}

n: number of embryos; % IVF: fertilization rate; developmental rates are refered to fertilizated oocytes; values are mean ± SEM. Values with different superscripts within columns differ significantly: a vs b $p < 0.05$; x vs y $p < 0.1$.

No differences were found in development after 3 days of culture and Day 6 blastocysts rate was higher in the group cultured in mSOF, and Day 7 blastocysts rate was slightly higher in mSOF group ($p < 0.1$). These results indicates an increased development ability up to the blastocyst stage for embryos cultured in mSOF.

The authors thank Dr Baro for their help.

Notes

APOPTOSIS IN *IN VITRO* vs *IN VIVO*-DERIVED BOVINE EMBRYOS

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Apoptosis has been detected from the 9-16 cell stage in *in vitro* bovine embryos (Byrne et al. J Reprod Fert, 1998, 117:97-105) but no data are available for *in vivo*-derived bovine embryos. The aim of this study was to compare the incidence of apoptosis in *in vitro* and *in vivo*-derived bovine embryos from the morula to the blastocyst stage.

In vivo derived bovine embryos were recovered at Day 7 or Day 8 post insemination from 7 dairy cows after ovarian stimulation. Only morulae and blastocysts were used. *In vitro*-derived bovine embryos were obtained from abattoir oocytes after in vitro maturation and fertilisation and culture in SOF medium containing 5% foetal calf serum under a gas atmosphere of 5%CO_2, 5%O_2 and 90% N_2. Morulae were collected at Day 5 and Day 6 post-insemination. Blastocysts were collected at Day 6, 7 and 8. Collected embryos were fixed in 2.5% paraformaldehyde for 20 min and then submitted to the TdT-mediated dUTP-biotin nick end labelling (TUNEL) technique (kit - Boehringer) to evaluate the number of apoptotic nuclei. Nuclei were counter-stained with Hoechst 33342 for evaluation of total cell number.

In vivo-derived morulae contained more cells than their *in vitro* counterparts, but due to differences in kinetics of development, they were not collected at the same time (table). At the same embryonic stage, the number and rate of TUNEL positive nuclei were higher in *in vivo*-derived embryos. If the comparison is made on the basis of embryo age, the number and rate of TUNEL positive nuclei were similar at Day 7 and at Day 8 between *in vivo* and *in vitro*-derived embryos.

Table: comparison between *in vivo* and *in vitro*-derived bovine embryos for the number and rate of apoptotic nuclei evaluated by the TUNEL technique.

	In vivo embryos				*In vitro* embryos			
	N	Total cells	TUNEL+ nuclei N	TUNEL+ nuclei %	N	Total cells	TUNEL+ nuclei N	TUNEL+ nuclei %
Stage								
Morula	27	$78 \pm 2.8^{1,a}$	$3.8 \pm 0.4^{1,a}$	$4.9 \pm 0.5^{1,a}$	15	$33 \pm 3^{1,b}$	$0.7 \pm 0.2^{1,b}$	$2.8 \pm 1.1^{1,b}$
Blastocyst	15	$93 \pm 3.6^{2,a}$	$8.1 \pm 1.0^{2,a}$	$8.9 \pm 1.2^{2,a}$	29	$91 \pm 6^{2,a}$	$4.3 \pm 0.5^{2,b}$	$4.7 \pm 0.5^{2,b}$
Age								
Day 5	ND				10	26 ± 3^{1}	0.6 ± 0.3^{1}	3.3 ± 1.5^{1}
Day 6	ND				12	59 ± 5^{2}	1.3 ± 0.3^{1}	2.3 ± 0.6^{1}
Day 7	25	$79 \pm 2.8^{1,a}$	$4.1 \pm 0.4^{1,a}$	$5.1 \pm 0.5^{1,a}$	10	$73 \pm 4^{2,a}$	$3.5 \pm 0.7^{2,a}$	$5.0 \pm 1.1^{1,a}$
Day 8	18	$89 \pm 4^{2,a}$	$7.1 \pm 1^{2,a}$	$8.1 \pm 1.1^{é,a}$	12	$120 \pm 8^{3,b}$	$6.5 \pm 0.5^{3,a}$	$5.8 \pm 0.7^{1,a}$

[1,2] Significant difference between different superscripts in the same column (within stage or age); [a,b] Significant difference between different superscripts between in vivo and *in vitro* embryos

In conclusion, this study shows for the first time that apoptosis is a physiological process in bovine embryos at the morula and blastocyst stage. At the same time post insemination, the rate of apoptotic nuclei seems similar between *in vivo* and *in vitro*-derived embryos in our culture conditions, although the kinetics of development is different.

Notes

PREGNANCY-ASSOCIATED GLYCOPROTEIN CONCENTRATIONS IN N'DAMA COWS FOLLOWED SEVERAL MONTHS AFTER PARTURITION AND MAINTAINED WITH A FERTILE BULL

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The bovine Pregnancy-Associated Glycoprotein 1 (boPAG-1) is a placental glycoprotein produced by binucleate cells of the trophectoderm. This protein is released in the maternal circulation and can be detected in maternal blood as early as 3 to 5 weeks after fertilization. Therefore, the determination of the plasmatic concentration of the PAG have been used as pregnancy marker and indicator of foeto-placental well-being.

The aim of this study was to analyze the PAG concentrations in a herd of 32 N'Dama cows maintained with a fertile bull during several months after parturition.

Peripheral blood (10 ml) was collected weekly from the jugular vein into heparinized tubes from the first week after parturition until the 6th–8th month postpartum. Plasma was separated by centrifugation (1 500 x g for 15 min) immediately after blood collection and stored at –20°C until assayed for PAG. A homologous bPAG-RIA (Zoli *et al*., 1992, Biol Reprod 46:83-92) was used to measure PAG concentrations in the plasma.

PAG concentrations decreased regularly in the postpartum period (Figure 1) to become under the threshold (0.8 ng/ml) around week 10 to 16.

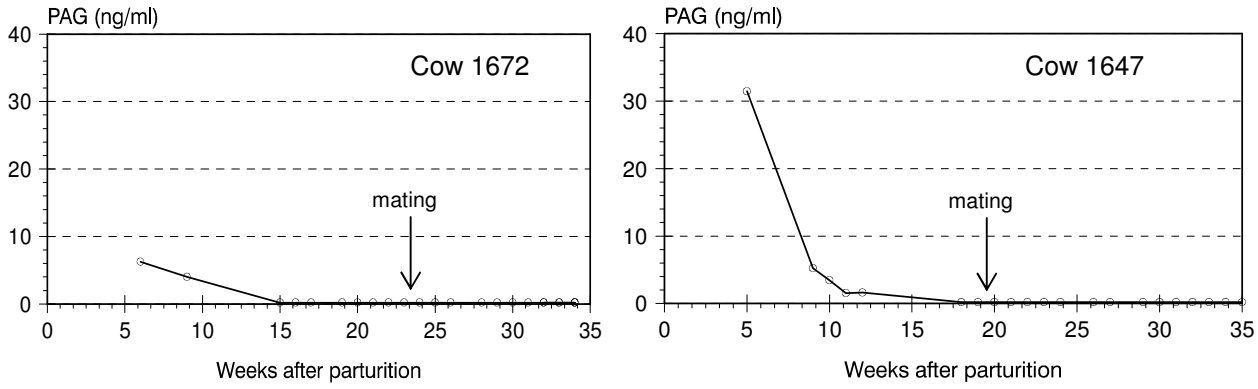

Figure 1. Plasma PAG concentrations of cows 1672 and 1647 during 34 and 35 weeks after parturition.

After the typical pattern of decrease in PAG concentrations during the postpartum period, PAG concentrations remained under the threshold until the last observation: blood sample collected after 10 to 39 weeks (24.37 ± 8.06 weeks, mean ± SD).

In conclusion, this study clearly illustrates that PAG assay is a useful and efficient indicator of infertility in Bovidae.

Notes

USE OF TWO SERUM REPLACEMENTS FOR PRODUCING *IN VITRO* BOVINE EMBRYOS

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The objective of this study was to analyse the effects of two serum replacements (CPSR3®, SIGMA and ULTROSER®, LIFE Technologies) added to the culture medium, in the production of *in vitro* bovine embryos.

Cumulus-oocyte complexes (CCOs) were matured in TCM-199+10% foetal calf serum (FCS) + FSHp (1 µg/ml)+LH (5 µg/ml)+estradiol-17β (1 µg/ml)+cysteamine 100 µM for 22-24 h. Matured COCs were incubated together with swim-up, frozen/thawed sperm in Fert-TALP + 10 µg/ml heparin for 18 h (Day 0). Presumptive zygotes were vortexed and cultured up to Day 8 in SOF (Holm *et al*., Theriogenology 52: 683-700) to which three different concentrations (2%, 4% and 6%) of ULTROSER® or CPSR3® were added 42 h postfertilization. Culture conditions were 39ºC, 5% CO_2 in air and hight humidity. Results are shown in tables 1 and 2. Data were analysed by Duncan's test and expressed as a mean±SEM.

Table 1: Development of embryos in SOF in presence of ULTROSER® (ULT), FCS or no supplements

Group	N	% Cleavage	% 5-8 D3	%M.D6	% Bl. D7	% Bl. D8
2% ULT	107	83.2±1.7^{a}	54.9±4.3	26.5±6.1	8.2±3.8^{ab}	8.2±3.8^{b}
4% ULT	112	84.8±1.7^{a}	56.4±4.3	23.6±6.1	5.5±3.8^{b}	6.4±3.8^{b}
6% ULT	99	85.3±2.0^{a}	55.8±4.9	13.5±7.0	1.2±4.4^{b}	2.0±4.3^{b}
5% FCS	109	81.0±2.0^{ab}	43.1±5.0	23.2±7.1	20.1±4.4^{a}	21.3±4.4^{a}
FCS(-) ULT(-)	65	77.3±2.4^{b}	50.9±4.0	19.5±8.4	1.9±5.2^{b}	3.2±5.2^{b}

Table 2: Development of embryos in SOF in presence of CPSR3® (CP), FCS or no supplements

Group	R	% Cleavage	% 5-8 D3	%M.D6	% Bl. D7	% Bl. D8
2% CP	138	86.9±3.6	50.5±5.3	26.7±4.6	15.1±3.8^{a}	22.8±3.7^{a}
4% CP	140	76.1±3.6	47.4±5.3	29.8±4.6	18.6±3.8^{a}	21.7±3.7^{a}
6% CP	159	79.7±3.6	46.5±5.2	28.0±4.5	17.9±3.7^{a}	20.6±3.6^{a}
5% FCS	147	82.4±3.6	46.1±5.2	27.6±4.5	21.1±3.7^{a}	22.1±3.6^{a}
FCS(-) CP(-)	65	79.6±4.9	53.4±7.1	24.9±6.2	6.6±5.0^{b}	7.3±4.9^{b}

Tables legend: R. replicates; % 5-8 D3: 5-8 cell-embryo rate at Day 3; % M D6: Morulae rate at D6; % Bl D7-8: Blastocysts rates at Day 7 and 8. Diferent superscripts within columns differ significantly ($p<0.05$)

In the two trials, no significant differences between culture groups were found on embryo development up to the morula morula stages. However, blastocyst rates in presence of ULTROSER® were lower ($p<0.05$) than after culture in SOF + FCS. The addition of CPSR3® to SOF produced blastocyst rates similar to those obtained with FCS.

Project FEDER 1 FED97-0023.

E. DIAZ is a fellowship holder from INIA.

Notes

IN VITRO DEVELOPMENT OF DIPLOID RECONSTRUCTED RABBIT EGGS USING HAPLOID 32- AND 64-CELL PARTHENOGENETIC NUCLEI

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In mice, the combination of haploid nuclei from 2- to 16-cell parthenogenetic embryos with haploid male recipient eggs reconstructs the normal heteroparental ploidy and derives in live offspring (Surani *et al*., 1986; Cell, 45: 127-136); however, no development is obtained with more advanced donor stages. In the rabbit, full-term development has recently been obtained after transferring 1/32 haploid nuclei (Escribá & García-Ximénez, in preparation). To determine whether fully transcriptional developmental stage of the maternal genome (64-cell parthenogenetic stage) affects the functional integration with the resident pronucleus is pursued in this study.

Haploid donor embryos at the 32- and 64-cell stage were obtained by electroactivation of young rabbit ova and subsequent *in vitro* cultured for 32h and 48h (Escribá & García-Ximénez, 2000; Reprod Nutr Dev, in press). Couplets formed by haploid female donor cell and manipulated zygote recipient (with clear cytoplasm) were electrofused (2.2kVcm^{-1} for 60 μsec. duration each, 30 min apart) and the resulting nuclear configuration determined. The percentage of reconstructed zygotes that after fusion contain two pronuclei was stimated at 71% (32/45), regardless of the donor cell stage (32 or 64-cell).

Table. Pre-implantation *in vitro* development of reconstructed eggs from 1/32 and 1/64 blastomers according to the nuclear configuration after fusion.

Embryonic haploid nuclear donor stage	Final nuclear configuration*	Initial nº of reconstructed eggs	Nº development to (% of cleaved)		
			Nº cleaved (%)	Morula/ Blastocyst	Hatched Blastocyst
1/32	0+1	3	3 (100)	0	-
1/64		4	3 (75)	0	-
1/32	1+1	19	19 (100)	10 (53)	2 (11)
1/64		13	13 (100)	7 (54)	4 (31)
1/32	2+1	2	2 (100)	1 (50)	0
1/64		4	4 (100)	4 (100)	2 (50)

*Reconstructed eggs were categorised as: 0+1 eggs: Haploid reconstructed eggs formed by entirely enucleated recipient eggs plus a haploid female nucleus introduced by fusion. 1+1 eggs: Diploid eggs reconstructed by combining recipient eggs containing a single pronucleus with a female haploid nucleus. 2+1eggs: Triploid eggs.

Despite the reduced number of implied eggs, these results suggest that the *in vitro* developmental ability of 1/32 and 1/64 reconstructed diploid eggs are comparable (53 and 54% morulae and blastocysts, respectively).

The parthenogenetic haploid embryo development up to advanced stages, even to blastocyst is possible (Escribá & García-Ximénez, 1999, Theriogenology 51, 963-973), and results presented here, could indicate that 1/64 haploid nuclei are also able to integrate functionally with the resident pronucleus, allowing experimental reconstructed diploid eggs to proceed on pre-implantation *in vitro* development efficiently. Subsequent efforts should be applied to determine the ability of cited experimental embryos to develop to term. Moreover, the use of more advanced parthenogenetic donor stages should also be addressed, in order to clear up whether in the rabbit, the nuclear organisation of the maternal haploid genome is altered some cellular divisions after the onset of major genome activation, usually described as occurring at the 8-, 16-cell stage in this species.

Notes

OXIDATIVE STRESS: PREVENTIVE EFFECT OF ß-MERCAPTOETHANOL AND TROLOX®

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A previous study (Feugang *et al.*,Theriogenology 2000;53:352) demonstrated that exogenous {AAPH; 2,2' azobis (2-amidinopropane) dihydrochloride, a radical initiator} or endogenous {BSO; buthionine sulfoximine, an inhibitor of gluthatione synthesis} oxidative stress initiators induce degeneration of bovine blastocysts at Day 8 pi when added from Day 5 pi. Here, we evaluate the protective effect of ß-mercapthoethanol (ß-ME, a precursor of gluthatione synthesis) and Trolox® (a water soluble analog of vitamin E) on these inducers.

After IVM/IVF, bovine oocytes were cultured in SOF medium containing 5 % FCS, at 39°C under 5 % O_2, 5 % CO_2 and 90 % N_2. Morulae were recovered (Day 5 pi) and distributed into groups of 5 embryos, in 20-µl drops of fresh culture medium. Three conditions were tested: 1) medium alone; 2) 0.01mM of AAPH and 3) 0.4 mM of BSO. ß-ME was added from Day 5 pi and during exposition to reactive oxygen species (ROS) inducers (from Day 6 to Day 8 pi), and Trolox® was added simultaneously with the two ROS inducers (from Day 5 to Day 8 pi). The rate of blastocysts was evaluated at Day 8 pi and blastocysts were stained with Hoechst 33342 for cell number evaluation.

Blastocyst degenerescence was observed in the groups treated with oxidative stress inducers in the absence of ß-ME or Trolox® (Table). The presence of ß-ME or Trolox® in the culture medium significantly reduced the rate of degeneration. ß-ME but not Trolox®, seemed to increase the mean cell numbers (ANOVA, P = 0.07) and hatching rate (47% vs 30%; Chi square, P = 0.02) of Day 8 blastocysts.

Table: Protective effect of 0.1 mM ß-ME and 0.4 mM Trolox® on blastocyst development and cell number at Day 8 pi (total of 6 replicates).

Groups	ß-ME	Intact blastocysts n (%[1])	Mean cell number (± sem)	Trolox®	Intact blastocysts n (%[1])	Mean cell number (± sem)
Control (0 mM)	-	24 (80) ^{a}	124 ± 8 ^{a}	-	27 (90) ^{a}	119 ± 8 ^{a}
	+	23 (77) ^{a}	150 ± 8 ^{a}	+	28 (93) ^{a}	126 ± 7 ^{a}
AAPH (0.01 mM)	-	12 (40) ^{b}	123 ± 11 ^{a}	-	12 (43) ^{b}	104 ± 12^{a}
	+	24 (80) ^{a}	135 ± 8 ^{a}	+	20 (70) ^{c}	105 ± 8 ^{a}
BSO (0.4 mM)	-	8 (27) ^{b}	138 ± 14 ^{a}	-	11 (37) ^{b}	130 ± 12^{a}
	+	19 (63) ^{a}	144 ± 9 ^{a}	+	24 (80) ^{a}	109 ± 8 ^{a}

[1]Proportion from morulae (30 morulae in each condition).

^{a,b,c} values with different superscripts differ significantly within the same column.

Theses results show that (1) Trolox® and ß-ME can prevent degenerescence of IVP bovine blastocysts induced by oxidative stress generated by two different ways and (2) ß-ME could increase the quality of blastocysts.
Protective mechanisms (extracellular or intracellular) of those two molecules when added in the culture medium need further investigations.

Notes

COMPARISON OF TWO FSH TREATMENTS FOR SUPEROVULATION OF EWES

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The aim of this work was to study the efficiency of the administration of equal dosis of FSH versus decreasing dosis of FSH plus eCG in superovulatory treatments of ewes.

A total of 183 dry, adult Rasa Aragonesa ewes (51 donors and 132 recipients) were used in different assays. Oestrus synchronization in donors was achieved by an 14 d-treatment with vaginal sponges containing 40 mg FGA (Chronogest, Intervet) plus a cloprostenol injection (0.4 ml Estrumate, Mallinckrodt Vet.) on Day 12. Sponges were changed at Day 7. Superovulation was obtained by 8.8 mg of NIADDK-oFSH-17 (10 ml Ovagen, ICP) distributed into 8 i.m. injections every 12 h starting 60 h before sponge removal. Recipients were synchronized by FGA sponges and 400 IU of eCG at sponge removal, together with donors.

In 24 donors, FSH was injected in decreasing doses as follows: 2 x 1.32, 4 x 1.1, and 2 x 0.88 mg. An injection of 200 IU of eCG (Foligon, Intervet) was also given at the time of the 6th FSH dose (Group 1). In Group 2 (n = 27), FSH was administered in 8 equal doses of 1.1 mg/1.25 ml and eCG was not used. All donors were inseminated in uterus under laparoscopic control 50 h after sponge withdrawal with 10^8 spermatozoa/horn of refrigerated semen (15^{o}C).

Embryos were surgically obtained on Day 7 after sponge removal. 192 morphologically good fresh embryos were transferred (2 embryos/recipient) and 72 were frozen and transferred later. Ewes of Group 1 displayed oestrus earlier than Group 2 (24.2 ± 0.11 vs. 31.2 ± 1.12 h after sponge removal; $p<0.01$) and oestrus were more grouped ($p<0.001$). No differences were found in ovulation rate. The mean of total and viable embryos in Groups 1 and 2 are shown in Table 1. No differences between groups have been found in the reproductive results obtained after transfer of fresh or frozen embryos (Table 1).

In conclusion, in our experiments, association of eCG with a decreasing dosage of FSH resulted in a more homogeneous superovulatory response and in 1.64 additional transferable embryos per donor, without changing the reproductive efficiency after transfer.

Table 1- Reproductive performance after transfer of embryos obtained by two different treatments

	Ovulation rate± SEM	Unfertilized ova ± SEM	Embryos (± SEM) Total	Viable	Transferred embryos (n)	Lambing (%)	Lambs/emb. (%)
Group 1	11.54±0.78	0.08±0.06	8.58±0.97	7.12±0.87	Fresh (120)	73	65
					Fozen (44)	41	36
Group 2	11.26±1.34	1.41±1.00	6.93±1.31	5.48±1.10	Fresh (72)	75	66
					Frozen (28)	43	32

Supported by INIA and AECI

ADENOSINE TRIPHOSPHATE-CONTENT OF *IN VITRO*-PRODUCED DOMESTIC CAT EMBRYOS

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Most of the 36 species of wild cats are classified as threatened, vulnerable or endangered due to poaching and habitat loss. Therefore, the domestic cat is a useful model for the establishment of assisted reproductive techniques in endangered felid populations. Adenosine triphosphate (ATP) is the universal energy carrier produced by mitochondria. It has been demonstrated that during oogenesis expansion of cytoplasmic volume is accompanied by an increase in the number of mitochondria, but this number does not increase during embryonic development up to the blastocyst stage (Cummins, Rev Reprod 1998;3:172-82). The aims of this study were the evaluation and comparison of the ATP-content of 1) different developmental stages recovered at the same day and 2) of the same developmental stages recovered at different days of in vitro culture.

Cumulus-oocyte-complexes (COCs) were delivered by mincing of ovaries after ovarectomy, selected and matured in TCM 199 supplemented with BSA, LH and FSH. After 24 to 28 h, COCs were inseminated in a TALP medium containing heparin and $2x10^5$ motile spermatozoa per ml recovered from excised epididymes. After in vitro fertilisation presumptive zygotes were cultured in 400 µl of SOF medium supplemented with essential, nonessential amino acids, and 10% (v/v) estrous cow serum. Different stages of Day 6 and Day 8 embryos were used for determination of the ATP-content using firefly luciferin-luceferase reaction measured by luminometer (Stojkovic *et al*., Biol Reprod 1999;61: 541-547). Based on at least three replicates, means of the measurement were compared by Student`s t-test. The results are presented in Table 1.

Table 1: ATP-content of *in vitro*-produced domestic cat embryos measured at different development stages and days of *in vitro* culture and rate of development to each stage.

Day of culture	Embryo stage (n)	Developmental rate (%)	Mean ATP-content pmol ± SD
6	Morula (8)	5.1	0.16 ± 0.12^a
6	Blastocyst (31)	19.7	0.96 ± 0.54^b
6	Expanded blastocyst (28)	17.8	1.22 ± 0.53^b
6	Hatching blastocyst (7)	4.4	2.68 ± 1.51^c
8	Morula (9)	3.9	0.34 ± 0.26^a
8	Blastocyst (14)	6.1	0.95 ± 0.68^b
8	Expanded blastocyst (34)	14.9	1.25 ± 0.71^b
8	Hatching blastocyst (23)	10.5	3.01 ± 2.67^c

[a-c]Different superscripts indicate significant differences ($P<0.05$)

The results demonstrate that later developmental stages of embryos contain more ATP at the same day (differences between blastocysts and expanded blastocysts not significant) and that same developmental stages at different days (6 and 8) contain nearly the same ATP. Further studies should investigate if the differences could be due to different metabolic activity and or in an increase in the number of cells or mitochondria.

Notes

SEX DETERMINATION IN BOVINE EMBRYOS BY MICRO-ASPIRATION. RESULTS OF FIVE YEARS PRACTICE UNDER FARM CONDITIONS

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Sexing of *in vivo*-produced embryos by PCR usually requires a biopsy of 4-5 cells. The method of Micro-aspiration has been chosen in this project because this technique causes less damage to the embryos. After this procedure it is still possible to split the embryos with a normal pregnancy rate (50% from a half embryo). A Leitz Labovert inverted transmitted-light microscope with 2 electronic micromanipulators and one hand-operated manipulator (Leitz, Germany) was used. The first electronic micromanipulator holds the embryo with a pipette of 110 µm external diameter and 25 µm internal diameter. The second one is connected with a micro-aspirator (Narishige, Japan) and an aspiration pipette of 28 µm external diameter and 25 µm internal diameter. The micro-aspirator syringe is filled with 0.5 ml air and 2 ml Fluorinert FC-77 (Sigma, Belgium). Fluorinert was chosen instead of oil because it keeps the cells from sticking into the pipette. Futhermore the aspiration pipettes can be used several times after washing without any risk of DNA contamination. These 2 micromanipulators are used for the selection of biopsies from morulas. Once blastocysts or expanded blastocysts are obtained, the third hand-operated manipulator gets used with a closed glass pipette of 3 µm diameter. After aspiration of 5 cells, the closed glass pipette serves as a "knife" in between the embryo and the aspiration pipette. This manipulation is necessary because of the rather strong cellular adhesion in blastocysts.

On farm sex determination was carried out by detection of male specific Y-chromosomal DNA sequences using PCR. The test was done with the "Y.C.D." technique as developed by AB Technology (Washington, USA).

From January 1996 until May 2000, 1.057 embryos were sexed. During the first part of the project (1996-'97) 291 biopsies collected on farm were sent to the ET-station Nückel in Germany. During the second part (1998-2000) 766 embryos were sexed under farm conditions (immediately after recovery) by the VRV-ET-team.

The efficiency of the sex diagnosis increased from 80% in 1996 to 91.6% in 2000.

The accuracy of the sex diagnosis was 99.5%; out of 200 calves only one calf was found to be of the wrong sex (analysed female, born male). The pregnancy rates were 45 % for frozen sexed embryos (grade I and grade II) and 56% for freshly sexed embryos (grade I, II and III).

As a conclusion, we may state that sexing of bovine embryos is possible under farm conditions and has become a routine part of the embryotransfer technology for the Flemish cattle breeders, clients of the VRV-ET-team.

Notes

EFFECTS OF CITRATE PLUS MYO-INOSITOL AND ACETOACETATE ON *IN VITRO* BOVINE EMBRYO DEVELOPMENT IN MEDIUM CONTAINING BSA

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Citrate and myo-inositol have been demonstrated to improve bovine embryo development when added to a simple, defined medium containing PVA (Holm *et al.*, 1999,Theriogenology, 52: 683-700). However, the simultaneous embryotrophic effect of both compounds was not investigated in medium containing BSA, probably due to citrate has been found to contamine albumin preparations (Gray *et al*., 1992, J. Reprod. Fert. 94: 471-480). In addition, internal citrate increased in the presence of acetoacetate and β-D-Hydroxybutyrate in somatic cells *in vitro* (Yudkoff *et al.*, 1997 J. Neurochem. 69: 682-692). On the other hand, the effect of acetoacetate improving bovine embryo development *in vitro* in SOF medium as modified by Takahashi and First (1992, Theriogenology 37, 963-978), was shown to be serum-dependent (Díez *et al*., 2000, Theriogenology, 53:293). As a consequence, we designed this experiment to test bovine embryo development *in vitro*, in medium containing BSA and no serum, in the presence of combinations of the compounds cited above.

In vitro produced bovine denuded zygotes were cultured *in vitro* in SOFaaci (Holm *et al*., 1999 Theriogenology, 52: 683-700) with 8 g/l BSA and under 5% CO_2 in air and 90% humidity at 39ºC, for 8 days after fertilization. Substrates and concentrations used were: lactate 7.02 mM + pyruvate 0,72 mM; citrate 0.34 mM + myo-inositol 2.77 mM; and acetoacetate 3.6 mM. Droplets of culture medium were kept under mineral oil and renewed twice throughout culture. Development was checked two times according to the results shown in table.

Table: Effects of citrate and myo-inositol on *in vitro* development of bovine embryos.

Energy substrates	Citrate + Myo-inositol	n	R	% Morulae	%Blastocysts Day 7	%Blastocysts Day 8
Lactate/Pyruvate	+	92	4	31.9 ± 8.3^{a}	23.6 ± 5.7^{a}	25.5 ± 6.8^{a}
Lactate/Pyruvate	-	67	3	33.0 ± 1.2^{a}	22.6 ± 1.7^{a}	22.6 ± 1.7^{a}
Acetoacetate	+	52	3	7.4 ± 3.6^{b}	0.0^{b}	0.0^{b}
Acetoacetate	-	47	3	9.2 ± 4.1^{b}	0.0^{b}	0.0^{b}
No substrate	+	87	4	9.7 ± 3.9^{b}	5.1 ± 3.6^{b}	6.3 ± 3.2^{b}
No substrate	-	93	4	9.5 ± 3.2^{b}	1.0 ± 0.9^{b}	1.0 ± 0.9^{b}

n = number of oocytes. R = number of replicates. ANOVA and REGWF test ($p<0.05$)

As observed, citrate and myo-inositol did not to improve blastocyst development in any experimental group. On the contrary and according to previous reports, acetoacetate showed severe embryo toxicity, which suggests to investigate specific conditions to test this compound in the absence of serum. In addition, albumin should be dialyzed to remove citrate in order to perform a more defined study of the citrate and myo-inositol effects in medium with protein.

Project FEDER 1 FED97-0023

E. DIAZ is a fellowship holder from INIA.

Notes

EFFECTS OF FOLLICULAR POPULATION ON ENDOCRINE AND OVARIAN RESPONSE IN SUPEROVULATED EWES

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This study had the objective to determine the contribution of different preovulatory events to the variability observed in the response of sheep to superovulatory treatments. Follicular development, onset of the estrus behaviour, timing of the preovulatory LH surge and ovulation rate (OR) were determined in 7 Manchega ewes superovulated with 8 decreasing doses (1.5 ml x 3, 1.25 x 2 and 1 x 3) of Ovagen™ injected twice daily from 60 h before to 24 h after the withdrawal of a 40 mg FGA sponge (Chronogest®). Development of all ≥ 2mm follicles from the first FSH injection to progestagen withdrawal (0 h) was determined just prior every FSH injection by 7.5 MHz transrectal ultrasonography (Aloka SSD-500). Estrus detection and jugular blood sampling for LH radioimmunoassay were performed every 3 h from 14 to 53 h after sponge removal and OR was determined 4 days after. Pearson correlation procedures showed that the number of follicles with 2-3 mm in size at first FSH dose (mean ± S.E.M.= 10.4 ± 1.5) was positively correlated with the number of ≥ 4mm follicles at 0 h (19.0 ± 2.7, $P < 0.01$, $r = 0.7$). Results also showed that a higher number of ≥ 4mm follicles at 0 h was related with a higher OR (18.2 ± 3.8, $P < 0.005$, $r = 0.93$) and an earlier appearance of estrus (31.5 ± 1.5 h, $P = 0.08$) and LH surge (45.0 ± 2.3h, $P < 0.005$, $r = -0.92$). In the other hand, there were no significant differences between ewes bearing or not a large follicle (≥ 6 mm) at first FSH dose on number of ≥ 4 mm follicles at 0 h (15.3 ± 6.4 vs 21.7 ± 15.3), timing of estrus (32.0 ± 3.0 vs 31.4 ± 1.9 h), onset of LH surge (44.0 ± 6.0 vs 48.2 ± 2.9 h) and CL number (13.5 ± 1.5 vs 20.2 ± 5.2). This results confirms that superovulatory response in ewes is related with the number of 2-3 mm in size follicles present on the ovary at first FSH injection (Gonzalez-Bulnes *et al*., J. Reprod. Fert. Abstract Series, 1999, 24: 6). These follicles are able to grow in response to exogenous stimulation until reach estrogenic competence, causing an earlier onset of estrus, LH surge and ovulation in high-responding ewes.

Notes

DETECTION OF PREGNANCY-ASSOCIATED GLYCOPROTEIN (PAG) AND ULTRASONOGRAPHY FOR DIAGNOSIS OF PREGNANCY, HYDROMETRA AND EMBRYONIC MORTALITY IN DAIRY GOATS

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Early pregnancy diagnosis has a considerable value in the reproductive management of dairy goat. With a transabdominal ultrasonographic probe a reliable pregnancy detection on the basis of uterine fluid was possible by day 27 of pregnancy; fetuses were visualized in all does by day 34 (Padilla & Holtz 2000; 7th Int Conf Goat, France 1: 483-484). Proteins secreted by the placenta that enter in the maternal circulation can be useful indicators of pregnancy. In 1998, Garbayo *et al.* (Biol Reprod 58: 109-115) purified 3 pregnancy-associated glycoproteins (PAG) from caprine placenta that allowed to develop sensitive RIA systems to detect pregnancy in goats as soon as 21 d after breeding (González *et al.* 1999; Theriogenology 52: 717-725). Hydrometra is an accumulation of aseptic fluid in the uterine lumen in the presence of a persistent corpus luteum (Pieterse & Taverne 1986; Theriogenology 26: 813-821). It is a major cause of infertility in dairy goats that reduces significantly the productive potential of herds (Hesselink 1993; Vet Rec 132: 110-112).

The aim of this study was to compare the reliability of caprine PAG detection, transabdominal ultrasonography and both methods as a whole for pregnancy and hydrometra diagnosis in dairy goats.

Dairy goats (n = 94) were synchronized by insertion of an intravaginal sponge (45 mg of FGA) for 11 d, followed by administration of PGF2α analogue (7.5 mg of luprostiol) and eCG (250 IU) two days before sponge removal. The day 0 was established at 2 days after sponge removal. Pregnancy or hydrometra was tested in all goats at days 28 and 35 by transabdominal ultrasonographic scanning (Toshiba Sonolayer, 5 MHz); afterwards, the goats diagnosed as nonpregnant on day 35 were tested again on day 65. Blood samples were collected at day 28. Plasma was removed by centrifugation and stored at –20ºC until assayed for PAG. The PAG concentrations were determined by a semiheterologous RIA, according to the method previously described (González *et al.* 1999).

Table 1. Number of goats diagnosed as pregnant, nonpregnant and hydrometra

	PAG on day 28	US on day 28	PAG plus US on day 28	US on day 35	US on day 65
Pregnant	60	42	60	55	58
Nonpregnant	34	49	32	37	34
Hydrometra	--	3	2	2	2

US = Ultrasonography

Table 1 shows the number of goats diagnosed as pregnant, nonpregnant and hydrometra with each method used. There were 60 pregnant and 34 nonpregnant goats on day 28 as detected by PAG concentrations. Ultrasonography on day 28 detected only 70 % of pregnant goats diagnosed by PAG and classified as hydrometra one goat that was pregnant. Nevertheless, the measurement of PAG plus ultrasonography on day 28 as a whole appears to provide a reliable diagnosis of pregnancy as well as hydrometra. Ultrasonography on day 35 showed that 5 goats that were pregnant on day 28 showed no reliable signs of pregnancy. Among these 5 goats, ultrasonography on day 65 revealed 2 pregnant goats showing fetuses of about 9 wk, 1 pregnant goat showing fetuses of about 6 wk and 2 nonpregnant goats, suggesting the occurrence of 2 cases of early embryo mortality. Therefore, the detection of PAG on day 28 followed by ultrasonography on days 35 and 65 might also be used to investigate embryonic mortality in dairy goats.

Notes

SOURCES OF VARIATION OF EMBRYO PRODUCTION AFTER SUPEROVULATION IN PRIM HOLSTEIN DAIRY COWS

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Data originating from 1695 donor females and representing a total of 2683 embryo collections (EC) performed during 4 years were used in this study. The effects of the year season, superovulation treatment (Group 1: n = 1191, 4 days FSH, 8 i.m injections decreasing doses, total dose 350 and 500 µg for heifers and cows respectively, STIMUFOL ND, Merial Fr), 1rst FSH between d 9 and 12 post oestrus; Group 2: n= 502, same as Group 1 + 5 days progesterone implant, CRESTAR ND, Intervet Fr, starting 2 days before 1rst FSH; Group 3: n= 990, 1rst FSH between d 13 and d 18, + 10 days progesterone implant starting 6 days before 1rst FSH), parity (heifers, 1rst, 2d, 3rd, 4th or more lactations), rank of collection for a given donor and bull of AI (n=170, 25 with more than 30 EC) as well as genetic effects (father, n=184 and grand father, n=206 with respectively 24 and 23 with more than 30 EC) were analysed on total (TOT), transferable (TRA), grade 1 (GR1), degenerated (DEG) embryos and unfertilized oocytes (UFE). ANOVA were performed using SAS (proc GLM) with genetic effects considered as random and other effects as fixed. Individual differences between groups were further investigated by Student Newman Keuls tests.

Significant effects of the type of superovulation treatment used were found on TOT and TRA embryos. Values for Group 2 (7.69 TOT and 5.64 TRA) were significantly higher ($p<0.05$) than for Group 1 and 3 (6.93 and 6.56 TOT, 4.67 and 4.24 TRA). Mean numbers of transferable embryos were 4.77, 5.33, 5.62 and 4.0 in heifers, and cows in 1rst lactation, 2 d lactation and 3 lactation or more respectively. Values for heifers, cows in 1rst or 2 d lactation were significantly higher than in cows with 3 lactations or more ($p <0.05$). All variables except GR1 and DEG were affected by the rank of collection. Mean TRA numbers decreased significantly with the rank of collection (4.81, 4.67, 4.26 respectively for 1rst collections, collections 2 to 4 and rank > 4) whereas UFE number was significantly increased in females collected more than 4 times ($p <0.05$). A significant random effect of the bull of AI has been found on grade 1 embryos but not on other variables ($p <0.05$). Mean GR1 values ranged from 1.1 to 3.7 depending on the bull used for AI. Other random effects for the father (f) and grand father (gf) of the donor cow significantly affected almost all variables ($p <0.05$) except DEG. Mean TOT values ranged from 3.68 (f12) to 10.3 (f22). Corresponding values for TRA and GR1 were respectively of 2.63 and 1.3 (f12) and 7.63 and 4.6 (f22). In only one case (f5), GR1 number was lower than for f12 (1.16) despite a higher number was observed for TOT (4.85). Similarly, TOT values ranged from 4.01 (gf8) to 8.96 (gf22). Corresponding values for TRA and GR1 were respectively of 2.46 and 1.13 (gf8) and 6.43 and 3.86 (gf22). In only one case (gf20) the production of GR1 embryos was higher than for gf22 despite a lower number was observed for TOT (8.12). Short term progesterone supplementation starting 2 days before first FSH injection may improve embryo production following superovulation. Embryo production was strongly affected by individual factors (parity, rank of collection) and by the genetic origin of donor cows which seems to influence mainly the response to superovulation.

Notes

AFLP-BASED ANALYSIS OF SEX SPECIFIC GENETIC EXPRESSION OF BOVINE EMBRYOS IN PREIMPLANTATION STAGE

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This research was carried out to study if there is a differential genetic expression between male and female bovine embryos produced *in vitro* during preimplantation stages of development. Bovine blastocysts were obtained by *in vitro* maturation, *in vitro* fertilization and *in vitro* culture. Biopsies were taken and sex of each embryo was examined by PCR. The remaining embryo was frozen and when sex was determined, several embryos of each sex were grouped and RNA was extracted. Afterwards by means of RT-PCR and AFLP differential display (a PCR-based method whereby cDNAs made from two mRNA samples are compared on side-by-side tracks of a sequencing gel), six differential genetic markers were isolated and cloned. Four of these markers were specifically expressed in the female embryos and two were expressed only in the male embryos (Table 1). The comparison of these sequences with the gene bank database showed that meanwhile some of them are homologous to mRNA already described, others show percentages of homology lower than 30% with known RNA sequences. Our study is the first report of the presence of specific mRNA in female bovine blastocysts. One reason for the specific female expression could be the expression of imprinting genes that only are expressed from a parental allele.

Table 1. Sex specific genetic markers identificated in bovine embryos and their homology with sequences of Gene Bank

Marker	Sex	Sequence partial-homology to
AX1	X	Homo sapiens splicing factor Prp8 mRNA
AY1	Y	Knowles Solter mouse blastocyst B1 Mus musculus cDNA clone mRNA sequence
AY2	Y	Bovine SRY mRNA
AX2	X	MARC 1 BOV Bos taurus cDNA 5´ (cDNA identificado de la combinación de cuatro librerías de cDNA)
AX3	X	Bovine lung prostaglandin synthetase (PGF) mRNA
		Cow prostaglandin F synthetase II (PGFS) mRNA
AX4	X	Human Xp26.1-2; Human Xp22

Notes

DEVELOPMENT OF GAMETE RECOVERY AND FOLLICULAR TRANSFER (GRAFT) FOR BOVINE OOCYTES OF DEFINED ORIGIN

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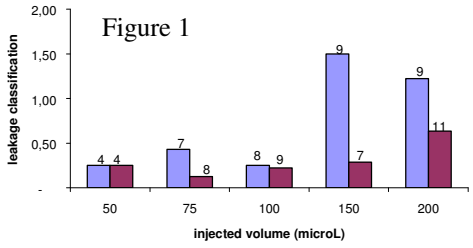
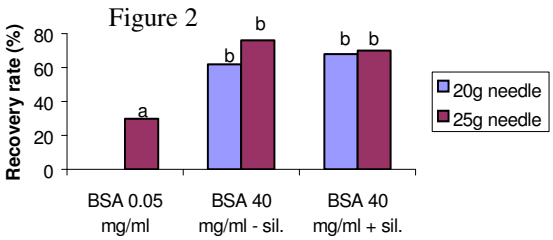
In the cow, oocytes in preovulatory follicles have undergone "prematuration" before the LH surge initiates final maturation. The generally limited yield of embryos in IVP may be attributed to the use of immature oocytes. Temporary hosting immature oocytes in a preovulatory follicle could provide the environment required for "prematuration". Therefore, we studied a technique to transfer immature oocytes into preovulatory follicles.

A system to inject fluid into follicles was used similar to Bergfelt *et al*. (Theriogenology 1998;50: 15-25). It consisted of tubing connecting a needle with 2 syringes, one of 10 ml (to fill the system) and one of 1 ml for injecting small volumes. In experiment 1, leakage of recipient follicles (>10 mm) was measured using 20 g and 25 g needles with various volumes of a solution of methylene blue. Ovaries were placed in water and follicles were approached from the stroma. Leakage was classified into 4 classes. Class 0: dye not visible in water; 1: some immediate dye in the water; 2: immediate formation of a blue spot on the floor of the water container, and 3: severe continuing stream of dye and collapse of the follicle. A mean leakage classification >0.5 was defined as not useable. In experiment 2, 5-20 immature slaughterhouse oocytes in 100 µl volumes of 0.9% saline with BSA were injected into follicles > 10 mm *in vitro*. Oocytes were recovered after 24 h by puncturing and flushing the recipient follicle. Sessions were performed with and without silicone coating of the tubing and needles. In experiment 3, 20 g, 21 g, 24 g and 25 g needles were tested on capacity to pass the vaginal wall by transvaginal puncture *in vitro*, using an ultrasonography probe with needle guide. To test *in vitro* results *in vivo*, 10 oocytes in 100 µl of 0.9% saline with 40 mg/ml BSA and 20 IU heparin to prevent blood clothing were injected into preovulatory follicles at 48 h after prostaglandin im using a 21 g siliconized needle in 4 cows. After 24 h, oocytes were recovered by ovariectomy (n = 2) or by OPU (n = 2).

Leakage was negligible for both needles with volumes up to 100 µl (Fig. 1) and BSA in the medium improved recovery (Fig. 2) while siliconization prevented loss of oocytes in the system. The 24 g and 25 g needle could not be used to properly pass the vaginal wall. *In vivo*, the size of the recipient follicles was not affected, but in only 1 of 4 cows n > 1 oocytes were retrieved. In conclusion: *In vitro* the GRAFT-system worked well, but for the *in vivo* application further research is required.



Leakage of dye from cow follicles > 10 mm. Number of follicles is written above bars.



Recovery of oocytes after 24 h incubation in recipient follicles in vitro. [a,b] significant difference.

Notes

PREGNANCIES AFTER BOVINE OVUM PICK-UP IN SPAIN: PRELIMINARY RESULTS

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The production of embryos from prepubertal calves and cows, as a way to shorten the generation interval and improve annual genetic gain, is a very interesting reproductive tool.

Ovaries from six unstimulated Asturiana de los Valles cows were punctured twice a week during seven weeks (14 sessions). Oocytes were collected by ultrasound-guided follicle aspiration using a 6.5 MHz transductor (SA 600, Medison Co., Korea) attached to a 17G gauge needle (Cook Veterinary Products, Australia) and to a teflon tubing with vacuum pressure of 85 mm Hg (flow rate 14 ml/min).

As a previous approach, COCs were matured *in vitro* (MIV) in TCM 199 + 10% FCS +10 ng/ml of EGF for 24 h in 5% CO_2 in air and high humidity. Matured COCs were fertilizated with swim-up separared, frozen-thawed sperm in Fert-TALP + 10 µl/mg heparin for 18 h. Presumptive zygotes were vortexed and cultured *in vitro* (CIV) in SOF (Holm et al., Theriogenology 52: 683-700) + 5%FCS added 66 h after fertilization. Results are shown in table 1.

Table 1. Embryo development of CCOs after MIV in the presence of EGF and CIV in SOF

n	R	% Cleavage.	%5-8c.	%Morulae D6	%Blastocyst D7
10	5	50	33.3	10	0

n: number of oocytes; R: replicates; %5-8 c: 5-8 cell embryos at Day 3

Consequently, in another attempt, we changed our maturation conditions (1 µg/ml FSH +5 µg/ml LH + 1µg/ml 17β-estradiol + 100 µM cysteamine in VERO cells, instead of EGF). Fertilization procedure was the same. For *in vitro* culture, we used two systems: SOF to which 6% CPSR3® (a serum replacement, SIGMA) was added 66 h post fertilization, and B2 + FCS with VERO cells. Table 2 shows the results obtained. Data were analysed by ANOVA and Duncan's tests.

Table 2. Embryo development of CCOs after MIV in presence of VERO cells and CIV in SOF or B2

Culture	n	R	% Cleavage	%5-8c.	%Morulae D6	%Blastocyst D7
SOF	31	5	76.5 ± 7.0	38.6 ± 4.9	32.5 ± 7.9	16.7 ± 8.9
VERO+B2	56	9	71.9 ± 7.1	49.4 ± 5.3	31.9 ± 8.0	26.9 ± 8.6

n: number of oocytes; R: replicates; %5-8 c: 5-8 cell embryos at Day 3. Results are Mean±SEM

No significant differences were found, but a tendence to improve the blastocyst rates is appreciated when maturation and culture take place with VERO cells. Sixteen blastocysts were transferred to synchronous recipients (1 embryo/recipient). Pregnancy rate at 21 days (progesterone levels>2 ng/ml) was 66.7%. Pregnancy rate at 35 days (ultrasonographic diagnosis) was 33.3%. At this moment, on going pregnancies are two.

Project FEDER 1 FED97-0023. Authors thank Itziar Fernández and José Manuel Prendes (Coop. Agricultores de Gijón) for their help.

E. DIAZ is a fellowship holder from INIA.

Notes

VARIABLES INFLUENCING EMBRYO COLLECTION RESULTS IN SUPEROVULATED GERMAN HOLSTEIN CATTLE

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Data (1934 embryo recoveries in superovulated German Holstein donors) were collected from a commercial ET-Center (VOSt-Georgsheil) from March 1991 until April 1999 to determine the effects of age of donors (heifer: n = 337; 1st calf: n = 257; >= 2 calfs: n = 293), no. of superovulations (one: n = 1359; two: n = 282; >= three: n = 293), time after calving, herd, gonadotrophin (pFSH: FSH-P, n = 470, Folltropin®, n = 332; oFSH: Ovagen®, n = 85; eCG: Intergonan® + Anti-PMSG, n = 110), type of insemination (single bull once: n = 21; single bull twice: n = 1294; different bulls twice: n = 276), year and season on the number and quality of embryos recovered on Day 7. Additionally, the bull-influence on the sex of calves was investigated: following insemination with different bulls paternity was detected by PCR in the laboratory of the RPN Verden. Data were analysed by analysis of variance followed by Tukey´s procedure, *t*-test, Wilcoxon´s rank sum test, and correlation and regression analysis (SAS/Stat software). Results concerning the numbers of collected, degenerated and transferable embryos as well as unfertilized oocytes are shown in Table 1.

Table 1: Influences on embryo collection results

Variables	Embryos collected	Embryos degenerated	Oocytes unfertilised	Embryos transferable
Year	-	-	+++	-
Season	-	-	-	-
Age of donors	+++	+++	-	+++
Time after calving	-	-	-	-
No. of superovulations	-	+	++	-
Gonadotrophin	+++	-	+	++
Type of insemination	-	-*	-*	-*
Bull	-	++*	++*	++*
Herd	+++	+	-	+++

+++: $P < 0.001$; ++: $P < 0.01$; + $P < 0.05$; - : not signif.; * concerning the percentage of embryos

Cows with two or more calvings showed the best results. The number of unfertilised oocytes ($P = 0.035$) and degenerated embryos ($P = 0.004$) rose significantly with increasing numbers of superovulation. FSH-P® and Folltropin® were highly superior to Ovagen® and Intergonan®.. Field fertility of a bull was not correlated with his fertility in the ET-programme. Bulls significantly influenced the quality of embryos represented by the percentage of transferable and degenerated embryos or unfertilised oocytes. No influence of the investigated bull-population on the sex of calves could be found, although there were some bulls who sired significantly more male calves (over 60%). A highly significant effect of the herd of donors existed on the total number and number of transferable embryos.

In summary, the study demonstrates that herd, age of donors, gonadotrophin and bulls are major factors influencing the success rate of embryo recovery and suggests that a proper management and a preselection of donor cows and sires could lead to higher numbers of transferable embryos.

Notes

THE OPS VITRIFICATION OF GV-OVINE OOCYTES WITH DIRECT REHYDRATION: IS IT WORKING QUICK COOLING OR QUICK THAWING OR BOTH?

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The following literature data were the ground for performing these investigations:

1) The effectiveness of elevated both cooling and warming rates for cryopreservation of oocytes and embryos by direct plunging into liquid nitrogen using small volume of vitrifying (cooling/warming) solution (Martino *et al*., 1996: Biol. Reprod. 54, 1059-1069; Vajta *et al*., 1998: Mol. Reprod. Dev. 51, 53-58).
2) The effectiveness of elevated warming rate at thawing of vitrified oocytes (Isachenko *et al*., 1998: Cryobiology 36, 250-253) and absence of visible crystal formation when warming/cooling rate was higher than 1.3 (Isachenko *et al*., 1999: Cryobiology 39, 35, abstr.).
3) Decreasing viability of the vitrified oocytes with decreasing the diameter of glass capillaries (decreasing the volume of vitrifying solution, and respective decreasing of thawing/cooling rate) [Hochi *et al*., 2000: Theriogenology 53, 255 abstr.].

   The objective of our experiments was to test the protocols for vitrification of GV-ovine oocytes with three cooling/warming regimes: slow cooling-quick thawing, quick cooling-slow thawing and quick cooling-quick thawing

Excellent (0.7 per slaughter house ovary) cumulus-oocyte complexes (COCs, n = 269) were aspirated using 19-G needles, divided into three treatment groups, and vitrified in open pulled straws (Vajta *et al*. 1998: Mol. Reprod. Dev. 51, 53-58) as follows. After exposure at 38°C in 40% ethylene glycol + 0.75 M sucrose, prepared on culture medium (CM) containing TCM-199 + 20% fetal calf serum, COCs were directly plunged into liquid nitrogen. After thawing COCs were expelled from straws directly into CM at 38°C. The cooling-warming rates of COCs in group 1 (n = 87), 2 (n = 54) and 3 (n = 128) were approximately: 3000°C/min-16.000°C/min; 20.000°C/min-2000°C; 20.000°C/min-16.000°C/min, respectively. Then the COCs were transferred for 24 h to TCM-199 + 100 µl/ml ovine follicular fluid + 1 µg/ml FSH at 5% CO_2. After 24 h of maturation, COCs were denuded and stained by Hoechst 33342.

The fluorescent microscopy evaluation showed that the MII stage was reached by 0%, 0% and 35% COC ($P<0.001$) in group 1, 2 and 3, respectively.

In conclusion, these experiments have demonstrated that the effectiveness of OPS vitrification of GV-ovine oocytes is explained by the combination of elevated rates of cooling and thawing.

Supported by INIA.

Notes

INHIBITION OF MESSENGER RNA POLYADENYLATION PREVENTS ASSEMBLY OF THE SPINDLE APPARATUS IN BOVINE OOCYTES

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During the growth phase of oocytes mRNA synthesized in the nucleus is stored in the cytoplasm after specific deadenylation. During onset of oocyte maturation this "dormant" mRNA is specifically polyadenylated. This process catalyzed by poly(A) polymerase transfers the mRNA into a translatory active state. In our experiments we examined the effects of cordycepin, a specific inhibitor of poly(A) polymerase, on *in vitro* maturation of bovine cumulus oocyte complexes (COCs).

Maturing COCs in mod. TCM 199 (TCM 199 + 3 mg/ml BSA and 13.4 µg/ml LH) supplemented with 5 µg/ml cordycepin for 24 h blocked all oocytes in germinal vesicle (GV) stage whereas 77.5 ± 9.9 % of control oocytes (24 h in mod. TCM 199) exhibited metaphase 2 (M 2) stage. MAP kinase (MAPK) and histone H1 kinase (H1k) activities in inhibited oocytes decreased significantly ($P < 0.01$; t-test) (H1k: 228 ± 52 cpm; MAPK: 962 ± 238 cpm) compared with COCs matured for 24 h in mod. TCM 199 (H1k: 422 ± 212 cpm; MAPK: 3211 ± 1012 cpm).

In further experiments COCs were matured first for increasing periods (6, 9 and 12 h) in mod. TCM 199, then transferred to mod. TCM 199 supplemented with 5 µg/ml cordycepin and denuded after 24 h. 86.3 ± 15.8% of the oocytes transferred after 6 h (6- 18+)and 85.2 ± 10.6% transferred after 9 h (9- 15+) remained in the diakinesis stage. MAPK and H1k activities of these oocytes compared with the activities of M 2 oocytes (see above) were significantly lower ($P < 0.01$) [H1k: 193 ± 51 cpm (6- 18+), 154 ± 58 cpm (9- 15+); MAPK: 983 ± 283 cpm (6- 18+), 751 ± 116 cpm (9- 15+)] reaching the level of unmatured oocytes (H1k: 163 ± 42 cpm; MAPK: 888 ± 188 cpm). Increasing the inhibitor-free cultivation period to 12 h (12- 12+) reduces the amount of oocytes in diakinesis to 24.9 ± 10% whereas the kinase activities increased significantly($P < 0.05$) (H1k: 348 ± 62 cpm; MAPK: 1291 ± 251 cpm).

After fluorescent staining of oocytes in diakinesis with FITC-conjugated antibodies against α-tubulin (a major protein of the spindle apparatus) no spindle assembly could be shown.

The presented results show that the assembly of spindle apparatus could be prevented by inhibiting polyadenylation in bovine oocytes. Since inhibition of polyadenylation is accompanied with significant decreases of MAPK and H1k activities we suggest that these enzymes control spindle assembly.

Notes

TRANSMISSION OF CAPRINE ARTHRITIS-ENCEPHALITIS VIRUS (CAEV) BY EARLY EMBRYONIC CELLS FROM *IN VIVO*-PRODUCED GOAT EMBRYOS

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The aim of this study was to investigate whether caprine arthritis-encephalitis virus (CAEV) interacts with early embryonic cells from *in vivo*-produced goat embryos during *in vitro* co-cultures and whether the embryo zona pellucida (ZP) plays a protective role against CAEV infection.

ZP-free and ZP-intact 8-16 cells embryos were incubated for 2 h in infectious medium containing 10^4 tissue culture infective dose 50 ($TCID_{50}$/ml) of CAEV, washed 5 times in separate 2-ml volumes of Minimum Essential Medium (MEM) supplemented with 10 % fetal cattle serum (FCS). They were then cultivated in a co-culture system containing caprine oviduct epithelial cells (CEOC) and a goat synovial membrane monolayer (GSM: indicator cells) for 72 h at 38.5°C in a 5 % CO_2 atmosphere. The same conditions of culture were applied for non-infected embryos (ZP-free and ZP-intact) used as controls.

Analysis of washing fluids' infectivity based on the observation of cytopathic effects (CPE) on GSM cultures revealed the presence of viruses only in the first two washes issued from treated groups (ZP-free and ZP-intact). The mixed monolayers (COEC+GSM) on which the infected ZP-free embryos were cultured showed CPE. In contrast, ZP-intact 8-16 cell embryos inoculated with CAEV, like all control groups (ZP-free and ZP-intact), did not resulted in CPE. CAEV infection apparently did not block the ZP-free embryo's *in vitro* development up to 72 h post-infection when compared with untreated controls (34.6 % and 36 % blastocysts respectively, $p>0.05$).

These data suggest that ZP-free in vivo-produced goat embryos are able to be a vector of CAEV. The absence of interactions between ZP-intact embryos and CAEV *in vitro* suggests that ZP is acting as a protective barrier for embryos and not as vehicle for virus transmission.

Notes

NEW CBS STRAWS DO NOT AFFECT THE VIABILITY OF EARLY EMBRYOS: *IN VITRO* STUDIES ON BOVINE AND MOUSE EMBRYOS

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The aim of the study was to investigate any potential toxicity of the constituents of the CBS straws for embryos. Therefore, early embryos of two different species (cattle and mouse) were incubated in the CBS straws for a period of time at least three times greater than that required for the freezing/thawing process. The ability to develop further *in vitro* to the blastocyst stage was compared to that of control embryos cultured *in vitro* without any contact with the straws.

Bovine zygotes were produced *in vitro* through IVM-IVF and were isolated 20 h after insemination. Cumulus cells were removed and one-cell stage embryos were either used as controls (Group 1) or incubated into CBS straws for 2 hours at 39°C in B2 medium (Group 2). Both groups were then co-cultured for 7 days under similar conditions on vero cell monolayers to evaluate blastocyst formation and hatching .

Mouse embryos were collected from superovulated C57bl/6xCBA (F1) females at the 2-cell stage and randomly alloted to group 1 (control) or group 2 (experimental) in which they were loaded with M16 medium into CBS straws and then sealed and incubated for 2 hours at 37°C. To evaluate *in vitro* survival, the 2 groups of embryos were then simultaneously cultured for 4 days in microdrops of M16 medium, in the same incubator, using the same density of embryos per microdrop.

For each species, the blastocyst rate at the end of culture period was compared between groups using the Chi-square test. Survival rates are presented in Table 1.

Table 1: *In vitro* development of embryos after exposure to CBS straws

	Bovine		Mouse	
	Gr 1:control	Gr 2: CBS	Gr 1 :control	Gr 2 CBS
Nb of embryos (replicates)	172 (5)	140 (5)	176 (5)	165 (5)
blastocysts (%)	84/172 48.8%	69/140 49.3%	140/176 79.5%	126/165 76.4%

Not different from controls (P>0.05)

The results of this *in vitro* test confirm the absence of toxicity of the CBS straws for both bovine zygotes and mouse 2-cell embryos which are known to be very sensitive to any perturbation of the culture conditions at the early cleavage stages. The proportion of embryos that developed in vitro into blastocysts as well as the kinetics of development were not affected by an initial incubation of the embryos for 2h in the CBS straws. We can thus conclude that the use of this new CBS straw for cryopreservation should not have any deleterious effects on the survival rate of embryos.

Notes

EFFECT OF *IN VITRO* CULTURE IN SOF-BSA VERSUS SOF-SERUM ON THE DEVELOPMENT OF IVM-IVF BOVINE EMBRYOS UP TO DAY 12.

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The presence of serum during *in vitro* culture has been linked to the occurrence of the Large Offspring Syndrome both in sheep and in cattle. However, while in sheep there is a consistent body if evidence, in cattle the data in the literature are still controversial (Young *et al*., Large offspring syndrome in cattle and sheep. Rev.Reprod, 1998, 3:155-163).

In this study we investigated the effect of serum on the development of IVM-IVF bovine embryos up to day 7 *in vitro* and then up to day 12 *in vivo*. For this purpose bovine oocytes collected by dissection from ovaries of slaughtered donors were matured *in vitro* in medium 199 supplemented with 10% FCS, ITS (Sigma), heparin (10 µg/ml), LH and FSH (0,1 IU each, Pergovet Serono), L-IGF I (Sigma, 50 ng/ml) and L-EGF (Sigma, 100 ng/ml), in a non static culture system at 38,5°C in 5% CO_2 for 18-22 h. Fertilisation was carried out in 5% O_2 and 5% CO_2 in medium SOF with essential (EAA), non essential aminoacids (NEAA), glutamine, glycine (10 mM),6 mg/ml fatty acid free (FAF) BSA, 1 µg/ml heparin and PHE. Following fertilisation all embryos were cultured in microdrops of SOF-AA-FAFBSA (16 mg/ml) or SOF-serum (human, 20 %). From day 5 to day 7 the number of compacted morulae and blastocysts was recorded (Table 1). In order to evaluate the effect of the different media on subsequent development grade 1 blastocycts derived from embryos that had undergone compaction on day 5 were transferred in synchronised recipient heifers and allowed to develop up to day 12 when they were collected by non surgical flushing and their size was recorded (Table 2).

Tab.1. Effect of culture system on embryo compaction and blastulation

IVC	cleaved	cleavage	Comp.Mor. D+5 (%)	Freez.Blast. D+7 (%)	Tot.Blast. D+7 (%)
SOF-BSA	436/604	72%	152 (34.8%a)	108 (24.7%a)	134 (30.7%a)
SOF-serum	456/670	68%	56 (12.3%b)	36 (7.9%b)	130 (28,5%a)

T Student test . Values within columns with different letters are statistically different ($p<0.05$).

Tab.2. Embryo development up to day 12 and average size of embryos

type of *in vitro* culture up to day 7	N. transf. on D+7	N. recov. D+12	% recovery	average size of embryos ± SD: length (mm)	width (mm)
SOF-BSA	49	29	58.9%	0.93 ± 0.67	0.65 ± 0.37
SOF-serum	36	21	55.3%	0.80 ± 0.32	0.65 ± 0.24

The data in Table 1 indicate a significant reduction in compaction rate at D + 5 of embryos developed in SOF-serum as compared to SOF-BSA. The percentage of high quality blastocysts (freezable blastocysts) on day 7 was also significantly lower in SOF-serum while the total number of blastocysts forming on day 7 was not different. The results shown in Tab. 2 indicate that the viability up to day 12 of embryos that have undergone compaction in the two in vitro system is similar . The size of D + 12 embryos is not different between the groups suggesting that serum supplementation during IVC does not affect the growth rate of cattle embryos up to day 12.

This work was supported by the EC contracts n° CT 98 0032 and PL 98 4339.

Notes

COMPARISON OF BOVINE BLASTOCYST QUALITY AFTER SOMATIC NUCLEAR TRANSFER OR *IN VITRO* FERTILIZATION

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Full term development of somatic nuclear transfer embryos is still low and less than 10% of blastocyts can develop to term in cattle (Renard *et al*., Contracept.Fertil. Sex 1999; 27: 405- 411). Experiments were designed to examine the developmental rate and the total cell number in day 7 embryos derived from somatic nuclear transfer (NT) and *in vitro* fertilization (IVF) as controls.

Same batches of *in vitro* matured cumulus-oocyte complexes were allocated to two groups. Group 1 for nuclear transfer: enucleated oocytes were used as recipients for adult skin fibroblast as donor nuclei as described earlier (Vignon *et al*., Theriogenology 2000; 53:245). Group 2: oocytes were used for IVF as controls and inseminated with the semen of a single bull. Reconstructed NT embryos and IVF embryos were co-cultured under the same conditions on VERO cell monolayers in B2 medium containing 2.5% fetal calf serum at 39°C in humidified air with 5% CO_2. After 7 days of *in vitro* culture, morphologicaly evaluation of the embryos and development rates were observed. Only embryos morphologically qualified as grade 1 blastocysts were used for cell counting. The total cell number was quantitated using fluorescence labelling of the nuclei with bisbenzimide (Ho 33342). Blastocyst formation and quality were analysed using X^2 test and differences in cell number were assessed using Student's t-test.

Table 1: In vitro development and cell number of NT and IVF bovine blastocysts.

Origin of embryos	Blastocysts / Fused or fertilized embryos (%)	Grade 1 blastocysts / Total blastocysts (%)	Mean cell number X ±SEM (n = nb analysed)
NT	63/156 (40.4)[a]	35/63 (56.0) [c]	101.9 ±17.5[e] (11)
IVF	107/153 (70.0)[b]	80/107 (74.7) [d]	153.8 ±10.3[f] (15)

a,b:P< 0.05; c,d P< 0.05 ; e,f P<0.05

Results presented in Table 1 indicate that the rate of successful development to the blastocyst stage by day 7 was significantly different between the two groups. Blastocyst quality in term of morphological evaluation and total cell number was also significantly different between NT blastocysts and IVF blastocysts. This may account for differences in rates of implantation (30% for NT embryos vs. 60% for IVF embryos) after transfer to the uterus of recipient heifers. Studies on cell number in inner cell mass and trophoblastic cells in NT and IVF embryos are currently under investigation.

Notes

DETECTION OF SINGLE NUCLEOTIDE POLYMORPHISM MARKERS ON BIOPSIES FROM *IN VITRO* PRODUCED BOVINE EMBRYOS

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Nowadays, selection processes of cattle are money-consuming and time-consuming. Progress made in the field of quantitative genetics as well as in the field of *in vitro* fertilization let us foresee selection scheme that would greatly improve time required as well as cost efficiency. Such selection would be made partly on the basis of markers linked to Quantitative Trait Loci (QTL), genes of economical interest (Markers Assisted Selection).

Here we report the successful detection of Single Nucleotide Polymorphism markers on 15 cells bovine embryo biopsies, using different genotyping methods, based on Single Nucleotide Polymorphism (SNP) detection.

SNP markers were detected by mean of fluorochroms, using Oligonucleotide Ligation Assay (Grossman *et al*., 1998).

Two different systems have been used:

- one method implies a 3 markers multiplex linked to the α-MSH gene (Charlier *et al*., 1996), governing colour polymorphism.
- the other uses a 9 markers multiplex, linked to a chromosome 14 QTL influencing the milk composition, (Coppieters *et al*., 1998).

Several parameters of the detection have been explored. We could demonstrate that biopsy size was no obstacle, pre-treatment of the sample was important with respect to genotyping process. Nested PCR showed no advantages in detection. We could also demonstrate the importance of enzyme and additives brand.

We can foresee that the multiplication of markers of interest will increase the amount of DNA required, while the biopsy size must remain as minimal as possible not to compromise blastocyst survival.

We did use whole genome pre-amplification (aspecific amplification of the largest possible part of the genome) prior to SNP detection. We tested Primer Extension Preamplification (PEP, Zhang *et al*., 1992) and Degenerate Oligo Primed PCR (DOP-PCR, Thelenius *et al*., 1992). PEP seemed to be of little interest, while detection were possible after DOP-PCR which seems to be the method of interest.

This work is supported by a grant of the belgium "Ministère de l'Agriculture et des Classes Moyennes".

Notes

STUDY OF THE RELATIONSHIP BETWEEN CAPACITATION STATE OF SPERMATOZOA AND *IN VITRO* FERTILIZATION OF PORCINE OOCYTES.

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Polyspermy in pig oocytes fertilized in vitro is still unacceptably high. At the time of insemination in vitro, an increased number of spermatozoa per oocyte is suspected to be associated with a higher incidence of polyspermic penetration. Furthermore, the duration of coincubation of porcine oocytes matured in vivo with spermatozoa affects the rate of sperm penetration and the frequency of polyspermic penetration. It is also well known that only capacitated spermatozoa may attach to and penetrate zona pellucida. In this study, we first evaluated the effects of gamete coincubation time, then we attempted to determine if proportion of capacitated spermatozoa could be predictive of fertilizing ability of frozen-thawed semen in vitro. Oocyte-cumulus complexes were collected from slaughtered prepubertal gilt ovaries and matured in vitro for 44h in TCM199, with 10 ng/ml EGF, 400 ng/ml FSH and 10% follicular fluid. They were then denuded and cocultured in tris buffered medium (TBM, Abeydeera and Day, Theriogenology 1997, 48: 537) with 2.10^5 frozen-thawed spermatozoa/ml for 0 to 22h in experiment 1, for 22h in experiment 2. In experiment 2, capacitation state of spermatozoa was determined according to the CTC staining method described by Mattioli et al (Theriogenology 1996, 45:373). Staining pattern were defined as follow: type1, intact non-capacitated spermatozoa, fluorescence uniformly distibuted on the sperm head; type 2, capacitated spematozoa, fluorescence concentrated in the acrosomial region; type 3, acrosome reacted, fluorescence concentrated in the post-acrosomial region and/or damaged acrosome. Results are shown in tables 1 and 2.

Table 1: Effect of duration of oocyte-spermatozoa coincubation on penetration, polyspermic and monospermic fertilization.

	Oocytes						
	Examined	Penetrated		Polyspermic		Monospermic	
Coincubation time (h)	n	n	%	n	%	n	%
2	110	20a	18	5a	4	12	11
3	61	21b	34	3a	5	8	13
4	191	112cd	59	73b	38	27	14
6	106	58c	55	38b	36	20	19
22	175	118d	67	88c	50	29	17

a,b,c,d: significant difference within columns ($p<0,05$, Fisher's t test)
Until 4 hours of gamete coincubation, penetration rate increases,
then polyspermic fertilization increases over all.

Table 2: Penetrated and polyspermic penetrated oocytes after 22 h of coculture, and CTC staining patterns of spermatozoa at the moment of insemination in 5 independant replicates.

	Oocytes			Spermatozoa			
Replicate	n	Penetrated (%)	Polyspermic (%)	n	Type 1 (%)	Type 2 (%)	Type 3 (%)
1	32	31	30	219	23	37	40
2	16	56	67	252	12	21	67
3	42	64	48	228	14	25	61
4	24	88	81	221	27	27	46
5	44	89	97	238	17	46	37

There is no correlation between CTC staining pattern of spermatozoa and fertilization results. There is a strong correlation between total penetrated and polyspermic penetrated oocytes (Kendall rank test, $r^2= 0,8$, $p<0,03$).

In conclusion, penetration of pig oocytes as well as polyspermic fertilization occur since 2 hours after insemination. A strong correlation between penetration and polyspermic fertilization rates has been demonstrated, but there is no correlation between proportion of capacitated spermatozoa at the time of insemination and the fertilization rates.

Notes

NEW ARTIFICIAL INSEMINATION (A.I.) APPROACH FOR EWES: TRANS-VAGINAL LAPAROSCOPY.

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The aim of this study was to develop a new technique of insémination in ewe without surgery, then faster with frozen semen using laparoscopy and a trans-vaginal approach.
One hundred thirty ewes (61 Cross-bred, 16 Dorset, 42 Polypay, 11 Romanov) were selected on 6 farms. They were less than 5 years old, dry since at least 20 days before sponging, had lambed without any problem at least 70 days before sponging and had a body condition of at least 2.5 at selection. The synchronization protocol was the following: day 1 sponging (Veramix 60 mg USP) of a maximum of 30 ewes per group; day 14 sponge removal and injection of 500 I.U. of PMSG (52 Equinex, 56 Folligon, 22 PMSG-5000). On day 16, day of the A.I. (54 hours after sponge removal), about 10 minutes before the beginning of the intervention, the ewes have received a sedative and analgesic I.M., 6-8 mg of xylazine (Rompun, Haver) , afterwards each ewe was suspended by her hindquarters to a portable rail at 50 cm from the ground. A retracting perforation trocar of 455 mm was introduced in 2 way rigid liner (one for the trocar and the other for the catheter) and this liner was introduced using a speculum and a light source, into the vagina in the direction of the right or left side of the cervix. Following the perforation of the vagina, the trocar was removed and the endoscope with an angle of 30º was introduced. It was guided into the abdominal cavity to the A.I. site a second half of uterine horn. The frozen semen was chosen according to the breed and was thawed at the same time as the intervention. One straw with 10^7 of spermatozoons was used for each ewe. After thawing semen was aspired into a 50 cm catheter (double sided monojet) with a 25 gauge needle that was adapted for the liner. This one passed threw the second canal to deposit the semen inside the horns.

Lambings per flock and fertility percentage following the trans-vaginal A.I.

Flock	1	2	3	4	5	6	Total
# Ewes	13	21	35	9	35	17	130
Lambings	0	3	2	3	2	2	12
%	0 %	14.3 %	5.7 %	33.3 %	5.7 %	11.8 %	9.2 %

A total of 12 ewes from the 130 that were A.I. using this protocol lambed. In the first flock there were no gestations, but after a few corrections, at least 2 or 3 ewes per group have lambed. In conclusion, the results indicate that it is possible to obtain up to 33% of gestations with this new AI technique, without surgery. New trials must be done to solve different problems met during this project in order to obtain similar results as with abdominal laparoscopy and the speed of the operation improved.

Supported by CDAQ (Conseil pour le développement de l´agriculture du Québec, CANADA).

Notes

IT IS POSSIBLE TO INCREASE THE NUMBER OF VIABLE EMBRYOS USING PROGESTAGEN EAR IMPLANTS 1-3 DAYS BEFORE STANDARD SUPEROVULATION (SOV) WITH LOW RESPONSE COWS OR LOW FERTILITY COWS

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The aim of this study was to compare over 3 years' results in terms of viable embryos with the same cows having low response or low fertility with the standard 4day SOV or using ear implant (CRESTAR, Intervet) 1 to 3 days before the standard SOV.

The 8 holstein cows were located in farms and were between 4 and 13 years old. They were all SOV in mid-cycle 8 to 12 days with Folltropin V 360 mg for 4 days with decreasing dosis. They were SOV 1 to 3 times with this standard SOV before changing to the crestar program. The dominant follicle was aspirated in most of the cows 1.5 to 2 days before the begining of standard SOV or at the time of inserting the ear implant.
Results are shown in the table.

		STANDARD SOV					1 TO 3 DAYS IMPLANT				
Cow	Age	N°R	T.E.	V.E.	D.E.	O.	N°R.	T.E.	V.E.	D.E.	O.
1	13	3	26	12	1	13	4	30	21	0	9
2	6	2	9	2	3	4	2	30	2	3	25
3	7	3	17	13	0	4	1	8	8	0	0
4	4	1	3	1	2	0	2	12	9	2	1
5	8	2	21	0	6	15	3	47	18	12	17
6	7	3	17	3	1	13	5	36	7	8	21
7	7	2	5	0	1	4	2	10	7	2	1
8	9	2	32	0	0	32	1	24	3	0	21
Total		18	130	31	14	85	20	197	75	27	95
Average		2.25	7.22	1.72a	0.78	4.72	2.5	9.85	3.75b	1.35	4.75

N°R: number of recoverie;T.E.: transferable embryos; V.E.: viable embryos;
D.E.: degenerated embryos; O.: ova
Significant difference were observed between a and b by ANOVA ($p<0.02$).

These results indicate that it is beneficial to use the progesterone ear implant 1 to 3 days before the beginning of a standard SOV, not only because of the improved fertilisation rate (61.9% v.s. 76.2%), but also because of the increase in the number of viable embryos (1.72 v.s. 3.75).

Notes

COMPARISON OF THREE DIFFERENT CULTURE MEDIA FOR PORCINE *IN VITRO* PRODUCED (IVP) EMBRYOS: EFFECTS OF BOVINE SERUM ALBUMIN (BSA), FETAL CALF SERUM (FCS) AND PORCINE SERUM (PS) ON EMBRYO DEVELOPMENT

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Numerous approaches have been made in IVP of porcine embryos in the last decade. Suitable media are recently available for in vitro maturation, *in vitro* fertilization and *in vitro* culture of porcine zygotes.

In our study we compared three different culture media for porcine IVP-zygotes: i. North Carolina State University medium 23 (NCSU 23) supplemented with 4 mg/ml BSA (NCSU/BSA) , ii. NCSU 23 supplemented with 10% heat-inactivated FCS (NCSU/FCS) and iii. NCSU 23 supplemented with 10% heat-inactivated PS (NCSU/PS).

Ovaries of prepubertal gilts were collected at a local abattoir and transported within one hour to the laboratory. Oocytes were aspirated through 20 gauge needles and washed two times in TL-Hepes supplemented with 10% heat-inactivated FCS. Cumulus oocyte complexes (COC´s) were washed and cultured for 39 hours in NCSU 23 supplemented with 10% porcine follicular fluid, 10 IU hCG/ml, 10 IU eCG/ml and 0,57 mmol/l cysteine (maturation medium). Following maturation, COC´s were denuded with hyaluronidase (0.1%), washed in modified Tris-buffered-medium (mTBM/ fertilization medium) and transferred to mTBM. Fertilization was performed by incubating the oocytes with fresh semen in a concentration of 5×10^5/ml. Six hours later, putative porcine zygotes were washed and cultured in culture medium i., ii. or iii. for 168 hours.

Embryo development and cell number of morulae and blastocysts was evaluated by Hoechst-staining. Embryos with more than 8 cells were considered as morulae and embryos which started to cavitate were classified as blastocysts.

Results are shown in table 1.

Table 1. Embryo development in three different culture media

Cm	Zygotes cultured	Cleaved n (%)	> 4 -cells n (%)	Morulae n (%)	cell number Morulae	Blastocysts n (%)*	cell number Blastocysts
NCSU/BSA	238	163 (68)^a	48 (29)^a	8 (5)^a	10 ± 2,4	22 (13)^a	28^c ± 16,4
NCSU/FCS	249	164 (66)^a	33 (20)^a	17 (10)^a	10 ± 1,7	25 (15)^a	14^d ± 8,1
NCSU/PS	248	103 (42)^b	22 (21)^b	1 (1)^b	9	0 (0)^b	

Within whole columns, numbers with different superscripts are significantly different (a,b: Chi-square-test, p<0,001; c,d: students t-test, p<0,001). * = calculated over cleaved embryos

There are no significant differences between NCSU/BSA and NCSU/FCS regarding embryo development and blastocyst rate. However, NCSU/BSA seems to promote a higher cell number of blastocysts. It can be concluded, that NCSU/PS exerts a negative influence on the development of porcine zygotes, whereas NCSU/BSA and NCSU/FCS can both be used for the culture of porcine zygotes.

Notes

PCR SEXING OF DAY 7 BOVINE *IN VIVO* AND *IN VITRO* EMBRYOS

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Determination of the sex of embryos can be of great advantage for both the farmer and breeding organisations because it allows the optimal use of embryos. A method often used for sex determination is PCR amplification of part of the Y-chromosome after embryo biopsy. Here we report the accuracy of the PCR method and the pregnancy rate after transfer of the embryos after biopsy.

From July 1997 till March 2000 a total of 1951 Day 7 IETS Grade 1 and 2 bovine embryos (1395 *in vivo* and 556 *in vitro,* from a BRL/M199 or SOF culture system) were biopsied for sex determination. Using a micro-manipulator controlled microblade, a small part of the morulae or the trophoblast of blastocysts was removed. Biopsies were evaluated by PCR using Y-chromosome specific DNA primers and the presence of product (the Y-chromosome) was monitored under UV light without the need for gel electrophoresis (Bredbacka *et al*. Theriogenology 44, 167-176, 1995). A selection of the biopsied embryos (based on their breeding value and sex) was transferred fresh or frozen [using 1.5M ethyleneglycol as cryoprotectant and directly transferred upon thawing (Otter *et al*., AETE 1998, p222)].

When all embryo developmental stages were analysed, the sex ratios are in the expected range: 48.7% male/51.3% female for *in vivo* and 50.9% male/ 49.1% female for *in vitro* embryos. We found no shift towards more males at more advanced embryo stages.
The pregnancy rates (at 8 month) of the biopsied embryos are shown in Table 1.

Table 1: Pregnancy rates of biopsied day 7 bovine *in vivo* and *in vitro* embryos

		Pregnancy rate		
Embryos	Transfer location	Biopsied	Unbiopsied	Difference
In vivo frozen	A	33.1% (n = 251)	38.3% (n = 188)	5.2 %
In vivo frozen	B	43.7% (n = 231)	51.6% (n = 8543)	7.9 % [a]
In vitro fresh	A	42.2% (n = 64)	47.6% (n = 382)	5.4 %
In vitro forzen	B	33.3% (n = 60)	35.3% (n = 1242)*	2.0 %

**embryos frozen in glycerol.* [a] *Significant difference (P<0.05, Chi-square)*

Until now 114 calves have been born from the sexed embryos: 16 males and 98 females. Seven of them did not correspond to the PCR-outcome, thus indicating an accuracy of 94%. All errors are false negatives, i.e. bull calves born while PCR gave female, which is what you expect since there is no check if the biopsy is really in the PCR tube.

It can be concluded that the embryo PCR sexing technique can be routinely used in an embryo transfer programme giving satisfactory accuracy and acceptable pregnancy results.

Notes

SOURCES OF VARIATION OF PREGNANCY RATES AFTER TRANSFER OF BOVINE FROZEN EMBRYOS

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Transfer of frozen embryos has been simplified in previous years by using ethylene glycol as cryoprotectant together with the "direct" transfer method. To improve pregnancy rates, sources of variation have to be characterised. Particularly, controversial effects have been reported for the synchronisation between donors and recipients. Moreover, genetic components such as the paternal origin of the embryos have been rarely studied. Therefore, this field survey aimed to investigate the effects of different sources of variation on pregnancy rates after transfer of frozen embryos in dairy cattle under farm conditions.

A total of 2134 transfers realized in France between 1996 and 1998 were included. Embryo characteristics such as stage (4 to 7), quality (according to IETS criteria), paternal origin (26 sires of AI with more than 28 transfers/sire), freezing method (ethylene glycol for direct transfer or glycerol sucrose for classical transfer) were recorded together with the following recipients data : breed, date of transfer, parity, type of oestrus (spontaneous or induced), quality of the corpus luteum, time of oestrus in comparison with the donor cow. The operator at transfer was also noted. Pregnancy was assessed by ultrasonography or rectal palpation. A multivariate model of mixed logistic regression with the potential variation factors was performed including paternal origin as a random effect.

Pregnancy rates (PR) averaged 50.5% and were significantly influenced by the method, operator, recipients parity and breed, CL quality. Whatever the year, transfer of ethylene glycol embryos resulted in higher PR than glycerol sucrose embryos (55.4% (n = 869) vs 47.2% (n = 1265) ; OR = 1.54; P = 0.03). Many characteristics of the recipients influenced PR after transfer. Multiparous recipients decreased strongly success of transfer when compared to heifers and primiparous (heifers = 52.0%; primiparous = 51.0%; multiparous = 40%; P<0.01). There was a significant effect of the breed, with higher PR observed in Normande than in Prim'Holstein females (OR = 2; 65.7% (n = 70) vs 49.6% (n = 1926); P = 0.02), even if the small number of Normande recipients could have biased the results. There was an effect of the CL quality assessed by rectal palpation on PR: most of the CL were defined as "very good" (88.4%) and were associated with higher PR (51.2%) than "fair" CL quality (45.1%; OR = 1.59; P = 0.03). The degree of synchrony between donors and recipients did not influenced PR after transfer. However, this factor was probably not discriminant, because the maximum degree of asynchrony was shorter than 36 hours and 75.1% of the recipients were transferred without asynchrony. The type of oestrus used as a reference before transfer was not related to success of transfer (spontaneous = 49.8% vs induced = 52.4%, P>0.05).

No significant effect of paternal origin of the embryos was observed, although PR ranged from 36% to 71% between sires. The stage and quality of embryos were homogeneous (94.1% of grade 1 embryos and 89.4% of morulae and young blastocysts) and did not significantly affect PR. On the contrary, the E.T. operator was an important source of variation of PR (P<0.001).

To conclude, the ethylene glycol method has improved success of transfer, but the choice of recipients is still an important source of variation of PR. On the contrary, a slight degree of asynchrony between donor and recipients, the type of oestrus before transfer and paternal origin did not affect significantly the results.

Notes

IN VITRO PRODUCTION OF BOVINE EMBRYOS IN DIFFERENT CULTURE SYSTEMS AND THEIR SURVIVAL FOLLOWING VITRIFICATION

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Despite progress in terms of increasing the yield of blastocysts from immature oocytes, the survival of *in vitro* produced embryos following cryopreservation has continually lagged behind that achieved with *in vivo* derived embryos. However, with the advent of simple procedures for the vitrification of such embryos, many of the detrimental effects of chilling injury can be circumvented. The aim of this study was to assess the effect of the *in vitro* culture system used in the production of bovine embryos on blastocyst yield and quality

In Experiment 1, IVM/IVF zygotes were cultured in either (1) synthetic oviduct fluid, (SOF, n = 552), in 5% CO_2, 5% O_2, 90% N_2, or (2) TCM+ granulosa cells (GC, n = 600), cultured in 5% CO_2 in air. *In vivo* blastocysts produced by superovulation were used as a control. Culture in SOF resulted in a significantly higher blastocyst yield on both Day 7 (31.3 vs 13.2%, P<0.001) and 8 (36.8 vs 23.7%, P<0.001) than TCM-GC. There was no difference in blastocyst cell number. Following vitrification/warming using the OPS technique (Vajta *et al*., 1998, Mol Reprod Dev 51: 53-58), survival at 72 h of *in vivo* produced blastocysts was significantly higher than both *in vitro* groups, while significantly more blastocysts produced in TCM+GC survived compared to those produced in SOF (0, 43.5, 78.3 % for SOF, TCM+GC and *in vivo*, respectively P<0.01).

Based on the results of Experiment 1, it was hypothesized that culture in SOF (to maximize blastocyst yield) in combination with GC (to optimize blastocyst quality) would lead to a high yield of high quality blastocysts. Thus, zygotes were cultured in SOF (n = 871), or SOF+GC (n = 863), in either 5% CO_2 in air, or 5% CO_2, 5% O_2, 90% N_2, giving rise to 4 treatments. In agreement with Experiment 1, culture in SOF in 5% O_2 resulted in significantly more blastocysts at Day 7 (26.4 vs 17.3%, P<0.01) and Day 8 (31.5 vs 23.2%, P<0.01) than SOF+GC. However, survival at 72 h post vitrification/warming was significantly higher for SOF-GC (44 vs 8.3%, P<0.001). Increasing the O_2 concentration to 20% significantly reduced the blastocyst yield from SOF (31.5 vs 17.3%, P<0.001). In addition, the quality of blastocyst produced was reduced in terms of survival post vitrification/warming (8.3 vs 0%, P<0.05). In contrast, there was no difference in blastocyst yield (23.2 vs 25.2%) or survival (44.0 vs 36.9%) in SOF+GC, irrespective of O_2 concentration.

Experiment 3 examined the duration of exposure to GC necessary to acquire improved blastocyst quality. Zygotes were cultured in (1) SOF, (2) SOF until Day 3, followed by SOF+GC for the remainder of the culture, (3) SOF until Day 5, followed by SOF+GC for the remainder of the culture, or (4) SOF+GC for the entire culture. Survival at 72 h post vitrification/warming was significantly higher (P<0.05) in Groups 2 (50.0%, 13/26) and 4 (55.3%, 26/47) than Groups 1 (21.7%, 10/46) and 3 (10.8%, 4/37).

In conclusion, culture in SOF results in more blastocysts than culture in GC, but the blastocyst quality is inferior in terms of survival following vitrification/warming. The presence of GC in SOF overcomes the detrimental effect of culture in a high O_2 atmosphere in terms of blastocyst yield and quality. Exposure to GC for at least 5 days is necessary to acquire this improved quality.

Notes

REDUCED *IN VITRO* FERTILIZATION OF PREPUBERTAL GOAT OOCYTES AFTER MATURATION IN AN EGF-BASED MATURATION MEDIUM

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We have recently reported that EGF has no a beneficial effect on developmental competence of prepubertal goat oocytes in a serum-free maturation medium in absence of hormones (Rodríguez-González *et al*., 2000; Therio., 53:468). In that study, we demonstrated that oocytes matured with EGF exhibited lower rates of normal fertilisation and embryo development when compared to an IVM medium supplemented with serum and hormones. The purpose of this study was to investigate the implementation of hormones to an EGF-based maturation medium with the objective of using EGF as a substitute of serum in order to employ a chemically defined IVM medium for maturation of prepubertal goat oocytes.

Ovaries were obtained from slaughtered prepubertal goats (45-60 days old). Cumulus-oocyte complexes were cultured in TCM 199 + hormones (1 µg/mL 17β-estradiol + 10 µg/mL oFSH + 10 µg/mL LH) supplemented with 10 ng/mL EGF (EGF treatment) or 10% steer serum (SS treatment). After 27 h of IVM, the oocytes were inseminated with capacitated with heparin (50 µg/mL) fresh semen (3.5×10^6/mL) in TALP medium supplemented with 1 µg/mL hypotaurine. Oocytes were fixed at 17 h post-insemination to evaluate the fertilization parameters with lacmoid staining.

The highest penetration observed when serum was added to the IVM medium (84.4%) compared to EGF treatment (25.3%) was due to higher normal penetration (24.0% versus 4.4%) and polyspermy rates (42.9% versus 6.3%), calculated from inseminated oocytes. Table 1 shows the effect of the addition of EGF or serum to the IVM medium on *in vitro* fertilization parameters with respect to penetrated oocytes.

Table 1: Effect of IVM suplementation (EGF or SS) on *in vitro* fertilization parameters (replicates = 3)

				Monospermy			Polyspermy	
Treatment	N	Pene-trated (%)	With MPN (% of penetrated)	Total (% of penetrated)	FPN+MPN (% of penetrated)	FPN+ non-decondensed sperm head (% of penetrated)	Total (% of penetrated)	Mean number of spermatozoa in penetrated oocytes
EGF	158	40 (25.3)b	8 (20.0)b	30 (75.0)a	7 (17.5)	23 (57.5)a	10 (25.0)b	2.1
SS	154	130 (84.4)a	83 (63.8)a	64 (49.2)b	37 (28.5)	27 (20.8)b	66 (50.8)a	2.4

MPN: Male pronucleus; FPN: Female pronucleus
a,b within columns differ significatly (P<0.01)

Results show that EGF, at least in the concentration used in this study, is not a useful compound for replacing serum in a IVM system of prepubertal goat oocytes. Although we can observe a higher polyspermy in the oocytes matured in presence of serum, a higher percentage of oocytes were able to decondense the sperm head and form the male pronucleus. Probably, serum contains substances that can improve the cytoplasmic maturation and subsequent in vitro fertilization of prepubertal goat oocytes. With respect to EGF, receptors for this growth factor could be not present or inactive in prepubertal goat oocytes.

We conclude that EGF, added to an IVM medium supplemented with hormones, can not improve the in vitro fertilization of prepubertal goat oocytes. Further experiments are designed to evaluate the presence of EGF receptors in cumulus-oocyte complexes obtained from prepubertal goats.

Notes

EFFECT OF GONADOTROPIN-RELEASING HORMONE ON CLEAVAGE RATE AND EMBRYO DEVELOPMENT OF BOVINE OOCYTES FERTILIZED *IN VITRO*

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Notes

INSEMINATION STRATEGIES TO CONTROL HE SEX RATIO IN CATTLE

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Timing of AI before anticipated ovulation as well as oocyte maturational state have been reported to determine the gender of offsprings (Gutierrez-Adan *et al*., 1999: Therio, 51: 397). A retrospective study was conducted on sex ratio of calves born during the last 8 years at Kapuskasing Beef Research Farm to examine effects of ovulation synchronization program and of time intervals either between PG-induced luteolysis or onset of estrus and AI. Crossbred beef females kept under similar rearing conditions were distributed to five mating programs. In the control group, AI was performed using the am/pm schedule after onset of spontaneous estrus (SE). In the treated groups, females received GnRH (2 ml of Cystorelin, Merial) and 6 days later prostaglandin (PG, 2 ml of Estrumate, Mallinckrodt Veterinary inc., Canada) and then were inseminated after onset of induced estrus (GPE) or using fixed time AI protocols. In these timed programs, females were inseminated once between 48 and 64 h after PG injection with (GPGT), or without (GPT) a simultaneous injection of GnRH, or were administered a second GnRH injection between 48 and 60 h after PG and were inseminated 12-16 h later (GPG-T). Estrous behaviour was monitored and recorded twice daily (0600 to 0900 and 1800 to 21h00) using vasectomized teaser bulls. Pregnancy diagnosis was performed by rectal palpation 45 to 60 days after AI and calvings were recorded. Only data from AI that resulted in calves of known sex were considered in this study (n = 885). Time of PG injection, of estrus detection, and of AI and calf sex were recorded by experienced technicians. Females that showed standing estrus (n = 714) were divided in 3 groups according to the time interval from onset of estrus to AI: -10 to +10 h, 10 to 14 h and 14 to 22 h. PG-treated animals (n = 526) were divided in 3 groups according to the time interval from injection to AI: 24 to 60 h, 60 to 72 h and 72 to 120 h. Statistical analysis used the Chi-Square test with continuity correction.

Pregnancy rate (PR,%), number and proportion of females calves (F) according to treatment, to time interval from onset of estrus and AI (h) and to time interval from PG injection to AI (h).

	Treatments					Estrus-AI interval			PG -AI interval		
	SE	GPE	GPT	GPGT	GPG-T	-10-+10	10-14	14-22	24-60	60-72	72-120
Cows	512	509	125	142	301	451	493	408	362	351	292
PR	65.2	60.5	45.6	50.7	56.2	48.3	62.9	54.2	51.4	55.8	57.5
Calves	327	302	44	69	143	193	304	217	178	187	161
F	162	153	23	44	71	110	152	98	105	99	70
%	49.5^{a}	50.7^{a}	52.3^{ab}	63.8^{b}	49.7^{a}	57.0^{c}	50.0^{cd}	45.2^{d}	59.0^{e}	52.9^{ef}	43.5^{f}

The proportion of female calves was higher in GPGT than in all other treatments ($P < 0.04$) except in GPT. The proportion of female offsprings was higher when the estrus-AI interval was between –10 and +10 h than between 14 and 22 h ($P < 0.02$) after estrus. Furthermore, this proportion was higher when AI was practiced between 24 and 60 h than between 72 and 120 h ($P < 0.01$) after PG injection.

Since the sex ratio was skewed by the type of mating program and the timing of AI in relation with PG injection and onset of estrus, it is concluded that insemination strategies, using the GnRH-PG-GnRH program for fixed time AI, should consider the time interval between PG injection or onset of estrus and AI to enhance in cattle the number of offsprings of the desired sex.

Notes

COMPARISON OF GROWTH CURVES OF MOET, IVP CO-CULTURE AND IVP SOF CALVES

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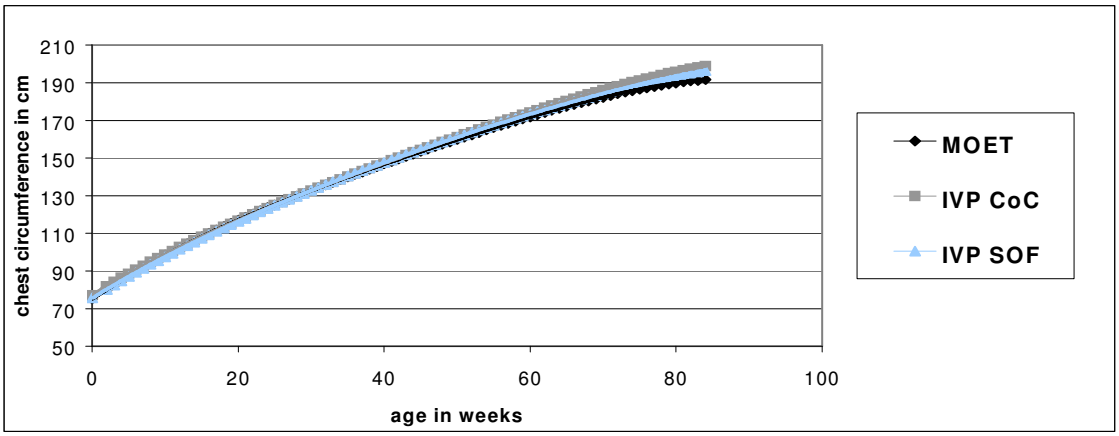
The quality of calves produced by different reproduction techniques is of great importance to farmers and breeding organizations. In an earlier study we reported increased birth weight and other deviations of IVP calves compared to MOET calves (van Wagtendonk-de Leeuw *et al*, Theriogenology 53; 575-597, 2000). The aim of the present study is to investigate whether these birth weight differences between IVP and MOET calves are reflected in growth characteristics during the first 18 months after birth.

MOET and IVP embryos were transferred and calves were born in the same recipient and calving herd in the same time period. Growth of the IVP Co-Culture, IVP SOF and MOET female calves was monitored by measuring chest circumference using a measure tape. All calves were kept in the same rearing herd. Measurements were done by one person monthly starting at 1 month of age until 12 months. A total of 49 calves were evaluated (20 MOET, 16 IVP Co-Culture and 13 IVP SOF). Individual growth curves of IVP Co-Culture, IVP SOF and MOET calves were fitted by a third order polynomial regression. From these curves, predicted chest circumference observations at fixed ages (0, 10, 20, .. 80 weeks) were derived. Least squares means for chest circumference were estimated by PROC GLM of SAS.

Birth weights of IVP Co-Culture, IVP SOF and MOET calves were 45.6, 40.9 and 40.8 kg, respectively. Chest circumference at birth was significantly larger in the Co-Culture group as compared to the MOET and SOF group, but not at later ages.

Figure 1. Growth curves of IVP CO-Culture, IVP SOF and MOET Calves in the first 18 Months age



It can be concluded that despite the clear differences observed in birth weight and chest circumference between IVP Co-culture and the two other groups, no significant differences in growth characteristics could be observed between the three groups. Interestingly already at week 10 no differences in chest circumference could be observed. This would implicate that the increased birth weight found in Co-culture IVP calves is already normalized at 1 month after birth.

Notes

MEIOTIC MATURATION OF CANINE OOCYTES : KINASES ACTIVITIES AND EFFECT OF SPERM PENETRATION

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In contrast with most mammals, canine oocytes are ovulated as immature oocytes at the germinal vesicle stage and complete their meiotic maturation during 48 to 72 hours within the oviducts (4). In this species, whatever the culture conditions used, the rate of *in vitro* maturation remains low (3). The first aim of this study was to investigate changes in Mitogen-Activated Protein Kinase (MAPK) and Maturation Promoting Factor (MPF) activities during meiotic maturation of bitch oocytes (study I). Moreover, the bitch accepts to be mated while immature oocytes are still in the oviducts (1). In study II, the influence of fertilisation immature oocytes on the resumption of meiosis, and the evolution of both male and female chromatin were examined *in vitro*.

Canine ovarian cumulus-oocyte-complexes (COCs) were collected during routine ovariectomies and cultured in Medium 199 + 20% FCS at 38.5°C. In study I, after 24, 48 or 72 hours of culture, chromatin was stained with Hoechst 33342 and oocytes were individually classified according to chromatin configuration as "germinal vesicle" (GV), "dense chromatin" (DC), "metaphase I and II" (MI, MII). MPF and MAPK activities were measured as Histone H1 and Myelin Basic Protein kinase activities respectively, as previously described (2).

In study II, COCs were cultured for 24 hours, then incubated with fresh semen for 24 hours, washed, then cultured for 24 hours and fixed. Control oocytes were cultured in the same conditions but without spermatozoa for 72 hours then fixed. After fixation, chromatin was stained with propidium iodide, and examined using laser scanning confocal microscopy.

A total of 103 oocytes were assayed individually for MPF and MAPK activities (study I). The MPF and MAPK activities appeared concomitantly after the DC stage and were significantly higher in oocytes that reached the metaphase I or II stages than in oocytes that remained at the GV or DC stages after 24, 48 or 72 hours of culture ($p<0.001$). No significant difference in both MPF and MAPK activities was observed between 24, 48 and 72 hours of culture for oocytes arrested at the GV stage and for oocytes that reached the DC, MI or MII stage ($p>0.05$). This is the first report of kinase activities in canine oocytes.

Results of study II indicate that *in vitro* fertilisation can occur in immature oocytes and induces a resumption of meiosis. After 72 hours of culture, the percentage of oocytes at the GV stage was significantly less for fertilised oocytes (40% (n = 60) *versus* 60.3% (n = 285) for control oocytes ; $p<0.05$), and the percentages of stages beyond metaphase I was significantly higher for fertilised oocytes (28.3% *versus* 60.3% for control oocytes; $p<0.01$). Observation of chromatin areas in fertilised oocytes showed an overall parallel condensation-decondensation of both female and male chromatin from the GV to the pronuclear stage. This study points out the stimulating signal induced by sperm penetration for resumption of meiosis in this species. It would be of great interest to evaluate the profiles of kinases activities during meiotic maturation after fertilisation in immature canine oocytes.

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Notes

ENDOSCOPIC EMBRYO TRANSFER FOR THE PRODUCTION OF TRANSGENIC PIGS

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Transgenic pigs were produced by microinjection of different gene constructs into the pronuclei of fertilized eggs. Prepubertal gilts were superovulated, artificially inseminated and slaughtered 60 hours after injection of human chorion gonadotropin (hCG). The oviducts were flushed with PBS supplemented with 5 % heat inactivated lamb serum (LS) and 50 mg/l gentamicin and putative embryos were selected. Afterwards the embryos were transferred into PBS supplemented with 20 % LS. Twelve hours after onset of superovulation of the donor animals the prepubertal recipient gilts were injected with equine chorion gonadotropin and 72 hours later with hCG. The estrus behavior of the recipients was recorded. In contrast to mouse embryos the pronuclei of pig day one embryos are not visible under the microscope. Before DNA-microinjection the embryos had to be centrifuged due to the high contents of lipid granula in the cytoplasm. After centrifugation of the embryos for three min at 15000 g the lipid granula moved to one side and the pronucleus was detectable in the equatorial segment. Normally the pronuclei were injected until their diameter increased by approximately 50%. Around 2 hours after DNA-injection 30 injected embryos were pooled together and were loaded into the tip of an intravenous catheter (1.4 mm diameter, 50 cm length; Braun, Melsungen, Germany). Before and after the embryos air bubbles were aspirated.

The recipients were anaesthetised with 1,2 ml/10 kg 10% Ketaminhydrochloride (Ursotamin®, Serum Bernburg, Germany) and 0,5 ml/10 kg 2% Xylazin (Rompun®, Bayer, Germany). Endoscopic embryo transfer was done according to Besenfelder *et al*. (1997). For the transfer the endoscopic equipment consisted of a small cold light fountain, a light cable, a metal catheter for an oblique optic telescope, a metal catheter for an atraumatic forceps and a metal catheter for the venous catheter. Five cm caudal of the umbilicus the catheter for the optic was introduced into the abdomen and 10 cm lateral of the optic the catheter for the optic was brought in. After investigation of the ovary, which had to contain at least three day 1-2 corpora lutea, the infundibulum was fixed by the forceps. Afterwards the venous catheter with the embryos was introduced into the oviduct. Twenty one days after embryo transfer pregnancy were examined by ultrasound.

Forty embryo transfers with three different gene constructs were performed. Sixteen pregnancies (40%) were investigated on day 21. Some of these pregnant gilts delivered already and the litter size ranged between 2 and 10 piglets. Transgenic rates ranged depending on the construct around 15%.

The minimal invasive and highly efficient technique of endoscopic embryo transfer guaranties low stress for the animals. In order to assure the welfare of the recipient gilts this technique can be used for the production of transgenic piglets for several commercial and scientific purposes.

Reference:

Besenfelder U., Mödl J., Müller M. and Brem G. (1997): Endoscopic embryo collection and embryo transfer into the oviduct and the uterus of pigs. Theriogenology 47: 1051-1060.

Notes

APPLICATION OF OVAGEN™ IN SUPEROVULATION OF DAIRY AND BEEF COWS IN USA

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INTRODUCTION

Follicle Stimulating Hormone (FSH) has been used for superovulation of cattle for many years. Optimisation of treatment protocols has in general been based on ad hoc trials undertaken by individual practitioners. A recent, controlled trial in the United States of America, involving 269 cows, has provided results that indicate there may be opportunities to address and optimise superovulation and its outcomes.

MATERIALS AND METHODS

161 dairy cows and 108 beef cows were assigned to groups with the following parameters – 2 dosage levels of FSH (in the form of ovagen™), 2 synchronising strategies and 2 prostaglandin brands. Details of breed, time of heat, artificial insemination, ovulation rates, the number of transferable embryos etc were recorded and compared.

RESULTS AND DISCUSSION

- The number of transferable embryos was significantly lower for dairy cows (across all groups) than for beef cows (5.3 transferable embryos vs 6.9).
- Reducing the dosage of ovagen™ (from 17.6 mg, the standard dose, to 14 mg per cow) had no significant effect on the number of transferable embryos for either dairy cows (5.1 vs 5.5 transferable embryos) or beef cows (6.4 vs 7.4 embryos).
- In dairy cows there was no difference between doses or between prostaglandins. For beef cows however, the effect of using less ovagen™ may be enhanced by using Lutalyse (9.3 vs 4.8 transferrable embryos at 14 mg and 17.6 mg of ovagen™ respectively). Nearly half of the 21 animals treated this way yielded in excess of 10 transferrable embryos compared to 12% of those treated with a full bottle.
- Significant differences to the dosage of ovagen™ had no effect on the number of embryos generated, suggesting that the standard procedure (i.e. administration of 17.6 mg per cow) is not optimally cost-effective, as reducing the ovagen™ dosage (to 14 mg per cow) had no significant effect on the number of embryos. The effects of reducing the dosage, on the number of embryos generated, is influenced by the choice of prostaglandin and the synchronisation method employed. This effect is particularly marked in beef cows and offers a simple means to improve the outcomes of superovulation treatment.

Notes

PRELIMINARY STUDIES ON ADULT SOMATIC NUCLEAR TRANSFER IN PIGS: EFFECTS OF ELECTROFUSION PULSES AND POST-FUSION CYTOCHALASIN B TREATMENTS

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Adult somatic cloning could contribute to increasing genetic selection efficiency. In this study, porcine adult somatic nuclear transfer was carried out, using in vitro matured oocytes as cytoplasm recipients and cumulus cells as nuclear donors. The *in vitro* oocyte maturation procedure applied in our laboratory is based upon that described by Wang *et al*. (1997; J. Reprod. Fert. 111, 101-108) with hormonal modifications (Silvestre *et al*., 1999; II Congreso Ibérico de Reproducción Animal. 7-10 Lugo, España, 260-262). Due to the established relationship between cell size and cell-cycle phase (Wakayama *et al*., 1998; Nature 394, 369-374), medium-sized cumulus cells, likely to be at the early interphase cell-cycle, were selected as donors. Effects of electrofusion pulses and post-fusion CCB-treatment on nuclear transfer efficiency were examined. Two types of electrofusing treatments were performed. First treatment: oocyte-cumulus cell couplets (OCC) received one electrical set and only those non-fused were pulsed again 30 minutes after the first set; second treatment: all couplets, fused or not, were submitted to two electrical sets, 30 minutes apart. Each set consisted of two square wave electrical DC pulses of 1.5 kV/cm for 60 µs, at 1s apart in 0.3M mannitol solution containing 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.01% PVA (Du *et al*., 1999; Theriogenology 51, 201). Following these electrical stimuli, 7.5 µg/ml CCB-treatment was or was not applied for 1 hr. Finally, reconstructed embryos were cultured in Medium 199 with 10% FCS for 48 hr.

Table 1. Effect of two electrical pulse treatments on fusion efficiency of porcine cumulus cell to enucleated *in vitro* matured oocytes.

Electrical treatment	Number of OCC (%)		
	Initial	Fused	Immediate lysed
1st treatment	44	39 (89)	1 (2)[b]
2nd treatment	49	35 (71)	10 (20)[a]

[a, b] Within columns, values with different superscripts are significantly (P<0.05) different.

Table 2. Effect of electrofusing and CCB-treatments on porcine somatic nuclear transfer efficiency.

Electrical treatment	Post-fusion treatment	Number of fused OCC (%)		
		Total	Late lysed	Cleaved
1st treatment	NO CCB	22	0	14 (64)
	CCB	17	1 (6)	9 (56)
2nd treatment	NO CCB	17	4 (23)	8 (62)
	CCB	18	3 (17)	12 (80)

When the first electrical fusion treatment was performed, fusion rate was higher than that obtained after the second (89% vs. 71%; P = 0.07; Table 1), probably because following the first treatment, immediate lysis rate (assessed at 15 min after last electrical pulse) was lower (2% vs. 20% respectively; P<0.05). Late lysis rate is also higher after the second fusion treatment (Table 2). And so, it is not recommendable to repeat the electrical pulse set on already fused couplets, as this reduces the global nuclear transfer efficiency. On the other hand, to our knowledge, no paper on post-fusion CCB-treatment effects on porcine nuclear transfer has been published to date. In our conditions, post-fusion CCB-treatment does not cause appreciable effects on either late lysis or cleavage rates (Table 2). However, the observed pseudo-polar body extrusions (1st treatment-CCB: 4/17, -NO CCB: 7/22; 2nd treatment-CCB: 1/18, -NO CCB: 2/17; data non shown in tables) recommend lengthening the post-fusion CCB-treatment in subsequent works, more than the 1 hr or 2 hr periods required for PB2 extrusion (Procházka *et al*., 1992; J. Reprod. Fert. 96, 725-734).

Notes

ISOLATION OF THE PREGNANCY-ASSOCIATED GLYCOPROTEIN 1 (PAG-1) FROM ZEBU (Bos indicus) PLACENTA

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Characterised in the last 20 years, the pregnancy-associated glycoproteins constitute a large family of glycoproteins expressed in the outer epithelial cell layer of the placenta of ungulate species. Using molecular biological techniques, they were found to be members of the aspartic proteinase family, with more than 50% amino acid sequence identity to pepsinogen, cathepsin D and E (Xie *et al*., 1991, P.N.A.S. 88, 10247-51).

By using classical biochemical procedures (a series of extractions of soluble proteins, acid and ammonium sulfate precipitations, gel filtration and ion exchange chromatographies) some molecules of the PAG family were isolated from cotyledons of cows, ewes and goats.

The aim of this study was to investigate the presence of pregnancy-associated glycoproteins in zebu (Bos indicus) cotyledons by using affinity chromatography.

Uteri were collected from pregnant zebu females. The fetal cotyledons were immediately dissected, washed with 0.9% NaCl, and stored at –20°C until processed. Approximately 900 g of fetal cotyledons were used. The tissue was minced and homogenized with a hand mixer in KCl solution (0.3 M, pH 5). The homogenate was stirred slowly at 4°C for 3 h. It was then centrifuged at 27 000 x g, and the supernatant desalted, concentrated and lyophilised. Two hundred-milligram samples were loaded onto a pepstatin agarose column (1 cm x 5 cm; Sigma) previously equilibrated in 50 mM sodium acetate (pH 5.2). After elution of the unbound proteins, a linear pH gradient (pH 5.2-10) was applied at a rate of 0.5 ml/min.

The proteins eluted in the gradient were submitted to the two-dimensional electrophoresis and showed 3 major spots with molecular masses of 67 and 84 kDa. Microsequencage of the N-terminus of the 67 kDa spot revealed the R G S X L T T H P L R N I K sequence, corresponding to the bovine (Bos taurus) PAG-1.

This result shows that bovine PAG-1 is also present in the Bos indicus placenta. Investigations are in progress in order to isolate other pregnancy-associated glycoproteins and to compare the electrophoretic patterns of Bos indicus and Bos taurus placental proteins.

(Supported by Belgium Ministry of Agriculture and FNRS. The senior author received a scholarship from CAPES. We also thank the NIH Bettesd USA for gift of hormones).

Notes

COLLECTION OF OOCYTES BY OVUM PICK-UP FROM PREOVULATORY FOLLICLES OF COWS TREATED FOR SUPEROVULATION IN RELATION TO RECOVERY RATE AND DEVELOPMENTAL COMPETENCE

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In a previous study variable recovery rates (23-47%) were achieved using transvaginal ovum pick up (OPU) to collect cumulus-oocyte-complexes (COCs) in cows treated for superovulation (SO). An experiment was designed to optimise OPU in reference to recovery rate and subsequent developmental competence of COCs collected from *in vivo* during successive defined stages of maturation from cows treated for SO.

Normally cyclic cows were used for different puncture sessions after synchronization and treatment for SO with FSH (Ovagen, total dose of 17 ml im (15 mg), 8 injections at 12 h interval). To obtain preovulatory-sized follicles at a fixed time of development an ear implant with norgestomet (Crestar, Intervet International BV, Boxmeer, The Netherlands) was applied simultaneously with the first injection of FSH at Day 10 of the synchronized cycle (oestrus = D0). After 102 h the ear implant was removed and the LH surge was induced with GnRH (1.0 mg Fertagyl im; Intervet International). Thereafter, COCs were collected from follicles > 8 mm using OPU with a 7.5 MHz sector probe (Pie Medical, Maastricht, The Netherlands). In a previous experiment with 12 cows comparison of recovery rate using a needle diameter of 14, 16 and 18G resulted in selection of the 18G needle (vacuum 60 mm Hg). In a second experiment (n = 6 cows) this system was used to obtain both expanded and non-expanded COCs at two times of final maturation: collection of COCs started at 2 h (Pre-LH: left ovary) and subsequently in the same cows at 26 h (Post-LH: right ovary) after GnRH injection. The concentration of LH in the peripheral blood was estimated by RIA. Pre-LH COCs with a minimum of two layers of compact cumulus investment were selected and processed for IVM, IVF and IVC in 1 culture well per cow. Post-LH COCs with expanded cumulus cell investment were selected and processed for IVF and IVC. Results are presented in the Table. The separately cultured poor quality COCs (Pre-LH: n = 3; Post-LH: n = 10) did not develop into blastocysts.

Table Developmental competence of cumulus oocyte complexes (COCs) obtained by OPU from preovulatory cow follicles at defined stages in relation to the preovulatory LH surge

OPU relative to LH peak (h)	Follicles punctured (n)	COCs collected (n)	Recovery rate (%)[1]	COCs in IVF/IVC (n)	Cleavage at Day 4 n (%)[2]	Blastocysts at Day 9 n (%)[2]
Pre-LH 1.5±0.5	53	38	72%	35	29 (83%)	17 (49%)
Post-LH 25±1.0	75	53	71%	40	34 (85%)	21 (53%)

[1]COCs collected over follicles punctured; [2] Percentages over COCs in culture.

In conclusion, OPU can be applied as a tool to collect expanded and non-expanded COCs with high recovery rates from preovulatory-sized follicles without affecting the oocyte quality and developmental competence. Moreover, developmental competence of COCs was unaffected by the time at which the COCs were recovered in relation the preovulatory LH peak.

This study was supported by Holland Genetics, Arnhem, The Netherlands, in the framework of Eureka BTIP project

Notes

MITOCHONDRIAL REORGANISATION DURING IVM AND IVF OF PREPUBERTAL GOAT OOCYTES.

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Correct nuclear and cytoplasmic maturation are essential steps to acquire oocyte meiotic competence and the ability to be normally fertilised. Redistribution of mitochondria and other cytoplasmic organelles are essential for normal oocyte maturation. It is well known that intracellular distribution of mitochondria is related to the level of cell metabolism, proliferation and differentiation and those organelles move through the cytoplasm to the side where energy is required. It has been reported that the inability to disperse mitochondria during oocyte maturation is directly correlated to a block in the resumption of meiosis. In vitro maturation and fertilisation of prepubertal goat oocytes results in a low percentage of normal fertilisation and high percentages of non-decondensated sperm heads and polyspermy (1). These anomalies could be related to an incomplete or abnormal cytoplasmic maturation of these oocytes. The purpose of this study was to perform a time-dependent study of the mitochondria distribution during in vitro maturation and fertilisation of prepubertal goat oocytes.

Immature oocytes from prepubertal goats were aspirated from slaughterhouse ovaries and selected for *in vitro* maturation (IVM) in TCM199 with 10 µg/ml LH, 10 µg/ml o-FSH, 1 µg/ml 17β-estradiol and 10% foetal bovine serum for 27 hr at 38.5°C in 5% CO_2 in air. Oocytes were inseminated as described by Poulin *et al*. (2). At 0, 15, 20 and 27 h of IVM, and 20 h post-insemination, cumulus cells were removed with a narrow glass pipette and oocytes treated with Pronase to digest the zona pellucida. Oocytes were then washed in PBS and stained with 0.5 µg/ml Mitotraker®Green FM (Molecular Probes) for 1 hour. Following three washings in PBS, oocytes were stained with 10 µl/ml bisbenzimide Hoechst 33342 in PBS for 5 minutes to assess the nuclear stage after maturation and fertilisation. After that, oocytes were mounted between a coverslip and a glass slide supported by four columns of paraffin. The slide was sealed with nail polish. Control group consist on prepubertal goat oocytes fixed and stained with Hoechst. Stained oocytes were observed under a confocal laser scanning microscopy.

We have observed an uniform distribution of mitochondria on Germinal Vesicle oocytes. Mitochondria started to move to the centre of the oocyte in Germinal Vesicle Break Down stage. Oocytes in metaphase I stage showed the mitochondria disposed around the metaphase spindle. These results are in agreement with those observed by other authors on oocytes obtained from adult animals [human (3); mouse (4,5); bovine (6); goat (7)]. But, in contrast to them, we have also observed a peripheral distribution of mitochondria and a great concentration inside the polar body on metaphase II oocytes, and a peripheral mitochondria distribution on normal fertilised (2PN) and abnormal fertilised oocytes (polyspermic and asynchronic oocytes).

It has been postulated a correlation between *in vitro* developmental capacity and the energetic requirement of the cell (8). Low percentage of 2PN oocytes and anomalies after IVF of prepubertal goat oocytes maturated *in vitro* (1,9) could be related to a low energy level and the abnormal disposition of mitochondria in the cytoplasm. This could result in the incapacity of the oocyte to decondense the sperm head and finally to achieve a normal fertilisation.

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Notes

BULL EFFECTS ON TIME OF FIRST CLEAVAGE *IN VITRO* AND RELATIONSHIP WITH FIELD FERTILITY DATA

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An *in vitro* assay for determining bull fertility that could replace field fertility trials would be of great economic benefit to the AI industry. However, attempts to correlate field fertility with *in vitro* development have lead to conflicting results. We have previously demonstrated that there is a strong correlation between the time of first cleavage of an oocyte post insemination and its ability to develop to the blastocyst stage. The aim of the present study was to examine the relationship between field fertility and time of first cleavage and subsequent blastocyst yield.

Following *in vitro* maturation, cumulus oocyte complexes were randomly allocated to 1 of 6 groups. Semen from six bulls was used with 150 day non return rates (NRR) varying from 0.57 to 0.78. Each bull was represented in each of 9 replicates. At 20 h post *in vitro* fertilization, presumptive zygotes were transferred to droplets of synthetic oviduct fluid (SOF). The embryos were examined at 30, 33, 36, and 42 h post insemination, and the number of cleaved embryos at each time point for each individual bull recorded. Blastocyst yields from each bull were recorded on days 6, 7 and 8 post insemination. Data were analyzed using SAS (release 6.12 for windows).

Stepwise multiple regression analysis and variable selection procedures (requiring variables to be significant at 0.15) indicated that the best predictor of fertility was a model containing 33-hpi cleavage % only. The correlation coefficient between cleavage in vitro at 33 hpi and field fertility was $r = 0.689$ ($P<0.0001$).

Table 1. Effect of bull on cleavage rate and embryo development *in vitro*.

Bull No.	Field Fertility		Cleavage at 33 hpi		Day 7 Blastocyst %
	n	NRR %	Oocyte No.	%	
ELG	3443	78^{a}	528	63^{a}	21.8^{ac}
HTA	6805	76^{b}	571	67^{a}	27.0^{b}
LEW	4587	74^{c}	517	33^{b}	18.4^{c}
MTL	1255	68^{d}	499	49^{c}	22.2^{abc}
KKN	499	68^{d}	513	42^{d}	25.0^{ab}
TJD	562	57^{e}	536	15^{e}	9.1^{d}

^{abc}Values in the same column with different superscripts differ significantly ($P<0.05$).

In conclusion, these data confirm our earlier observations that earliest cleaving zygotes are more competent in terms of development to the blastocyst stage than those that cleave later. In addition, the results indicate that the sire can have a significant effect on the kinetics of early embryonic development as measured by time of first cleavage post insemination.and that the timings of the early embryonic cleavage divisions are a useful predictor of bull fertility under field conditions.

This research was supported by Enterprise Ireland.

Notes

IN VITRO DEVELOPMENT AND ABILITY TO HATCH OF ENCAPSULATED BOVINE EMBRYOS

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The objective of this study was to evaluate the effects of encapsulation of bovine embryos on *in vitro* development in co-culture with oviduct cells and the ability of such embryos to hatch outside the capsules. Excellent quality Day 4 embryos produced *in vitro* were randomly assigned to 2 groups: encapsulation (n = 110) and control (n = 106). For the encapsulation procedure, groups of five embryos were directly immersed in 1.5 % sodium alginate in physiological saline, rinsed three times, and collected into a fine-bore pipette, with a very small amount of sodium alginate. Pipette tips were placed into petri dishes containing 1.5 % calcium chloride in saline. Their contents were then expelled and on contact with the calcium chloride formed a semi-solid high viscosity mould (microgel) of alginate, which contained the embryos. Cylinders were cut to obtain five embryos per capsule and co-cultured with oviduct cells at 39ºC and in 5% CO_2 in air. Unencapsulated embryos were used as control. Day 7 embryos were graded according to their morphological appearance on the basis of the Linder and Wright embryo grading classification. Differences between groups were determined by Chi-square analysis.

Table 1 shows the effect of sodium alginate encapsulation of bovine embryos on rates of development and hatching at Days 7 and 9, respectively. Forty-seven excellent quality blastocysts developed from the encapsulated group and 36 from the control group at Day 7. Encapsulated embryos were able to hatch at Day 9, first from the zona pellucida and then from the alginate capsule. Although more control embryos which initiated hatching completed hatching compared to encapsulated, there were no significant differences between groups. It can be concluded that encapsulated bovine embryos develop *in vitro* to the blastocyst stage in coculture with bovine oviduct epithelial cells at least at the same rate as control. This finding may be of practical interest for in vitro embryo production, because encapsulation procedures can offer better identification of embryos and can reduce the effect of environmental factors.

Table 1: *In vitro* development and ability to hatch of encapsulated and unencapsulated bovine embryos co-cultured with bovine oviduct epithelial cells.

Group	n	Blastocyst Day 7 (%)*	Initiating hatching Day 9 (%)*	Completely hatched Day 9 (%)*
Encapsulated Day 4	110	47 (42.7)	31 (28.2)	20 (18.2)
Control	106	36 (34)	25 (23.6)	24 (22.2)

Differences between proportions were not significant.
* The percentage is calculated from total embryos.

Notes

PREGNANCY RATE OBTAINED FROM FROZEN-THAWED OVINE AND CAPRINE EMBRYOS, PRESERVED FOR SEVERAL YEARS IN LIQUID NITROGEN

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The aim of our study was to compare "*in vivo*" survival of frozen - thawing ovine and caprine embryos, preserved for several month or years in liquid nitrogen. For this purpose, some embryos were imported from France (1993) and some were collected from the local ovine and caprine breeds, synchronised and superovulated by usual methods. The embryos were transferred to synchronised recipients by surgically only during the breeding season.

All freezing solutions were prepared using Dulbecco's phosphate buffera saline (PBS) supplemented with 0.3 M sodium pyruvate, 3.3.mM glucose and 20% FCS. Selected embryos for freezing were exposed to PBS with 0.5M; 1M and 1.5 M glycerol, each bath for 8 minutes. All embryos were placed into the centre of 0.25 ml sterile straws, separated by air bubbles from two columns of PBS with 0.5 glycerol.

The freezing of the embryos was carried out following a classical curve, using a programmable freezer CE-3000 (2°/min from +20°C to +10°C, 1°/min from +10°C t -7°C, seeding at -7°C, cooling with 0.3°C/min until -18°C, 1°/min. until -35°C and finally immersion in liquid nitrogen at -196°C). After thawing, by immersion in water at 39°C for 30 seconds, all the embryos were placed in PBS (0.5M and PBS) for 5 minutes.

Results concerning pregnancy rates obtained after transfer of thawing ovine and caprine embryos are presented in the table.

Years	[a]Embryos n (%)	Breeds	Recipients (n)	Time of cryo-preservation (months)	Pregnant at term (n)	New Borns (n)	Embryo survival (%)
1996	6 (54)	*Lacaune	3	48	2	3	50.00
1997	5 (45)	*Ile-de-France	2	60	1	2	40.00
1998	12 (60)	Karakul	4	6	4	7	58.33
1998	6 (46)	*Angora	3	72	2	2	33.33
1998	13 (68)	Local goats	6	8	5	7	53.85
1999	11 (69)	Palas Merino ewes	6	24	4	6	54.55
2000	17 (74)	[b]Palas Merino ewes	7	3	5	9	52.94

[a] normal embryos after freezing-thawing, that were transferred to recipients;
[b] pregnancy test established by echography;
* embryos imported from France in September 1993

The embryo quality was appreciated by the morphological integrity of zona pellucida and blastomeres. After thawing, the proportion of normal embryos which were transferred, was 60-74% when the preservation in liquid nitrogen was 3-24 months and 45-54% when the preservation of embryos was 48-72 months. Embryo survival was 53%-58% for all embryos preserved under 24 months in liquid nitrogen and 33-50% for embryos preserved more than 48 months in liquid nitrogen.

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