Use of a synthetic substitute to animal products for rabbit and ovine embryo cryopreservation

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Use of a synthetic substitute to animal products for rabbit and ovine embryo cryopreservation

Embryo cryopreservation media usually contain animal-derived products, such as bovine serum albumin (BSA). These products present two major disadvantages: an undefined variable composition and a risk of pathogen transmission. The substitution of animal products of embryo cryopreservation media by synthetical products may improve procedure standardization (by avoiding variability in media composition) and avoid sanitary concerns inherent to animal-derived products.

We aimed to evaluate the effect of replacing BSA in rabbit and ovine embryo slow freezing and vitrification media with a synthetic animal products free medium composed of synthetic hyaluronic acid: STEM ALPHA.Cryo3 (« CRYO3 »).

Firstly, we evaluated the substitution candidates through a thermodynamic approach, using differential scanning calorimetry. In paralel, we adapted a mitochondrial activity evaluation protocol (JC-1) to rabbit embryo, which allowed us to complement morphological *in vitro* evaluation, and evaluate two ewe superovulation protocols.

Secondly, we used a biological approach to evaluate the replacement of BSA with synthetical products (containing CRYO3) in rabbit and ovine embryo slow freezing and vitrification media, using *in vitro* (slow freezing and vitrification) and *in vivo* (vitrification) evaluation methods.

Our results seem to demonstrate that the chemically defined substitute CRYO3 can successfully replace BSA during rabbit embryo and ovine embryo cryopreservation (slow-freezing and vitrification).

Keywords
Cryobanking, Animal genetic resources, Embryo, Animal-derived product, Synthetic medium, Differential Scanning Calorimetry, Slow-freezing, Vitrification

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Utilisation d'un substitut synthétique aux produits d'origine animale pour la cryopréservation d'embryons de lapin et de brebis (Summary in French)

La cryoconservation des embryons est essentielle à la conservation de ressources génétiques animales puis à leur mise à disposition. Associée aux techniques de transfert, elle a contribué à la diffusion des matériels génétiques de valeur dans le monde entier, en remplaçant le déplacement des animaux.

Les milieux de cryoconservation d'embryons contiennent généralement des produits d'origine animale, tels que l'albumine sérique bovine (BSA). Les produits d'origine animale semblent favoriser la viabilité et le développement des embryons, et posséder de nombreuses propriétés avantageuses pour la cryoconservation des embryons, telles que la chélation des métaux, la diminution de la tension de surface, la régulation de la pression oncotique et du pH, ainsi que l'élimination de toxines.

Cependant, les produits d'origine animale présentent deux inconvénients majeurs :

- une composition pas complétement définie et variable (qui peut être due à des variations physiologiques et biochimiques entre donneurs, à l'âge ou à la diète de ceux-ci, à la photopériode, ou même aux méthodes de préparation de ces produits) ;
- un risque de transmission d'agents pathogènes (tels que bactéries, virus, levures, champignons, mycoplasmes ou prions).

La substitution des produits d'origine animale par des produits synthétiques, dans les milieux de congélation lente et de vitrification d'embryons, pourrait contribuer à l'amélioration de ces procédures : d'une part, par la diminution de la variabilité de la composition des milieux de cryoconservation, qui permettrait une meilleure standardisation de ces techniques ; d'autre part, par l'amélioration des conditions sanitaires, en diminuant le risque de contamination des embryons.

L'acide hyaluronique est un glycosaminoglycane qui peut être synthétisé en laboratoire. Il est naturellement présent dans les fluides du tractus reproducteur de la femelle et sa concentration augmente dans l'utérus au moment de l'implantation. Après avoir été utilisé comme substitu de l'albumine dans des milieux de culture d'embryons, l'acide hyaluronique est devenu un candidat intéressant pour remplacer les produits animaux présents dans les solutions cryoprotectrices d'embryons.

Au cours de ce travail, nous avons cherché à remplacer la BSA par un milieu synthétique formulé sur une base d'acide hyaluronique (STEM ALPHA.Cryo3 - «CRYO3»),

et à en évaluer les effets sur les protocoles de congélation lente et de vitrification d'embryons cunicoles et ovins. Pour ce faire, nous avons procédé en deux étapes.

La première étape a consisté en de multiples expériences préliminaires, notamment :

- l'évaluation des propriétés thermodynamiques d'une solution de vitrification d'embryons de lapin classique, contenant des produits d'origine animale (0.4 % (*v/v*) BSA), et de deux solutions contenant le potentiel substitut - CRYO3 (D-PBS + 20 % CRYO3, et 100 % CRYO3). Ces mesures s'inscrivent dans la continuité de résultats obtenus précédemment au sein de notre équipe avec des solutions de congélation lente d'embryons de lapin et démontrent que la solution contenant 100% CRYO3 possède les caractéristiques les plus appropriées à l'obtention d'une vitrification pendant le refroidissement, tout en conservant la plus grande stabilité au cours du réchauffement ;
- l'adaptation d'un protocole de détection des altérations du potentiel membranaire mitochondrial (marquage JC-1) à l'évaluation de la fonctionnalité des embryons chez le lapin. Nous avons ainsi pour la première fois établi les conditions de marquage des embryons dans cette espèce, qu'ils soient frais ou congelés, et obtenu une méthode d'évaluation de leur viabilité et de leur fonctionnalité fiable ;
- la mise en place et l'évaluation de protocoles de superovulation de brebis, adaptés à nos conditions de terrain, afin de produire des embryons ovins viables. Les résultats que nous avons obtenus n'ont pas permis d'objectiver l'effet de chaque protocole sur le nombre d'embryons viables. Des travaux complémentaires, incluant un plus grand nombre de brebis et la possibilité de réaliser des inséminations intra-utérines, se sont rapidement avérés nécessaires pour tirer des conclusions solides. Toutefois, nous avons constaté que les deux traitements de superovulation peuvent être utilisés pour obtenir in vivo des embryons ovins exploitables, avec un taux de superovulation supérieur à 70 %.

Parallèlement, nous nous sommes également intéressés à l'utilisation d'un agent nucléant (Snomax®) pour l'induction du seeding dans les protocoles de congélation lente de l'embryon chez la souris. Ce questionnement est venu nourrir celui d'un doctorant en physique travaillant au laboratoire sur l'étude des propriétés thermodynamiques des milieux de congélation lente des embryons et a fait l'objet d'une publication.

La seconde étape de notre travail a consisté à évaluer les effets du remplacement de la BSA par des produits synthétiques (contenant du CRYO3) dans des milieux de congélation lente et de vitrification d'embryons cunicoles et ovins. Cette approche biologique comportait différentes étapes :

- la congélation lente d'embryons ovins, suivie de l'évaluation du développement embryonnaire in vitro ;
- la vitrification d'embryons ovins, suivie de l'évaluation du développement *embryonnaire in vitro* et *in vivo* ;
- la congélation lente d'embryons de lapin, suivie de l'évaluation du développement embryonnaire *in vitro* et de l'activité mitochondriale (marquage JC-1) ;
- la vitrification d'embryons de lapin, suivie de l'évaluation du développement embryonnaire *in vitro*, de l'activité mitochondriale (marquage JC-1) et du développement embryonnaire *in vivo*.

Après congélation lente des embryons de lapin avec 1.5 M DMSO, dans une solution de base contenant 0.4 % (*w/v*) BSA (SF1), du D-PBS avec 20 % de CRYO3 (SF2), ou 100 % de CRYO3 (SF3), nos résultats ont montré que, bien qu'il n'y ait pas eu de différence significative, les embryons appartenant au groupe SF3 ont présenté, en général, de meilleurs taux de développement *in vitro*.

Après vitrification des embryons de lapin dans une solution de base contenant 0.4 % (*w/v*) BSA (VS1), du D-PBS avec 20 % de CRYO3 (VS2), ou 100 % de CRYO3 (VS3), supplémentée par 20 % (*v/v*) de DMSO et 20 % (*v/v*) d'EG, une différence significative n'a été observée que pour les taux de survie *in vitro* (supérieurs dans le groupe VS3). Cependant, les taux de gestation du groupe VS3 ont montré une tendance à la supériorité.

Après congélation lente des embryons ovins dans une solution base contenant des produits d'origine animale (0.4 % (*w/v*) BSA – SF1) ou du D-PBS avec 20 % CRYO3 (SF2), supplémentée de 10 % (*v/v*) d'EG, nous n'avons pas observé de différence significative sur le développement embryonnaire post-décongélation.

Les taux de développement *in vitro* et *in vivo* des embryons ovins obtenus après vitrification en présence de 40 % (*v/v*) d'EG se sont avérés supérieurs pour une solution composée de 100 % de CRYO3 (VS2) par rapport à une solution de base contenant 0.4 % (*w/v*) BSA (VS1). Toutefois, les différences observées n'étaient pas significatives, probablement en raison du nombre réduit d'embryons utilisés. D'autres études devront être menées sur un plus grand nombre d'embryons, afin de mieux évaluer l'impact du milieu de cryoconservation sur les embryons ovins.

Les résultats obtenus avec les embryons cunicoles et ovins semblent démontrer que le CRYO3, dont la composition synthétique est entièrement chimiquement définie, peut remplacer la BSA dans les procédures de congélation lente et de vitrification.

L'utilisation de produits synthétiques chimiquement définis permet ainsi d'éviter les inconvénients liés aux produits d'origine animale auxquels ils se substituent, et d'obtenir des conditions expérimentales mieux définies, plus cohérentes et parfaitement reproductibles, en même temps qu'un risque de transmission d'agents pathogènes diminué. Parallèlement, elle permet de s'affranchir des préoccupations qui relèvent du bien-être animal et qui résultent des conditions de production de certains de ces produits d'origine animale.

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Mots clés : Cryobanque, Ressources génétiques animales, Embryons, Produits d'origine animale, Milieu synthetique, Calorimetrie differentielle à ballayage, Congélation lente, Vitrification

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List of abbreviations used in the present study

ATP:	adenosine triphosphate
BMC:	Blanche du Massif Central
BSA:	bovine serum albumin
CL:	*corporea lutea*
CO_2:	carbon dioxid
CPA:	cryoprotectant agent
CPAs:	cryoprotectant agents
Da:	dalton
DAPI:	4',6-diamidino-2-phenylindol
DMSO:	dimethyl sulfoxide
DNA:	deoxyribonucleic acid
DSC:	differential scanning calorimetry
D-PBS:	dulbecco's phosphate buffered saline
EG:	ethylene glycol
ET:	embryo transfer
FCS:	fetal calf serum
FDA:	fluorescein diacetate
Fig.:	figure
FSH:	follicle-stimulating hormone
HA:	hyaluronic acid
HAS:	human serum albumin
ICM:	inner cell mass
IIF:	intracellular ice formation
INA:	ice nucleating agents
IVC:	*in vitro* culture
IVF:	*in vitro* fertilization
IVP:	*in vitro* produced
LH:	luteinizing hormone
j/g:	joules per gram
LN_2:	liquid nitrogen
M:	molar
MMP:	mitochondrial membrane potential
min:	minute
mg:	milligrame
mL:	milliliter
n:	sample size
NCS:	newborn calf serum
O_2:	oxygen
pFSH:	porcine FSH
pLH:	porcine LH
PI:	propidium iodide
PVA:	polyvinyl alcohol
PVP:	polyvinylpyrrolidone
RBC:	red blood cells
s:	second
s.e.m.:	standard error of the mean
TE:	trophectoderm
T_m:	melting temperature
T_c:	crystallization temperature
T_d:	devitrification temperature
T_g:	glass transition temperature
v/v:	volume / volume
VS:	vitrification solution
w/v:	weight / volume
w/w:	weight / weight
WM:	washing medium
WS:	warming solution
ZP:	*zona pellucida*
°C:	celsius degree
°C/min:	celsius degree per minute
µL:	microliter
ΔH:	enthalpy of crystallization

Definitions of rates used in the present study

Blastocyst rate - the percentage of embryo that attained the blastocyst stage, determined by the presence of well-developed blastocoel cavity and a distinguishable embryoblast (per cultured embryo).

Expansion rate - the percentage of embryo that attained the expanded blastocyst stage, determined by the presence of a well-developed blastocoel cavity and a thin ZP, with an increase of embryo volume (per cultured embryo).

Hatching rate - the percentage of blastocysts with a trophectoderm herniating through ZP (per cultured embryo).

Survival rate - the percentage of embryo that were not morphologically damaged immediately after cryopreservation (per cryopreserved embryo).

Pregnancy rate – the percentage of females positive to pregnancy diagnosis per recipient

Implantation rate – the percentage of born pups (alive and dead) per transferred embryo on pregnant females

Live birth rate – the percentage of live-born pups per transferred embryo on pregnant females

General introduction

In 1992, Rio de Janeiro hosted a major United Nations conference - the **United Nations Conference on Environment and Development** (UNCED), also known as the Earth Summit. This conference assembled 193 countries and most of the world's nations committed to cooperate together internationally to protect the environment and nonrenewable resources. During this conference, the "**Convention on biological diversity**" was created and one of its main objectives was "the conservation of biological diversity, the sustainable use of its components, and the fair and equitable sharing of the benefits arising out of the utilisation of genetic resources" [1].

In this context, France created the **French National Cryobank** - a scientific research group (Groupement d'Intérêt Scientifique) in 1999, to cryopreserve genetic resources from French livestock breeds [2]. It was co-funded by a total of 12 partners, with the French Agricultural Ministry being the principal signatory. Some of the other partners were the INRA (French national institute for agronomics research), the IFREMER (French Research Institute for Exploration of the Sea), technical institutes for the various species of interes, and the French breeds association (Races de France).

The French National CryoBank aims to guarantee a very long-term conservation of genetic biodiversity of all French livestock species (cattle, sheep, goats, donkeys, rabbits, poultry, fish species, etc) that belong to one of these types:

- Type-I: genetic material from animals that belong to niche breeds that are in danger of extinction.
- Type-II: material from animals that belong to breeds that are under selection programmes and are insufficiently disseminated as breed-standard stock although they have outstanding traits (eg. particular genotype sets, remarkable pedigrees, etc).
- Type-III: genetic material from animals that are representative of their breed for a defined period of time, in order to have a "snapshot" of the breed variability.

For security reasons, the collections (semen, embryos, cell types) are duplicated and stored in two distinct locations.

In 2010, the **Nagoya Protocol** was adopted, as a supplementary agreement for the "Convention on biological diversity", in order to provide clear guidelines for the effective implementation of the fair and equitable sharing of benefits obtained from the use of genetic

resources. At that moment, a special attention starts being provided to the international exchanges of biological material, and to the sanitary quality of this material.

In 2012, the project **CRB-Anim** (Center for Biological Resources of Domestic Animals) was created, as a program of the "National Biology and Health Infrastructures" financed by the program “Investissements d'Avenir” until 2020. This project aims to connect and support the existent domestic animals biobanks in France, in order to build a single, coherent, nationwide infrastructure. CRB-Anim’s goal is the improvement of the comprehension of relations between genotype and phenotype, avoiding biodiversity loss of domestic animals. To do so, CRB-Anim works in two main strategies: enhancement of the collections by combining genomic resources with reproductive resources, and **improvement and standardization of cryopreservation procedures**, by assessing post-thawing viability.

In order to cryopreserve biological resources from specimens from a particular species or breed, three approaches can be used:

- male resources preservation, with sperm collection and cryopreservation;
- female resources preservation, which includes oocyte, embryo or ovarian cortex;
- somatic tissues preservation, such as skin or cartilage fragments, that can produce induced pluripotent stem cells.

Those techniques are widely used to cryopreserve biological resources. However, cryopreservation protocols usually include cryopreservation media containing **animal derived products** (such as animal serum). These products present two major drawbacks: **a variable composition, which may originate variable results**, and **a risk of disease transmission**.

These difficulties constitute serious ob**stacles to international exchanges of cryopreserved biological material**. In order to improve and secure international movement of biological material, a standardization of these techniques is required, in parallel with the use of products that do not generate variability, and do not present a risk of pathogens transmission.

In this sense, the **substitution of animal products of embryo cryopreservation media by synthetical products** may improve procedure standardization (by avoiding variability in media composition) and avoid sanitary concerns inherent to animal-derived products.

This PhD thesis project comes from this need to **standardize cryopreservation protocols, replacing animal derived products by synthetic substituents, in embryo cryopreservation media**. This works gives continuity to a former PhD student work (Pierre Bruyère), who studied the replacement of animal products in rabbit embryo slow freezing media [4]. My thesis is conducted in parallel with a physicist PhD thesis (Hugo Desnos), whose main objective is the study of thermodynamic properties of embryo slow-freezing media.

During this project, we aimed to **evaluate the effect of replacing BSA** in rabbit and ovine **embryo** slow freezing and vitrification media with a **synthetic animal products-free medium** composed of synthetic hyaluronic acid: STEM ALPHA.Cryo3 (« CRYO3 »).

To this end, two main approaches were developed:

- **a thermodynamical approach**, including the evaluation of media used to vitrify rabbit embryos (containing animal products or synthetical products);
- **a biological approach**, including slow freezing and vitrification of rabbit and ewe embryos, using an animal products-based medium or a synthetic media, evaluated *in vitro* and *in vivo* in a synthetic cryopreservation medium.

Firstly, a "**Review of literature**" chapter will contextualize the challenges of embryo cryopreservation, of cryopreserved embryo evaluation, as well as the possible consequences of using animal derived products in embryo cryopreservation media.

Secondly, the **experimental part** will be composed of two subparts:

- The first one comprises preliminary studies, which allowed us to develop methods to be used in the main studies: evaluation of the thermodynamic properties of the substituents, development of a method of embryo activity assessment, and development of a method of ewe superovulation. A preliminary study (evaluation of the use of Snomax® as "Manual seeding" substitute in mouse embryo slow freezing) was conducted in parallel with Hugo Desnos thesis, and was not linked with the main studies. This study allowed Hugo Desnos to pursuit his thesis experiments.
- The second one comprises the **main studies**: rabbit and ewe embryo slow freezing and vitrification, and *in vitro* and *in vivo* evaluation of cryopreserved embryos.

In the **discussion** chapter, we will reiterate the principal findings of the preliminary studies, and discuss in detail the results obtained during our main experiments, which will be followed by **conclusion and perspectives**.

Chapter I - Review of literature

1. Embryo cryopreservation

The aim of embryo cryopreservation is to ensure high survival rates and high viability rates after storage at subfreezing temperatures. At temperatures near liquid nitrogen (LN_2; -195.8 °C), only solid phases exist and no biological reaction can occur. For these reasons, embryos can remain stored for long periods [5]. However, to reach these temperatures, embryos must traverse an intermediate zone of temperature (between -15 and -60 °C) through freezing and through warming, during which critical events can occur, compromising embryo survival [6].

Depending on the cell type, water may represent 60 to 85 % of the cell volume [7]. As water transition to the solid phase represents a volume increase of up to 9 % [8], cells can be irreversibly damaged during this process. Considering these facts, ice crystal formation is considered as a major critical step in embryo cryopreservation [9].

There are two main strategies to cryopreserve embryos: slow freezing and vitrification. During slow freezing, extracellular ice crystal formation occurs and embryos survive in a glassy state between ice crystals [10], while in vitrification the entire solution turns into a glass-like state without ice crystal formation due to the high viscosity of the medium [11].

1.1 Embryo slow freezing

Whittingham [12] and Wilmut [13] first reported the successful frezzing of mammalian embryo, in 1972. These authors slow froze mice embryo in the presence of dimethyl sulfoxide (DMSO), used as a cryoprotectant agent (CPA) [12,13]. Based on these methods, successful slow freezing of other mammalian species embryos were reported shortly after [14–16].

In embryo slow freezing, embryos are pre-equilibrated in a freezing solution containing low concentrations of cryoprotectant agents (CPAs), and then cooled to subfreezing temperatures with a controlled cooling rate, through an equilibrium process [17].

CPAs added to slow freezing media are frequently glycerol or DMSO (at a concentration of 1.35 M - 1.5 M) [18], or other penetrating agents such as ethylene glycol (EG) and propylene glycol [19,20] and non-permeating agents such as sucrose, glucose or fructose [21–23].

Usually, a 2 – 5 °C/min cooling rate is applied from room temperature to a temperature slightly below melting point, when nucleation is induced. After nucleation, a slower cooling rate of 0.3 to 0.5 °C/min is applied to temperatures below -30 °C, before plunging the embryo straws in LN_2 [23–27].

The main objectives of this procedure are to avoid water crystals formation and to limit this event to the extracellular compartment. To this end, knowing the principles of water crystallization, the possible causes of embryo damage and how to avoid them is of great importance.

1.1.1 Water crystallization

During slow freezing, the temperature of the system is lowered gradually in a controlled manner. Water molecules can cool below their melting temperature (0 °C), but remain in an unstable liquid state through a process called **supercooling**, until the **nucleation** (formation of an ice nucleus) occurs and the crystallization phenomenon starts. Without external perturbation, this transition may occur more than 10 °C below the maximal melting temperature [28]. Supercooling is the difference between melting temperature and nucleation temperature [29].

Numerous factors can influence the supercooling magnitude of a system, such as system temperature, cooling rate, volume, type of container, particles present in the liquid,

etc. Moreover, distinct supercooling degrees can be obtained, even when all these variables are meticulously repeated [30].

Nucleation processes are classified as **homogeneous** or **heterogeneous**. Homogeneous nucleation, also called spontaneous nucleation, occurs in the absence of foreign particles. A good example is pure water: although melting temperature of water is 0 °C (at atmospheric pressure), if no impurities or surfaces are involved, water can crystallize at -40 °C [31,32], being in a supercooling state between 0 °C and -40 °C.

On the other hand, heterogeneous nucleation is influenced by structural inhomogeneities, such as surfaces or impurities, which act as nucleation sites. This type of nucleation occurs at higher temperatures than homogeneous nucleation. In biological systems cryopreservation, nucleation is referred as heterogeneous, as the water contains foreign particles [33].

The temperature at which each solution crystallizes depends on the size of nucleation sites of the foreign particles present in the media [32]. In general, a solution containing particles with smaller nucleation sites crystalizes at a lower temperature than a solution with particles containing bigger nucleation sites [32]. In a supercooled system, the probability of nucleation increases as the system's temperature decreases, and the nucleation rate increases as supercooling increases [34]. CPAs are added to the cryopreservation media, to diminish the temperature at which ice crystallization first occurs, decreasing water melting point [35]. Once the crystallization process has started, the first ice nuclei provide a structural template upon which the other water molecules (present in the liquid state) will diffuse and attach to, contributing to the growing of the ice clusters [36].

Nucleation and crystal growth can both occur in simultaneous, and the interaction between these two parameters influence crystal size and distribution [36,37]. However, crystal growth and ice structure also depend on a set of other complex parameters such as cooling rate, nucleation temperature and supercooling degree [9,32,38]. **Cooling rate** is a crucial parameter that influences ice crystal size and distribution. A slower cooling rate will give rise to a small amount of big crystals [8]. On the opposite, a higher cooling rate will give rise to an important amount of small crystals, with an irregular surface [39]. As the solution temperature decreases, its viscosity increases, and water molecules diffuse slower towards the ice cluster, slowing crystallization [8]. **Nucleation temperature** and **supercooling magnitude** affect significantly crystal growth [29]. If nucleation takes place at higher subzero temperatures, closer to the melting point, ice crystals are bigger (~ 10-30μm) and are better organized in a dendritic structure [8,40], with smoother surfaces [39]. As the temperature of the media

decreases, the supercooling degree increases and the nucleation rate increases [34], ice crystals are smaller (~ 10µm) [38,40], and are distributed in a chaotic distribution [38]. For this reason, control of the nucleation temperature is crucial to prevent this damaging process.

1.1.2 Mechanisms of freezing injury

During cryopreservation, embryos can be damaged by a multiplicity of factors, some of which, are not yet completely elucidated [30,41]. Embryo response to these injuries may also depend on intrinsic factors, such as embryo quality [42], developmental stage [43,44], production method (*in vitro* / *in vivo)* [45], or particularities due to the species [46]. Some of the principal causes of embryo damage that can be associated to the cryopreservation process are:

- Intracellular ice formation

Embryo cryopreservation protocols aim to avoid intracellular ice formation (IIF) [47], since it is one of the principal causes of cellular death during slow freezing [48]. IIF is influenced by many factors, such as the presence of extracellular ice crystals, the cooling rate, the supercooling magnitude, the nucleation temperature, the dehydration degree and the temperature of the system .

A variable of major importance is extracellular ice crystals, since ZP is capable of exerting a resistance to the passage of extracellular ice crystals, but cannot completely block it [48]. Another physical parameter determining the degree of IIF is the supercooling. Several authors showed that the probability of IIF increases with the degree of supercooling [40,48,49].

To avoid IIF three major factors need to be controlled: the CPAs composition, the cooling rate and the nucleation temperature [50].

Embryo dehydration is dependent on cooling rate and on penetrating CPAs concentration. As mentioned earlier, there is a negative correlation between embryo dehydration and IIF, since if less intracellular water is present, less water will be able to crystallize inside the embryo.

Nucleation temperature affects IIF, as the gap between melting temperature and nucleation affect the supercooling magnitude.

Under -30 °C, intracellular structures can trigger heterogeneous nucleation and be responsible for IIF [7].

- Mechanical stress

The mechanical damage caused by the formation of ice crystals or ice front displacement can play a critical role on cellular viability after slow freezing. Ice crystals growth in the extracellular compartment and embryos are confined between ice dendrites, in non-crystallized liquid areas [41,51]. As seen on the "intracellular ice formation" section (in the previous page), ice crystal shape, size and disposition depend on several factors such as cooling rate, nucleation temperature and supercooling degree. Consequently, depending on these factors, embryos will face different types of mechanical stress.

Direct mechanical interaction between ice and imprisoned cells [52,53], reduced space between ice dendrites [54,55], or even cells engulfment by the growing crystals [56], can be responsible for cell damage and cell death.

Severe or quick embryo volume expansions and contractions, due to rapid ice formation or to CPAs movement, is a non-negligible factor of mechanical stress, and is a potential cause of post-thaw viability losses [57].

- Dehydration and CPAs toxicity

Before ice formation, the presence of CPAs in the freezing solution leads to an increase of the extracellular osmolarity, allowing the water to leave the embryo. If penetrating CPAs are present, they will enter the cells and avoid an excessive dehydration. After the nucleation process, the extracellular ice formation starts. As the water molecules crystalize, the quantity of free water declines, increasing the extracellular osmolarity, which will contribute to the cell dehydration [49,58]. If cooling rate is faster, the water does not have time to leave the embryo, and IIF probability is higher. If the cooling rate is slower, more water will leave the intracellular space. As less water is present inside of the cells, the probability of IIF is decreased [49,58]. Therefore, the presence of penetrating CPAs protect embryo from excessive dehydration and of IIF. However, the exposure to high concentrations of CPAs and solutes is toxic to the embryo [59], and an equilibrium between dehydration, toxicity and IIF possibilities must be attained [58].

- Osmotic injury

CPAs may also be a cause of cryoinjury during cooling and during thawing, if their addition and removal protocol is not optimal [60]. During these steps, CPAs can potentially induce osmotic injury to the cells, especially if important concentrations are present. Even if

permeating CPAs penetrate cells, it takes longer than water diffusion. Consequently, an important initial dehydration will occur, due to osmotic efflux of water, followed by rehydration due to inflow of the CPA and water inside the embryo [61]. The opposite will happen during the removal of a CPA at thawing: initially a swelling will occur, since extracellular medium will be less concentrated, and then the embryo will slowly return to initial isotonic volume as the CPA and water leave the cell. These repeated volumetric modifications can affect embryo functional integrity or induce death [61].

- Cell membrane injuries

Mammalian cells plasmatic membrane is a major site of damage during cryopreservation. Direct mechanic injuries due to ice growth can lead to membrane rupture [46]. However, less obvious membrane alterations may affect embryo viability after cryopreservation. During slow freezing, cellular membrane undergoes a phase transition from the liquid state to a solid state, with separation of the membrane lipid-protein complex components [62]. This phenomenon can result in *i)* membrane phospholipids losses, rendering the cell permeable to electrolytes and swelling [62], *ii)* in loss of hydrostatic pressure regulation capacity [63,64] or *iii)* in a loss of membrane fluidity [65]. Depending on the cooling rate, different membrane injuries can be observed [66].

These injuries can be classified in two categories: cold shock and chilling injury. **Cold shock** is defined as the damage caused by rapid cooling, and occurs at high cooling rates [67]. Some authors associated these damages with mechanical properties of the membranes. During cooling, a contraction of the membrane surface occurs, with a subsequent hydrostatic pressure increase, creating an osmotic force that leads water to leave the cell. If the cooling rate is high, the water can't leave the cell rapidly enough to prevent the tension accumulation in the membrane [68,69]. These alterations are immediate and are accompanied by membrane leakage (and lysis in severe cases) [66].

Chilling injuries are defined as the damages caused by the permanence of the cells at subzero temperatures for extended periods. Unlike cold shock, they occur at slow cooling rates [67]. Chilling damages are usually accompanied by a decrease of the selective permeability [66], ion homeostasis changes and transmembrane imbalances [70–73]. Reactive oxygen species production may follow and aggravate membrane damage, with metabolic alterations and disruption of the cytoskeletal matrix [70,74–77].

CPAs such as DMSO, are stated to increase membrane fluidity and reduce the temperature of membrane phase transitions [66].

- Storage

During storage in LN_2, the only reactions that may occur are photophysical events as a result of background ionizing radiation, that arise primarily through natural causes such as cosmic rays and terrestrial radiation sources – background ionization, which depend on the location on the planet [78]. When an atom or molecule absorbs enough ionization, it can eject electrons. This event can damage the embryo in a direct manner if its DNA absorbs the ionization; or indirectly, with the subsequent liberation of free radicals [79]. Basing on estimated background ionizing radiation, Mazur (1976) estimated the shelf-life of mammalian cells stored at −196 °C to be 200 to 3000 years [80]. Whittingham wanted to mimic extend periods of storage, and increased radiation exposure of mouse embryos by placing LN_2 containers next to a small radium source. After a background radiation equivalent to 2000 years, the authors didn't find mutagenic effect in offspring [81]. However, some investigators indicate a diminution of survival after long-term storage [71]. A plausible reason may be incorrect storage conditions, such as temperature fluctuations during transfer to LN2 tanks, sample storage, sorting and removal [82]. German studied the impact of small repeated cyclical temperature fluctuations on peripheral blood stem cells during storage, and observed a diminution of cell recovery, cell viability and functionality [82]. When referring to smaller volumes as vitrification devices, we can suppose that these fluctuations can have a greater impact on cellular survival and viability.

- Thawing / Warming

Although there are several potential mechanisms of cellular damage that may occur during thawing from a frozen state, cryoinjury may result in part from recrystallization. This event happens when smaller harmless extra- or intracellular ice crystals, formed during cooling, melt and merge into larger, more injurious crystals during thawing (classically during slow warming). Even if there is no variation in total amount of ice, damages are a consequence of modifications on crystals size and shape [59].

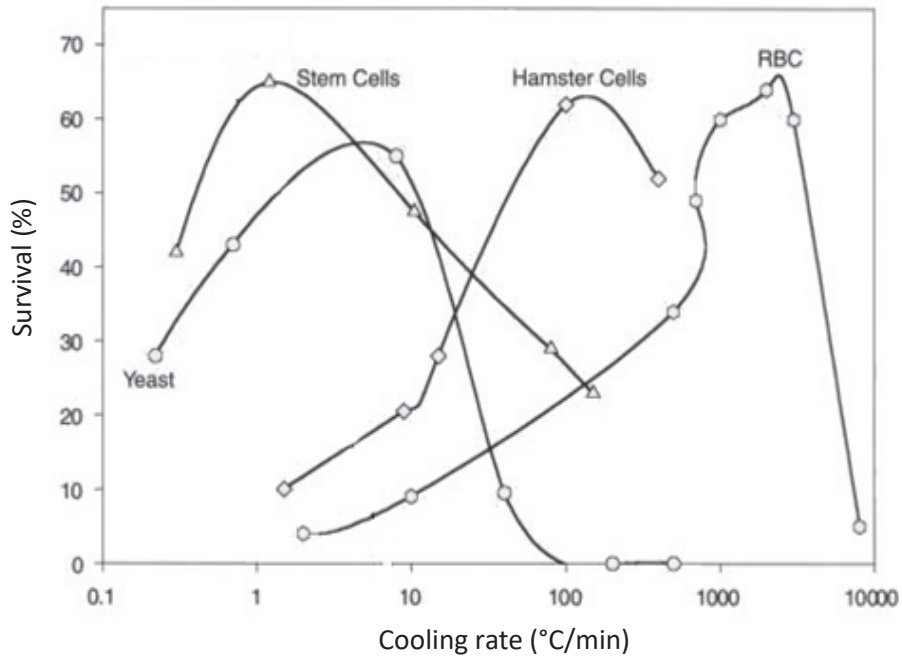
During thawing, nucleation rate increases faster than crystallization rate. For this reason, the probability of crystal formation is higher during thawing than during cooling. This observation is particularly valid when referring to highly concentrated media or at the end of slow freezing [83,84]. To avoid this event, thawing /warming rate should be superior to cooling rate.

1.1.3 Cooling rate

The optimal cooling rate is specific to each type of cells (Fig. 1), since it depends on biophysical factors such as membrane permeability to water and to CPAs, surface to volume ratio, energy activation, the temperature at which IIF occurs and osmotic limits of the cell [6,35,85,86]. Mazur was the first to demonstrate that these measurable parameters should be taken into account to estimate optimum cooling rate [6]. Among the numerated factors, water permeability and surface to volume ratio are of major importance. Cells with a low water permeability coefficient need slower cooling rates, since water will pass through their membranes more slowly. Embryos and lymphocytes have both a low water permeability coefficient (which explains the need of a slower cooling rate than red blood cells). However, embryos have a lower surface to volume ratio and, consequently, water has less relative surface to pass through [87]. This explains why embryos have better post-thaw viability rates after applying lower cooling rates [87]. This approach allowed the first successful cryopreservation of mouse embryos in 1972, where high post-thaw development rates were obtained after using cooling rates between 0.3 to 2 °C/min [12], a range that remained unaltered until now [23–25,87,88]. Based on this theory, the two-factor hypothesis of freezing injury was established: if cells are cooled too fast, IIF occurs; if cells are cooled too slowly, dehydration and long exposure to concentrated solutes may have a deleterious effect [89].

To control cooling rate during embryo slow-freezing, embryo straws are placed in the chamber of a programmable freezer that controls the system temperature by injecting liquid or cold nitrogen vapors [70].

Fig. 1. Evolution of the survival rate of different cell types as a function of the cooling rate. Adapted from [5].



1.1.4 Control of nucleation temperature

To minimize the supercooling magnitude and embryo injuries associated with this phenomenon, manual induction of the nucleation (**manual seeding**) needs to be performed during embryo slow freezing [90]. It is routinely performed by cooling a spot on the outside of the straw, with a cold instrument, which locally decreases the temperature of the sample and induces nucleation [28,29].

Manual seeding limitations

Manual seeding presents several drawbacks. When performed inside of a programmable freezer, manual seeding requires the manipulator's presence and rapid access to the samples through the opening of the freezer. It is also time-consuming, and it is difficult to seed more than one sample at a time. Moreover, opening the programmable freezer may result in a non-controlled temperature rise [29], compromising the standardization of this step. Some thermodynamic studies may require embryo slow freezing inside of other system configurations, such as differential scanning calorimetry (DSC) or cryomicroscopy. In these situations, access to the samples during the protocol is not possible, since cells are placed in a pan or between a microscope slide and a cover, so manual seeding cannot be applied.

Alternatives to manual seeding

Controlled ice nucleation can be induced by other methods such as:

a) Automatic seeding. Some programmable freezers include an option to induce automatic seeding. However, this is not routinely used in embryo freezing, since it was shown to have a deleterious effect on the embryo survival rate and development rate [91].
b) Electro-freezing, by applying a high voltage to a metal electrode [29], since the amount of surface charge seems to influence the nucleation temperature [31];
c) Mechanical induction by shaking, tapping or ultrasounds application [28]. A brief negative pressure of water is created by these events, which causes an increase of crystallization temperature [92]. Nonetheless, it is also hard to standardize this technique, and to integrate it into the programmable freezers [28];
d) Pressure shift, applied to freeze dried samples [28]. Briefly, the sample is cooled under compression to negative temperatures (high pressures low melting point, avoiding ice nucleation). Once the aimed temperature is attained, the pressure is released and crystallization occurs [93,94].
e) Adding to the media Ice Nucleating Agents (INA).

INA agents

To induce nucleation at higher temperatures, ice nucleating agents (INA) can be added to the freezing media [28,48]. They facilitate the orientation of water molecules in an ice-like structure to create active germ crystals. This effect promotes the freezing of supercooled water at temperatures higher than expected [95]. Among the numerous substances that can serve as ice nuclei, it can be found: *i)* inorganic crystalline solids, such as silver iodide [96] - successfully used to induce seeding in bovine and rabbit embryos [97,98]; *ii)* substances present in atmospheric aerosols, such as mineral dusts or metallic particles [99]; and *iii)* biological ice nucleating agents, which are produced by multiple organisms. The most frequently studied ice nucleating agents of biological origin are substances present in some bacteria membranes (i.e., *Pseudomonas syringae, P. viridiflava, P. fluorescens, Pantoea agglomerans* and *Xanthomonas campestris*). These bacteria are usually found in association with plants and are capable of inducing frost to their hosts at temperatures slightly below 0 °C [96,100].

Among the existent ice-active bacteria, *P. syringae* presents probably the greatest abundance and wider distribution [101]. The key of the ice nucleating activity of *P. syringae*

is a protein located at the outer cell membrane of *P. syringae*, INaZ, which can mimic the structure of an ice nucleus [102].

Snomax® (Snomax International Snowmakers AG, Steffisburg, Switzerland) is a commercial preparation of freeze-dried inactivated proteins extracted from *P. syringae*, sterilized by gamma irradiation. It is used as an additive to produce artificial snow. This commercial preparation has been used in DSC physical experiments [103,104], cryomicroscopy [48,105] and food industry experiments [106]. In 2015, our research team demonstrated that Snomax® can be a valuable tool to control, in a replicable manner, the crystallization temperature of a solution [104].

1.2 Embryo vitrification

Vitrification (from latin *vitreum*, "glass") is a cryopreservation technique where liquids solidify in an amorphous vitreous state, by extreme increase in the medium viscosity, in both extracellular and intracellular compartments [11]. This technique intends to overcome the chemical and mechanical dangers of ice formation during all stages of cryopreservation (cooling, storage, and warming), since its major particularity is the absence of crystallization [107].

Embryo vitrification was first accomplished by Rall and Fay, in 1985 [108], almost a half a decade after the first spermatozoa vitrification [109]. These authors vitrified mouse embryos in 0.25 mL straws, using a vitrification media containing DMSO, acetamide, and polyethylene glycol [108]. Numerous studies have reported the superiority of vitrification over slow freezing for embryo cryopreservation of different species [45,88,110–114]. Additionally, vitrification presents advantages such as not to require expensive equipment and to be less time-consuming [112].

Vitrification can be achieved either by using important cooling rates, or increasing CPAs concentration. Usually, both approaches are applied [115].

Vitrification media are usually composed of high concentrations of penetrating and non-penetrating CPAs, that increase medium viscosity [116,117]. In 1987, MacFarlane demonstrated that media containing more than 40 % (*v/v*) of CPAs are more likely to vitrify [117]. More recent studies corroborated this theory, showing that the probability of ice formation is inversely proportional to the CPAs concentration [118].

As the permeability of cells to water is generally higher than the permeability to penetrating CPAs, embryos exposed to these media start by experiencing an important osmotic shrinkage due to dehydration. Subsequently, penetrating solutes enter the embryos, that undergo a slower swelling to an equilibrium water volume [119]. The rate at which a permeable CPA enters and leaves the cells varies between CPAs and is temperature and concentration dependent. The higher the CPA concentration, the faster and more extensive the osmotic shock. This may be overcome either by gradual or stepwise addition and dilution of CPAs before freezing and after warming and by adding impermeable solutes such as sugars to dilution media [17,120,121]. As CPAs tend to enter the embryos more fast at higher temperatures, penetration rate depends on the temperature of the media [122].

High CPAs concentrations represent a greater risk of osmotic and toxic stress. To limit these injuries, the use of brief equilibration steps, mixtures of two or more CPAs, a shorter exposition to vitrification solutions, associated with a rapid cooling are generally used.

Additionally, washings are done after thawing, in order to completely eliminate CPAs [90,123–125].

Although its exact function is not fully understood, penetrating CPAs concentration may be reduced by adding high molecular weight polymers such as Ficoll and Dextran to the media [116,126].

1.2.1 Cooling rate

In contrast to slow freezing, where a slower cooling rate is preferred, during vitrification, faster cooler rates are desired. To achieve them, the samples are commonly plunged directly in LN_2, or put in contact with a cold metal surface [25,116]. Relying on the principle that the cooling rate increases as the sample volume decreases [108,127], the use of minimal volumes allowed successful vitrification with media containing less CPAs [128]. Small volumes allow a better temperature conductivity and, consequently, higher cooling and warming rates [128].

Over the past decades, numerous devices with very small volumes were created to allow cooling rates superior to 10 000 °C/min. Some devices allow direct contact with LN_2 - open systems, such as Cryotop [129], open pulled straws [130,131], microscope grid [132], or Cryoloop [133], to increase cooling rates to over 20 000 °C/min [134–136]. However, in open systems, the risk of contamination through LN_2 may be greater [137]. Closed systems involve a physical separation between biological material and nitrogen. In these systems, devices are heat sealed before contacting LN_2, to prevent contamination risks. Cryotip [138], Rapid–i® [136], CBS® High Security Straw [135] and Cryopipette [139] are examples of closed vitrification systems. However, due to thermos-isolation, cooling rates are reduced, in comparison to open systems [140]. A study compared cooling rate of a cut standard straw immerged directly in liquid nitrogen (open system) or encased in a standard straw (closed system), and obtained, with the first, a cooling rate 25 times superior (15 000 °C/min and 600 °C/min, respectively) [141]. A recent meta-analysis investigated seven studies that reported outcome after human blastocyst vitrification with open and closed systems, and, although a significant difference was not found, a tendency of lower live birth rates after closed vitrification was identified [140].

An intermediate system was developed, consisting in solid surface vitrification, where the device touches a metal block that is half-submerged into liquid nitrogen (Fig. 2), without direct immersion in LN_2. Cryohook [142] and Fibreplug [142–145] are examples of

commercial vitrification devices that use this method. If these devices are placed in a sealed sleeve, contact with LN_2 is avoided during storage, rendering it aseptic [144].

Fig. 2. Metal solid surface vitrification system with Fibreplug method.

A small droplet containing the embryo is transferred to the Fibreplug, that touches a metal block half-submerged into liquid nitrogen.

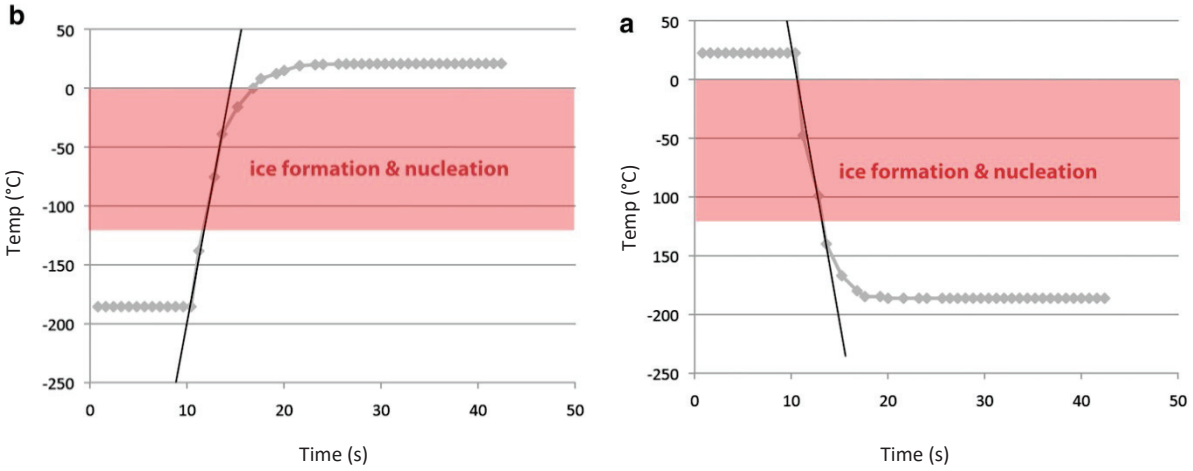
1.2.2 Warming rate

Critical warming rate (rate above which the risk of ice formation is avoided) is higher than cooling rate [83,84], and is influenced by this parameter: when cooling rate is increased from approximately 20 to 200 times, the critical warming rate diminishes by a factor of at least 15. This strong dependence is probably related to the rapid growth during warming of ice crystals formed during cooling [146]. A slower warming rate is, therefore, associated with recrystallization during warming [116,147]. Additionally, Huebinger showed that rates decelerate when getting next to the final temperature (Fig. 3). As the critical range temperature for ice formation (usually between 0 °C and –130 °C) is closer to biological temperatures than to LN_2 temperature, this rate slow-down will have a higher impact during warming [118]. As a smaller amount of bigger ice crystals are energetically more favorable than multiple small crystals, ice will recrystallize in larger ice crystals, altering embryo morphology. This recrystallization was directly associated with decreased survival rates [118].

Although most studies are based on the importance of high cooling rates, studies using mouse embryos and oocytes highlighted the importance of a rapid warming rate to obtain survival after vitrification [148–151]. However, due to the sensibility of cells to high temperatures, it is harder to attain high warming rates than high cooling rates [118].

Embryo vitrification protocols comprise commonly a warming step with a fast transfer of the vitrification device from the LN_2 directly to a washing medium [148,152,153]. To increase warming rate, warming media are often pre-warmed to 37 °C to 38.5 °C [114,154–156]. Seki and Mazur concluded that a warming rate of at least 3 000 °C/min was required to attain a survival rate of 80 % with mouse oocytes [147]. Besides, they observed that if the warming was fast enough, a reduced cooling rate (200 °C/min) should allow successful vitrification [147]. In 2014, Jin obtained approximately 100 % survival after vitrifying mouse oocyte with a medium containing 1/3 of usual CPAs concentration, slower cooling rates, and an extremely rapid warming rate (10 000 000 °C/min), obtained through a laser pulse [116]. These findings support the importance of the warming rate.

Fig. 3. Cooling and warming rates of a thermocouple plunged in LN_2 (a), and subsequently warmed in a water bath (b), simulating cell vitrification and warming protocols. Adapted from [118].



Although the initial rates are comparable, the sample takes nearly three times longer to traverse the critical temperature range in which ice crystals can be formed during warming (red area: 2.6 s (a) and 7.3 s (b)).

2. Embryo cryopreservation media

Embryo contamination and international movements

Over the past few decades, embryo cryopreservation has become crucial to the long-term preservation of genetic material in biobanks. Along with embryo transfer (ET), these techniques have contributed to the distribution of genetic information worldwide, replacing animal exchange [157]. The World Organisation for Animal Health assembled recommendations on risk management procedures concerning embryo collection and processing [158]. These recommendations refer to the donor selection, to general hygiene practices in the laboratory, to the sanitary handling of the embryos, and to the conditions of storage. They also describe a rigorous embryo washing procedure and a ZP inspection prior to cryopreservation or transfer [158,159] Even if these guidelines are the best way to reduce infectious disease transmission, embryo contamination is still of concern to health authorities [157]. Embryos may be contaminated during the different steps of production, cryopreservation or transfer. The infectious agents may come from the embryo (and resist to washing), from the media used for embryo handling, culture or cryopreservation, and from the nitrogen during storage [157].

During storage, large quantities of biological material are held together in LN_2 tanks, and infectious agents can be transmitted between samples (cross-contamination), if they are not correctly sealed or if a container is deteriorated. This concern is even of greater importance if the samples are stored in direct contact to LN_2, the case of open-vitrification systems [129,160].

Pathogens resistance to washing

ZP, especially in *in vivo* produced embryos, acts as a physical barrier to pathogens. Repeated washing of ZP seems to be an effective way to remove most infectious agents attached to the ZP or present in the flushing medium, including enzootic bovine leucosis, foot and mouth disease, blue tongue or *Brucella abortus*. However, there are still some agents, such as enveloped virus (i.e. herpesvirus) or some bacteria (i.e. *M. paratuberculosis* [157], *Haemophilus somnus* [161], Ureaplasmas [162], Mycoplasmas [163] or *Escherichia coli* [164], that can strongly attach to the ZP [157]. Furthermore, *in vitro* fertilized (IVF) embryos, ZP seems to be more immature and less compacted than in *in vivo* produced embryos. Consequently, viruses (i.e. bovine viral diarrhoea virus (BVDV), or *Leptospira hardjo* [165]) may adhere to the ZP or penetrate the embryo (Le Tallec et al. 2001). Several studies experimentally exposed oocytes or embryos to pathogenic agents, these include Bluetongue

virus [166], bovine herpesvirus-1 (BHV) [167,168], BVDV [168,169], bovine immunodeficiency virus (BIV) [168], foot and mouth disease virus [170], *Actinomyces pyogenes bovis*, *Escherichia coli* and *Streptococcus agalactiae* [164,171], that remained attached to IVF embryos after washing. In IVP (*in vitro* produced) embryos washing seems unsatisfactory to remove all pathogens.

Pathogens resistance to LN_2

Although the temperature of LN_2 is approximately −196 °C, several pathogens are capable of resisting these conditions. Enveloped viruses can resist to several freezing / thawing cycles, especially if the media contain stabilizers such as serum and sugars, or CPAs such as dimethyl sulfoxide (DMSO) [172,173]). Experimentally infected bovine embryos with BHV, BVDV and BIV resisted to cryopreservation and subsequent washings [168]. In 1995, five human patients were infected with hepatitis B virus after being subjected to cryopreserved bone marrow transplantation. An error in packaging and storage of samples resulted in a cross-contamination in the LN_2 tank [174]. Non-enveloped viruses can resist even without the presence of CPAs in the media [172]. Bielanski identified 32 bacterial and 1 fungal species from randomly drawn LN_2, frozen semen, and embryo samples stored in 16 LN_2 tanks [175].

In 2014, Pessoa analysed swabs from canisters and bottoms of reproductive resources LN_2 tanks. The authors identified 12 genera of bacteria and five genera of fungi, being *Bacillus cereus* the most prevalent bacterial contaminant (31.57 %). Even after decontamination, 12 % of the LN_2 tanks remained contaminated [176].

2.1. Serum-based media

Animal derived products such as bovine serum albumin (BSA), human serum albumin (HSA) or fetal calf serum (FCS) - also referred to as foetal bovine serum, are commonly added to animal embryo culture and cryopreservation media [25,152,154,177,178].

Serum albumin

Serum albumin is a large non-glycosylated protein (around 66 kDa), containing 583 (BSA) and 585 (HSA) amino acids [179]. It is the most predominant plasma protein in mammals. Albumin is synthesized in hepatocytes and secreted into the blood circulation, where it attains important concentrations (0.6mM in adults). This protein has a major role in maintaining plasma oncotic pressure, serum pH, and in the transport of diverse compounds, some of which are fatty acids, metals (the most important being Ca_2^+) and hormones [179–181]. Both origins albumin (BSA and HSA) share approximately 76 % sequence homology [179,182].

Serum derived products harvesting

BSA

Generally, BSA is extracted from bovine serum through cold ethanol precipitation, known as Cohn method [183]. Then, BSA is purified through different methods, including crystallization, preparative electrophoresis, ion exchange chromatography, affinity chromatography (e.g., ConA-agarose removes glycoproteins), heat treatment (removes globulins), low pH treatment, charcoal treatment (fatty acids removal), organic solvent precipitation (fatty acids removal), and low temperature treatment [184].

Human serum albumin

Several methods can be used to extract and purify HAS from human blood, being the most common: Cohn method [183], Cohn method combined with chromatography [185] and Chon method combined with liquid chromatography [186]. Other methods, including purification from placenta, ammonium sulfate precipitation combined with L-thyroxin ligand, heat shock, trichloroacetic acid / acetone precipitation, different dye ligand affinity chromatographies, and simulated moving bed chromatography (etc) are less efficient, being not suitable for commercial purposes [180].

Foetal calf serum

FCS is collected from alive bovine fetuses, taken from pregnant cows, during slaughter, by a cardiac puncture (through vacuum extraction, or through gravity and massage), without anesthesia. This production method can cause pain to the fetuses, and may raise moral and ethical questions [187]. In parallel, FCS price and availability depend on variations in worldwide livestock numbers, importation regulations, animal prices and livestock feed charges. A less expensive alternative to FCS is Newborn calf serum (NCS). It is collected from blood of calves less than 14 days old and filtered or heat inactivated in order to remove pathogens. It contains, however, higher protein and endotoxins levels [188].

Composition

Serum-derived products composition is poorly known. Media containing BSA or serum are classified as semi-defined or non-defined, respectively [189]. These products contain growth factors, cell attachment and spreading factors, hormones, carbohydrates, amino acids, proteins (such as albumin), vitamins and various undefined molecules [189,190]. Serum-derived products promote embryonic viability and development [189–194] and have numerous advantageous properties in cryopreservation media, some of which are: metal chelating activity, oncotic pressure regulation, pH regulation [195] and toxin-scavenging [196]. Citrate, also identified in BSA preparations, promotes cell proliferation and rabbit blastocyst expansion [197]. Additionally, animal/human sera have surfactant properties, which reduce the surface tension in the media, preventing embryos from floating or sticking to glass and plastic surfaces [195,198], and avoiding the adsorption of some media compounds (including hormones, growth factors and carrier proteins) to the material surfaces [199]. Moreover, the addition of serum-derived products to the cryopreservation media seems to protect embryos from possible toxic effects of CPAs during the cryopreservation process [176,200].

The optimal BSA concentration for embryo cryopreservation is 0.4 % *w/v* [201,202]. FCS is usually used at a proportion of 10 % [203,204] to 20 % *v/v* [156,205,206], in slow-freezing media, as in vitrification media.

Despite the numerous beneficial effects of serum on embryos during and after cryopreservation, negative effects have also been suspected. Ruminant embryos cultured with serum before blastocyst formation may present increased incidence of unusual development, accompanied by “large offspring syndrome”: high birth weight, prolonged gestation, frequent dystocia, elevated abortion rates and organ defects [207–211].

Serum-derived products present two major drawbacks: the variability of the composition and the sanitary risk of disease transmission.

2.2 Variability

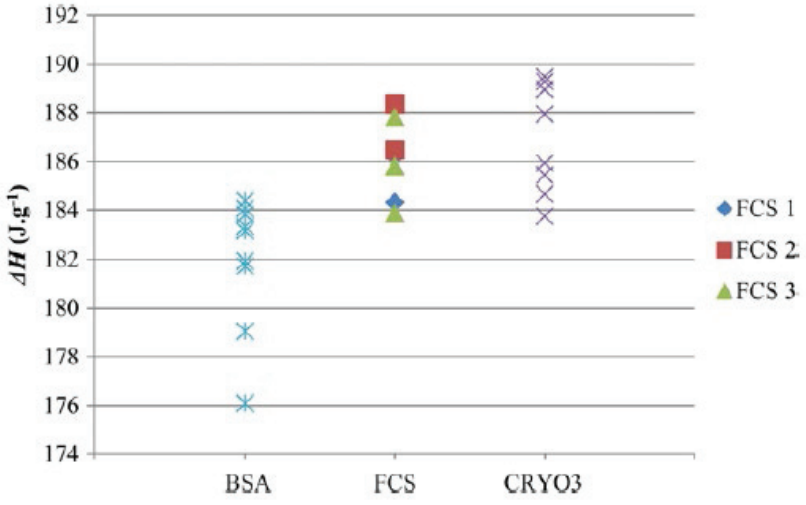
The exact composition of sera changes between batches and may be extremely variable [190]. This variation can occur as a result of physiological and biochemical differences between donors [212], and more generally with gender [213], age [214], diet [215], photoperiod [216] and preparation methods [217]. Regarding embryotrophic properties of BSA, some authors observed considerable variations between suppliers and even between distinct lots from the same supplier [192,218,219].

The impossibility of characterizing animal-derived products composition and its variability lead to unpredictable development rates and to experimental results that might not be reproducible.

Thermodynamic properties

Bruyère combined two approaches to study cryopreservation media containing BSA, FCS, or a synthetic animal products free medium composed of synthetic hyaluronic acid (HA), STEM ALPHA.Cryo3 (referred to as "CRYO3", Stem-Alpha, Saint-Genis-l'Argentière, France): a physical approach, with DSC, and a biological approach, with embryo development. On the physical approach, Bruyère investigated thermodynamic properties, including the end of melting temperature (T_m) or the enthalpy of crystallization. He obtained similar enthalpy properties between the synthetic media and FCS, whilst medium containing BSA presented significantly different enthalpy properties (Fig. 4), but similar melting temperatures [3].

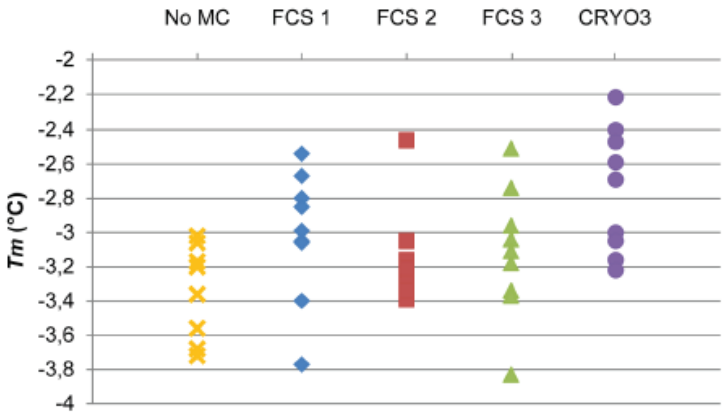
Fig 4. Diagram of enthalpy values for the cryopreservation solutions [3].



ΔH, enthalpy of crystallization; BSA, bovine serum albumin; FCS, fetal calf serum.

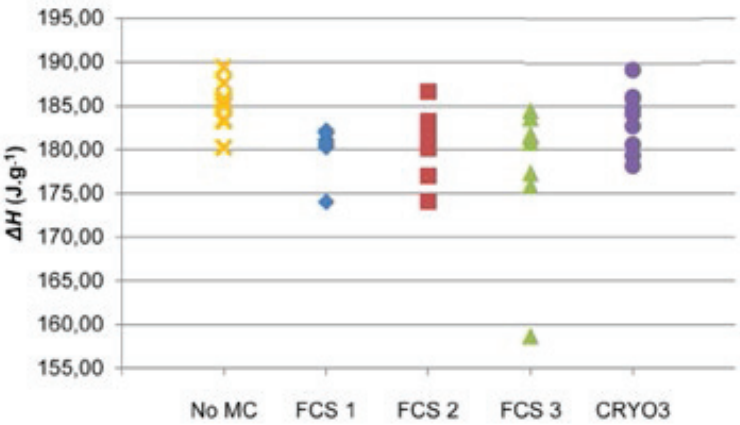
On a second study, Bruyère investigated FCS from three different suppliers, and compared them to the same synthetic medium (CRYO3). All the solutions presented similar mean thermodynamic characteristics but media containing FCS presented more variability, as well as aberrant values (Fig. 5 and Fig. 6), unlike CRYO3 medium, which appeared to be more stable [220]. He concluded that CRYO3 can thermodynamically replace solutions containing BSA or FCS [1,220].

Fig. 5. Diagram of the T_m values for the cryopreservation solutions [220].



No MC: No macromolecular component; FCS: Foetal calf serum.

Fig. 6. Diagram of the ΔH values for the cryopreservation solutions [220].



No MC: No macromolecular component; FCS: Foetal calf serum.

As animal-derived products, HSA also presents variability between donor blood composition and manufacturers processing techniques [221]. Consequently, when the goal is

to obtain defined media and standardized cryopreservation methods, all serum-derived products seem to be unsuitable.

2.3 Sanitary aspects

Although albumin is considerably better purified, tested, and controlled than serum, the use of any animal product raises sanitary concerns, limiting international embryo transportation. Sera can be contaminated with pathogenic agents such as bacteria, viruses (i.e. BVDV and bluetongue virus [222,223]), yeast, fungi, and mycoplasmas [224]. The possibility of contamination with prions (i.e. transmissible spongiform encephalopathies [225]), raises additional concerns, even if the risk of contamination seems to be low [226].

Commercial sera are usually declared to be pathogen-free. While irradiation of sera limits contamination, it is not always applied. In 1980, a study detected BVDV in 1 of 37 foetal bovine serum samples that had been subjected to heat inactivation and gamma irradiation [222].

Even if HSA is considered the most well defined commercial protein additive, its purification degree still remains unsatisfactory [221]. Although the risk of transmission of a virus through HSA products is almost insignificant, there remains the risk of contamination with agents as prions (Creutzfeldt-Jakob Disease) [227].

The most efficient alternative to avoid all the concerns related to animal products is the use of a synthetic chemically defined cryopreservation medium. The advantages of synthetic medium, with no animal derived products, are widely recognized as providing more defined, more consistent and more reproducible conditions, in addition to avoiding animal welfare and ethical concerns.

2.4 Substitutes

Animal derived products replacement (biological studies)

Numerous studies have aimed to replace animal products in cryopreservation media with media free of animal-derived products.

Silk protein sericin

Silk protein sericin is a protein with antibacterial, antitumor and anti-lipid peroxidation properties [228–230]. Primarily tested as a supplement in *in vitro* embryo culture [231], it was later studied in bovine embryo freezing media. Animal products were successfully replaced with various concentrations (0.1 %, 0.5 %, and 1.0 %) of silk protein sericin, obtaining satisfatory *in vitro* development rates (> 100 embryos per group), as *in vivo* development rates, after transfer (n > 900 embryos) [232].

Vegetal peptones

Vegetal peptones, 4 to 10 amino acids peptides extracted and purified from plants, with nutritive, antiapoptotic and growth stimulation properties [233,234], were firstly tested as sera substitute in animal cell culture [235]. Later, vegetal peptones were used to replace BSA in IVF bovine expanded blastocysts slow-freezing (30 embryos per group). No differences were observed regarding *in vitro* development after thawing [234].

Polyvinyl alcohol (PVA)

The defined nonorganic macromolecule PVA has been used to slow-freeze and vitrify embryos from different species, obtaining variable results.

After mouse embryos rapid-freezing, Gutiérrez found no significant difference on post-thaw survival or development rates in media containing PVA or FCS (n = 507) [236]. In the same species, Nowshari obtained equivalent embryo development rates using PVA, FCS or PVA in association with FCS medium (n = 537), as well as *in vivo* development rates (n = 528) [237]. After mice embryos slow-freezing, Palasz obtained equivalent post-thaw survival and development rates (n = 780) with PVA and with NCS [238]. On the other hand, another author reported inferior post-thaw mouse embryos development rates in PVA group than in the group containing animal products [239].

In the bovine species, after embryo slow-freezing with PVA, FCS and BSA (24 to 28 embryos per group), inferior gestation rates were obtained in the PVA group [240]. In two

other studies, PVA group post-thaw bovine embryos survival rates were significantly inferior after than in the group with animal products [238,241].

Concerning the ovine species, Leoni aimed to compare blastocyst formation and hatching rates, after vitrification with PVA or FCS. The authors reported significantly lower development rates in the PVA group, along with a diminution in protein synthesis after warming [242].

A more recent study, with porcine embryos, aimed to evaluate the effect of replacing serum in vitrification and warming media with different concentrations of PVA of *in vivo* produced embryos (*n* = 409). No significant differences were observed between animal sera group or PVA groups (0.1 %, 0.5 %, or 1.0 %), being 0.5 % the most interesting concentration [243] of PVA.

Polyvinylpyrrolidone (PVP)

PVP is a nonorganic macromolecule of variable molecular weight [244]. Studies using PVP tend to demonstrate a negative effect on cryopreservation media, with only one study showing similar post-thaw mouse embryo survival and development rates (*n* = 494) than FCS serum [236].

Seidel slow-froze bovine embryos with PVP, FCS and BSA based-media (24 to 28 embryos per group), and observed inferior *in vivo* development rates in PVP group [240]. Both authors observed that PVP had less surfactant properties than animal sera, having more difficulties in embryo manipulation [236,240]. Two authors obtained considerably lower development rates on mice embryos, and concluded that PVP is toxic to embryos [126,245].

Ficoll and Dextran

Ficoll is a synthetic macromolecule, commercialized in two high molecular weights: Ficoll 70 (70 000 Da) and Ficoll 400 (400 000 Da).

Gutiérrez aimed to compare mice embryo quick freezing in the presence of this compound (0.1 % Ficoll 400) with a medium containing FCS (*n* = 501), obtaining equivalent *in vitro* development rates [236]. In another study, mice embryos were vitrified with 18 % (*w/v*) Ficoll 70 as macromolecules source, obtaining promising post-warm *in vitro* development results (87 % expansion), but there was no comparison group with animal serum or albumin [246].

Kuleshova cryopreserved mouse embryos by rapid-cooling, using animal products free media containing 35 % polymers (dextran or Ficoll) and 25 % of penetrating cryoprotectants (EG), using a double straw arrangement to diminish contamination risk, obtaining *in vitro* development rates of 100 % blastocyst expansion and *in vivo* foetuses rates of 76 % [247]. One year later, the same authors aimed to reduce EG concentration, in mice embryo protein-free vitrification media, replacing a part of EG with Ficoll and dextran. Even if he didn't compare this medium with serum-derived medium, he obtained excellent *in vitro* (17-48 embryos per group, 96-100 % blastocyst expansion) and *in vivo* (62-76 % fetuses) development rates, rendering this macromolecules an excellent candidate for animal products replacement [126].

Lipid-rich albumin fraction of bovine serum

A chemically defined lipid-rich albumin fraction of bovine serum (Albumax I, Gibco) was used to slow-freeze *in vitro* and *in vivo* produced bovine blastocysts, comparing Albumax I with FCS and BSA. The authors concluded that survival wasn't affected by protein type during freezing [203]. Similarly, caprine expanded blastocysts were slow-frozen with Albumax I [248], but no sera-based group was tested. However, even if this product is better defined than BSA or FCS, it is not free of animal products.

Hyaluronic acid

Hyaluronic acid (HA) is a glycosaminoglycan that can be synthetized in its pure form [249]. HA can be found in follicular, oviduct and uterine fluids [250] and its concentration increases in the uterus by the time of implantation [251]. Human [252] and bovine [253] embryos contain receptors to this macromolecule during the preimplantation period. After successfully replacing albumin in embryo culture [254,255], HA became an interesting candidate for cryopreservation.

In 1990, Palasz obtained equivalent post-thaw murine and bovine embryo development rates after embryo freezing with synthetic HA and with NCS (n = 206) [256]. Joly observed equivalent post-thaw murine (n = 443) and ovine embryo (n = 120) *in vitro* development rates, after embryo slow-freezing using media containing HA or BSA [257].
Bioniche Life Sciences Inc. developed synthetic holding and freezing media (SYNGRO®) for bovine, equine, sheep and goat embryos, based on synthetic HA. However, few studies regarding cryopreservation were published with these commercial products [195]. Some authors used these media to slow-freeze equine embryos [258], and to slow-freeze and vitrify

bovine embryos [259], but these studies didn't aim to compare HA-based medium with animal derived products based media.

CRYO3

CRYO3 is a patented serum-free, protein-free and dextran-free medium, manufactured according to good manufacturing practices [cGMP-annex 1] in compliance with 2001/83/EC. CRYO3 is composed of synthetic hyaluronic acid of high molecular weight (> 10^6 Da), glucose, carbohydrates, amino acids, mineral salts, vitamins, fatty acids esters and buffers. This synthetic product was originally created for clinical applications, as a serum substituent in somatic and human adult stem cell freezing medium.

Bruyère compared post-thaw *in vitro* development of IVP bovine embryos, frozen with 18 % (*v/v*) CRYO3, 0.4 % (*v/v*) BSA and 18 % (*v/v*) FCS, and obtained superior development rates (CRYO3 81.5 %, BSA 42.2 % and FCS 58 %) and hatching rates (CRYO3 61.1 %, BSA 31.1 % and FCS 36 %) in 18 % (*v/v*) CRYO3 group [3]. The same author analyzed *in vitro* and *in vivo* post-thaw development of rabbit embryos frozen with 18 % FCS and 18 % CRYO3. Bruyère obtained similar blastocyst development rates (75.9 % for both groups) and significantly higher pregnancy rates (CRYO3 81.3 % and FCS 43.8 %), implantation rates (CRYO3 21.8 % and FCS 7.1 %) and live-fetuses rate (CRYO3 18.8 % and FCS 5.3 %) in the CRYO3 group.

3. Embryo evaluation

After cryopreservation, assessment of embryo viability and competence is essential. Suboptimal culture or cryopreservation conditions result in cellular stress, which causes retarded cleavage rates, cleavage arrest, abnormal genome activation and gene transcription, and altered patterns of energy metabolism. All these cellular stress consequences result in a loss of embryo quality [260]. One of the main challenges in embryo biotechnology research is to develop *in vitro* evaluation methods of embryo development competence.

In vitro assessment of embryo quality is important for the improvement of assisted reproductive technology.

3.1 Embryo morphology and embryo development

After fertilization, the zygote undergoes multiple cellular divisions (a process called cleavage), in a coordinated spatiotemporal program. The first cleavage takes place at about 16-20 hours after fertilization, into two, relatively equal-sized embryonic cells (blastomeres) [261]. As the cleavage takes place, the size of each blastomere is progressively reduced, contributing to reestablish the high cytoplasmic / nuclear ratio of the zygote to adult levels [262]. At around the 8 to 16-cell stage for most species, the embryo undergoes a compaction process, in which the round blastomeres flatten their shape to increase intercellular contact, and minimize intercellular space, with the loss of the individual blastomere outlines. The embryo yields the morula stage at this moment [263]. After compaction, at around the 32– to 64-cell stage in most species, the embryo goes through cavitation. The outer cells differentiate into a layer of trophectoderm (TE) cells, with tight junctions, that surround a cavity filled with fluid (blastocoelic fluid) – the blastocoel [263]. Inner cells differentiate into inner mass cells (ICM), at an eccentrically position [262]. At this phase, the embryo attains the blastocyst stage. The fluid cavity expands, increasing the embryo volume (expanded blastocyst stage), until the last phase before implantation - the hatching. To be able to implant, the embryo digests a part of the ZP (the extracellular matrix that involves the embryo), and emerges [261].

Good quality embryos display an optimal cleavage rate, specific to each species. Embryos dividing either too slow or too fast may have metabolic defects or chromosomal imbalance [264,265]. Recent time-lapse studies with human embryos indicate that not only the timing of cleavage, but also the synchrony of cleavage is important (in a perfectly synchronous embryo, 3-, 5-, 6-, 7- or 9-cell wouldn't be observed) [266,267]. The symmetry of blastomeres should also be considered, for instance: in a 4-cell embryo all the blastomeres should have equivalent volume, and if a 3-cell embryo is observed, it should contain a bigger blastomere (that is about to cleave), and two smaller blastomeres (that already went through cleavage) [262].

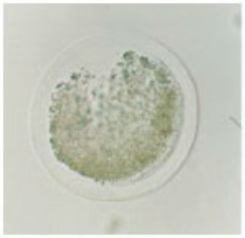
Fragmentation is also an important parameter during embryo morphological evaluation. Studies with human embryos showed that a fragmentation level superior to 10 %, even if reabsorbed during the course of embryo development, may significantly reduce pregnancy rate [268–270].

Several embryo morphology scoring systems, based on morphology, have been established [271]. The IETS scoring system is widely used in animal embryo evaluation. This

system involves a code for stage of development, ranging from “1” (unfertilized oocyte or 1-cell embryo) to “9” (hatched blastocyst), and a code for quality, based on embryos morphological integrity. Quality 1 embryos (excellent or good) have the highest probability to survive cryopreservation, and are recommended for international trade. They are uniform in size, color, and density blastomeres, with at least 85 % intact cellular material. Quality 2 embryos (fair quality) have moderate irregularities and at least 50 % intact cellular material. Although these embryos may present good survival rates ate transfer of non-cryopreserved embryos, they are less resistant to cryopreservation than quality 1 embryos. Quality 3 embryos (poor quality) present major irregularities, with at least 25 % of intact cells. Quality 4 embryos are dead or degenerating embryos [42].

Examples of bovine embryos classified according to the IETS system are illustrated in Fig. 7 and Fig 8.

Fig 7. Bovine embryos: examples of developmental stage and quality. Stages 1 to 5 [42].



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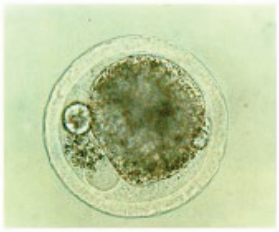
Quality Code:4



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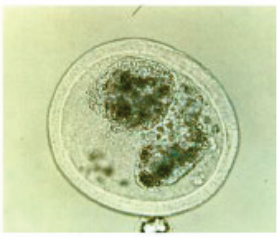

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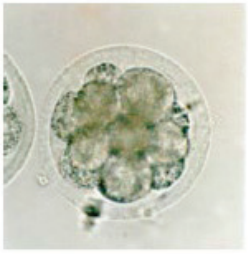
Quality Code: 4



Cycle Day: 7
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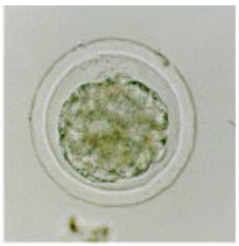
Quality Code: 4



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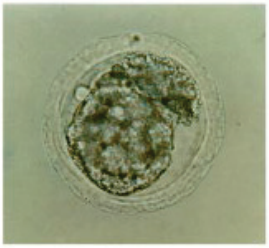
Qualiy Code: 1



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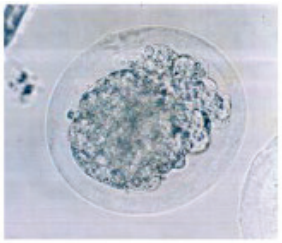
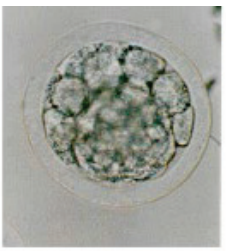


Cycle Day: 7
Stage Code: 4
Quality Code: 2

Cycle Day: 7
Stage Code: 4
Quality Code: 3



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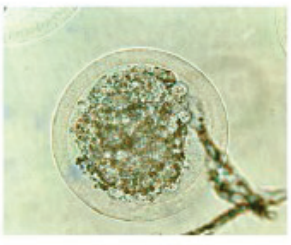
Quality Code: 3

Cycle Day: 7
Stage Code: 4
Quality Code: 3



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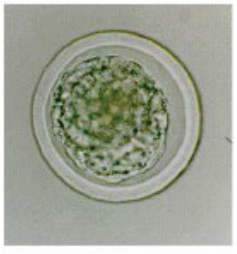
Quality Code: 3



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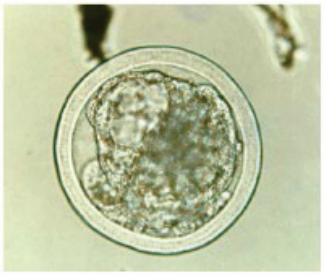
Quality Code: 1

Fig. 8. Bovine embryos: examples of developmental stage and quality. Stages 5 to 9 [272].



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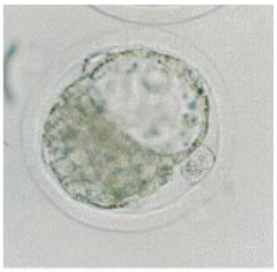
Stage Code: 5
Quality Code: 2



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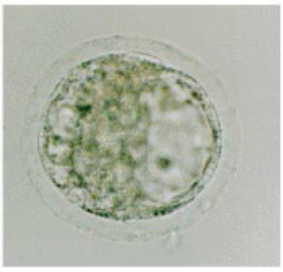
Quality Code: 1



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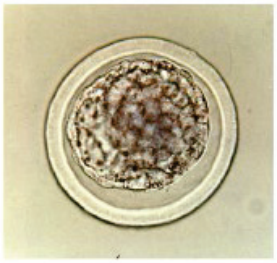
Quality Code: 2



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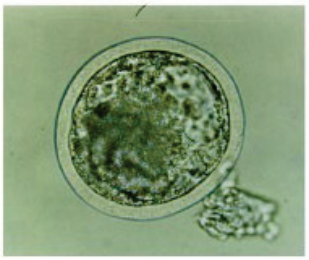

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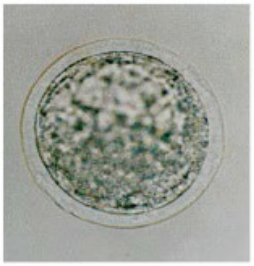

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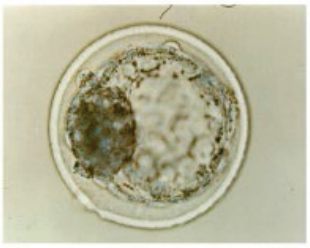

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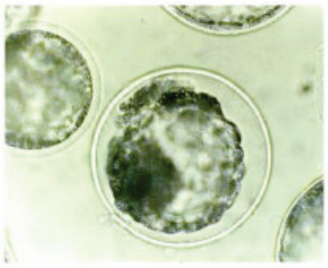
Quality Code: 1



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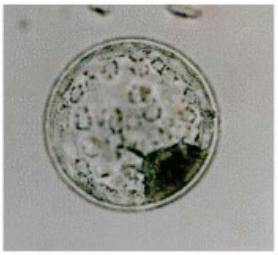
Quality Code: 2



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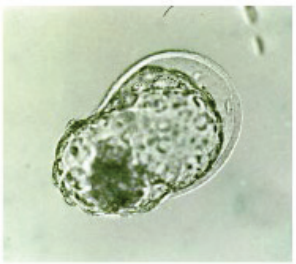
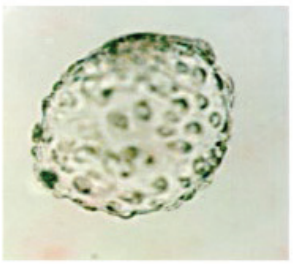


Cycle Day: 8
Stage Code: 8
Quality Code: 1



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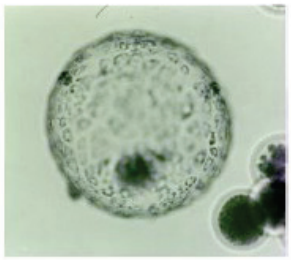
Stage Code: 8
Quality Code: 1



Cycle Day: 9
Stage Code: 9
Quality Code: 1

The ability of embryos to develop to the blastocyst stage and to hatch from the ZP provides strong evidence that the cultured embryos are viable.

Although morphology and cleavage rate are the *in vitro* criteria most commonly used to evaluate and select embryos [273–275], embryo development is a dynamic process, and static observations of embryo development may present limitations regarding blastomeres count, synchrony of cleavage, or even the comparison of two embryos at the same stage [274], and may be influenced by the operator experience.

Time lapse equipment

First embryo time lapse reports date back to 1929, with a cinematographic film of rabbit embryo development [276]. Since then, studies were done with embryos from other species under different conditions [277–280].

The introduction of time-lapse equipment helped assessing embryo development and embryo morphology during periods that were previously omitted, improving strength and efficiency of morphologic scoring systems [281]. Recent time-lapse technology comprises incubators, with a built-in microscope and an integrated camera, that allow a continuous monitoring of the embryos, without the need of evaluating the embryos outside of the incubator and, therefore, avoiding the exposition to undesirable changes in temperature, humidity and gas composition [282].

Different studies used this tool to estimate the optimal timing of the first cleavages [280,283,284], as well as compaction time [285] and time to hatching [286,287]. However, the kinetics of development may also be influenced by the laboratory environment, such as culture media, temperature and incubator oxygen conditions [266]. For example, Wale observed that mouse embryo cleavage was delayed in embryos cultured in atmospheric oxygen (approximately 20 % oxygen), in comparison to embryos cultured in 5 % oxygen [288]. Other studies observed that *in vitro* development rates are slower when compared to *in vivo* development, which can be explained by the difficulty to obtain optimal culture conditions, similar to those in the female tract [289].

A more continuous monitoring possibility may be an interesting alternative to the static evaluation, reducing its subjectivity. However, time lapse systems present some drawbacks: a long time is necessary to assess a video motion, and it still presents poor depth of view [290], along with the important acquisition cost. Even though time-lapse equipment enhanced morphokinetics-based embryo viability evaluation, this technique is still subjective, manipulator dependent and non-standardized [271].

3.2 Total cell count and differential cell staining

ICM cells will generate the embryonic tissues (and other parts of the extra-embryonic tissues), while TE will originate the other parts of the extra-embryonic tissues and the placenta [291]. Total cell number [292,293], ICM count [292,294,295], TE count [294] and ICM:TE ratio [296] have been considered viability markers.

Distinction between ICM and TE cells imply a differential staining. Differential staining technique was initially described by Gardner, after isolation of ICM and TE by micromanipulation [297], and by Solter and Knowles, through immunosurgery using complement mediated membrane lysis [298], almost five decades ago. More recently, this technique has been modified, with the introduction of staining with fluorochromes [299,300].

Numerous dyes allow the assessment of membrane integrity or intracellular enzymatic activity, or both simultaneously. Among the most used dyes are: 4',6-diamidino-2-phenylindol (DAPI) - a fluorochrome that penetrates both intact and dead (permeated) cells; propidium iodide (PI) - that only penetrates dead cells [301]; neutral red and fluorescein diacetate (FDA) - that only enters viable cells; trypan blue - which allows distinction based on the principle that viable cells exclude the dye; [302] and Hoechst - that stains the nuclei [303]. Commercial Live / Dead kits are available, and combine two probes (ex. calcein-AM, which stains viable cells, and ethidium homodimer-1 or PI, whic stains dead cells) that allow the discrimination between live and dead cells.

3.3 Assessment of metabolism

3.3.1 Assessment of energy metabolism and oxygen consumption

Energy metabolism substrates

Energy metabolism evaluation has been widely studied in embryos from numerous species, including mice [304], bovine [305], rabbits [306], and humans [307–311]. Time-dependent substrate intake and metabolite production has been related to embryo viability in the human [308,311], in the bovine [305] and in the mouse species [304,312]. The pre-implantation embryo depends on different substrates to provide metabolic energy, according to development stage and species. For most mammalian species, the early stages of development (1- to 8-cells) require pyruvate and lactate for adenosine triphosphate (ATP) production [304,311,313], as primary substrates for carboxylic acid-based metabolism, predominant in early stages [304,314]. Pyruvate was considered essential for the first cleavage of the mouse embryo [304]. Lactate seemed to have a deleterious effect during the first mouse cleavage [315], but its presence from the 2-cell stage was considered beneficial for embryo development, since it seems to regulate the uptake and metabolism of pyruvate [316]. A possible additional role of pyruvate is the protection of the embryo against oxidative stress, since it can be decarboxylated in the presence of hydrogen peroxide to produce water, carbon dioxide, and acetate [317]. Consequently, pyruvate uptake may reflect an oxidative stress augmentation, and not only embryo competence [318]. This indicates pyruvate uptake alone should be interpreted with precaution. After compaction, there is a switch from carboxylic acid to glucose metabolism and the uptake of pyruvate and lactate decreases, and glucose becomes predominant energy substrate [310,319]. Glucose was described by some author as being essential for the bovine [305] and mouse [320] embryo hatching process. This pattern was also described in ovine embryos [321,322] and in porcine embryos [323], in which glucose consumption increased significantly from the 8-cells stage to the blastocyst formation. Although glucose was initially considered of great importance for rabbit blastocyst development [324], Robinson suggested that glucose wasn't the main energy substrate on rabbit blastocyst, and that it can have a greater dependence on neutral glyceride pools [306]. High glucose uptake levels was correlated with embryo competence in bovine embryos [305,325], in caprine embryos [326] in mouse embryos [314] and in human embryos [327,328]. However, numerous studies reported successful *in vitro* development using culture media without glucose [325,329,330]. Curiously, Lane and Gardner found a negative correlation between glycolytic activity and embryo viability after transfer [292]. Despite these

promising results, several authors didn't find a correlation between substrate uptake and quality or implantation capacity [307,309,331,332].

Oxygen metabolism

Since oxygen plays an important role in energy production through oxidative phosphorylation and respiration in mitochondria, oxygen consumption rate was also evaluated in order to analyse embryo quality. Oxygen consumption rate doesn't fluctuate significantly during early development stages, and an increase is observed during blastocyst formation. This pattern was observed in mice [333], in porcine [323], in bovine [334] and in human [335] embryos. After the blastocyst expansion, oxygen consumption rate decreases to pre-cavitation values [323]. A positive correlation was found between *in vitro* oxygen consumption rate and embryo competence in porcine embryos [336,337], and in bovine embryos [338].

Secreted metabolites

Several studies focused on proteins secreted by the embryo into the culture media, but so far no single protein has yet been identified as a consistent marker of embryo quality [339–341].

3.3.2 Assessment of mitochondrial activity

Mitochondria have a crucial role in cellular energy production, through ATP synthesis by the respiratory electron transport chain. These organelles regulate calcium signalling and play a key role in apoptosis activation, by releasing numerous cell-death-inducing molecules [342,343]. For this reason, mitochondrial membrane potential (MMP) has been widely studied as a cellular health or injury indicator [344]. Disruption of MMP has been associated with metabolic stress and early cellular apoptosis [345,346]. In the reproduction field, oocyte and embryo MMP has been studied as predictor of developmental competence [344,347–349]. A metabolic disruption is often a consequence of exposition to sub-optimal environmental conditions [350,351].

3.3.2.1 Mitochondrial activity dyes

Different dyes have been studied to evaluate MMP, such as Rhodamine 123, DiOC6(3), Mitotracker, CMXRos, and JC-1 (5,5'6,6'-tetrachloro-1,1,3,3'tetraethylbenzimidazolycarbocyanine iodide), using flow cytometry [352–354] and confocal microscopy [355]. However, several studies indicate that JC-1 dye presents a more consistent behavior and has a higher sensitivity to MMP disruptors [352,356,357].

JC-1 dye

JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) is a dual emission potential-sensitive probe, which accumulates within active mitochondria according to the mitochondrial membrane potential (MMP). Mitochondria with high MMP accumulate more cationic dye (J aggregates) and exhibit red fluorescence, whilst mitochondria with low MMP accumulate J monomers, exhibiting green fluorescence [358]. Therefore, embryos with higher MMP present a higher red to green ratio than less active embryos. This dual wavelength emission represents an advantage over other MMP dyes with only one emission spectrum. As JC-1 stains high MMP and low MMP mitochondria simultaneously, it can be considered to function as its own control. An alteration of mitochondrial morphology or mass would have a smaller impact on the MMP than with other dyes, as this would have an impact on both high and low MMP mitochondria.

As JC-1 stains living embryos, *in vitro* culture after staining is possible. This approach does not seem to have a detrimental effect on the frequency of blastocyst formation [347]. This property is particularly interesting if other functionality or viability tests are considered.
JC-1 has been used to assess mitochondrial activity in mice [344,347,349,359,360], bovine [361] and human [347,355] embryos. However, current protocols do not allow JC-1 penetration through the mucin coat of rabbit embryo and no fluorescence is obtained (*unpublished studies*). Consequently, JC-1 has never been used in rabbit embryos.

3.4 Assessment of *in vivo* development

In vitro studies are necessary to identify the conditions that have best chances to work in *in vivo* conditions, reducing the use and the number of experimental animals. Even if *in vitro* development is a suitable alternative to embryo transfer [362], results obtained after *in vitro* evaluation do not always reflect *in vivo* embryo development competence [363]. Consequently, caution should be taken when interpreting *in vitro* results.

After *in vitro* validation of experimental conditions, such as a cryopreservation or culture medium, or even a cryopreservation technique, the evaluation of *in vivo* development of embryos is of major value.

To evaluate *in vivo* development, embryos are transferred to female recipients, and pregnancy rate or birth rate are assessed [25,364–366]. The analysis of these results allows an evaluation of the tested conditions. If the goal is to compare an existing method / medium with a new condition, the obtention of similar or better *in vivo* development rates with the tested condition would allow to validate it.

This premised leaded us to perform *in vivo* transfers in the main studies, after validating our conditions with *in vitro* development and complementary *in vitro* tests.

Chapter II - Experimental part

In this chapter four preliminary studies will be described:

- The first one ("Thermodynamic evaluation of the cryopreservation media: differential scanning calorimetry") analyses thermodynamic properties of three media: a medium used to vitrify rabbit embryos containing animal products, and two synthetic media, containing different proportions of CRYO3. This study evaluates the interest of these two synthetic media as candidates for embryo vitrification media and preceeds biological studies.
- The second study ("Mitochondrial activity evaluation: JC-1 dye") consists in the adaptation of a mitochondrial activity staining protocol to rabbit embryo. This protocol will be used in one main study as a complement to *in vitro* evaluation of rabbit embryo development.
- The third study ("Ice nucleation agents") investigates the use of an ice nucleating agent (Snomax®) as a substitute to "manual seeding", during embryo slow-freezing procedures. Although this study didn't aim to answer directly our main questions, it was conducted in collaboration with the physicist Hugo Desnos, and allowed him to pursue his experiments.
- The last preliminary study ("Ewe superovulation") arose from the need to obtain ewe embryos, to be used in the main studies, and evaluated two different superovulation protocols. The embryos produced during these experiments were used in the second part of this thesis.

II.I Preliminary studies: development of methods

1.Thermodynamic evaluation of the cryopreservation media: differential scanning calorimetry

Differential Scanning Calorimetry (DSC) is a thermoanalytical technique, developed in the 1960s, that has been used in cryobiology to evaluate thermal properties of cryopreservation solutions subjected to controlled temperature scans, particularly during phase transition (crystallization and melting). This technique is used as a tool to characterise cryopreservation solutions [220,367], to predict optimal cryopreservation conditions and protocols [368–370] and to improve understanding of ice formation in living cells or organisms [49,371–373].

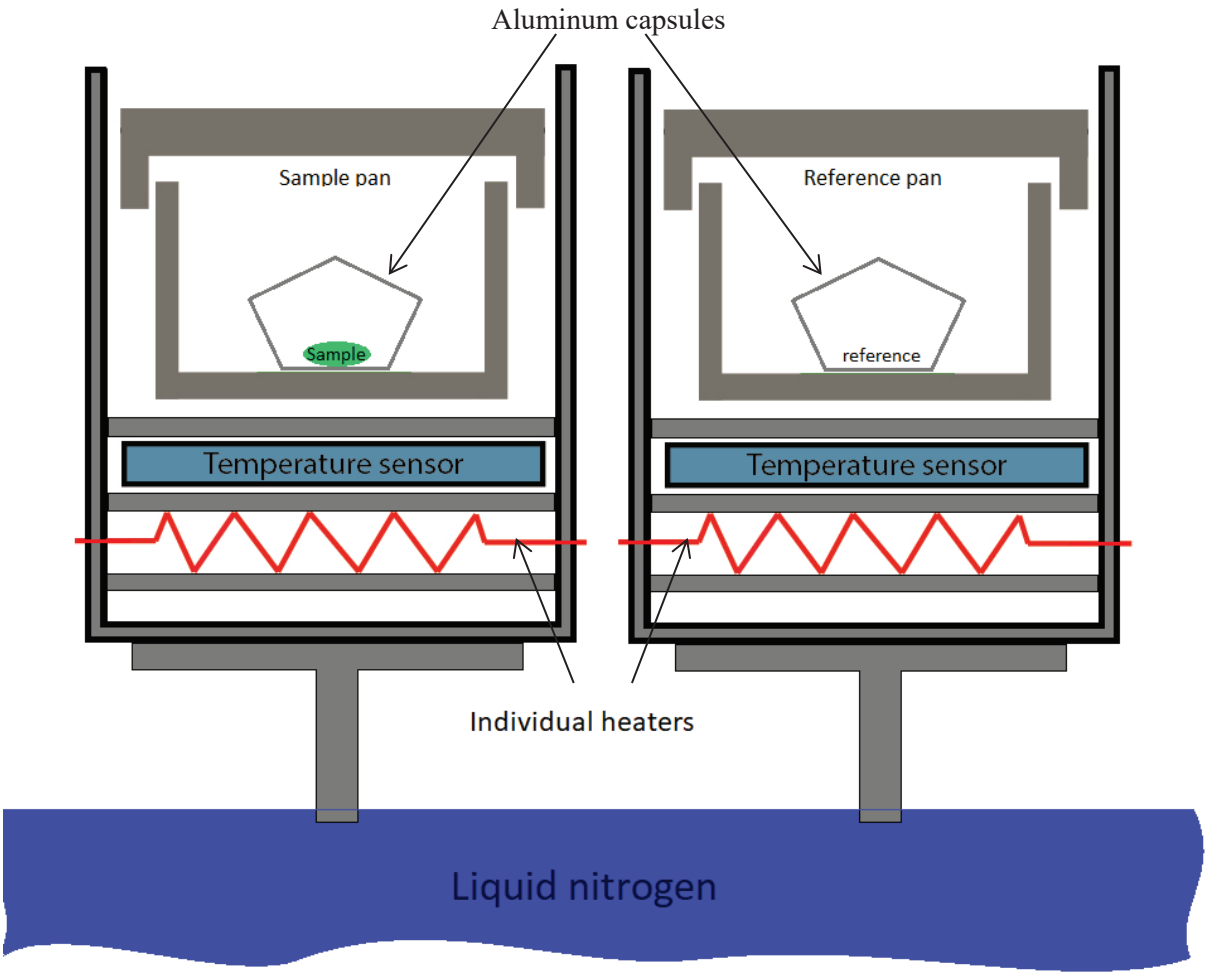
Currently, two distinct types of DSC are available: power compensation DSC and heat flux DSC. Heat flux calorimeters measure the temperature difference between the sample and the reference, whilst power compensation calorimeters measure the heat flow directly from the sample pan. Additionnaly, power compensation DSC allows the use of faster cooling rates [374]. In our experiments, we used a power compensation DSC (DSC Diamond, Perkin-Elmer, Waltham, Massachusetts, USA), in which heating or freezing rates of 0.1 °C/min to 300 °C/min can be programmed. For this reason, this type of DSC will be explained in greater detail.

A power compensation DSC system contains a power compensation unit that controls and monitors the temperature of two independent furnaces: the reference furnace and the sample one. It measures directly the heat flow of the sample by maintaining a zero temperature difference between both furnaces as the furnace temperature is scanned. The power compensation unit goes on by a cold finger which plunges in a liquid nitrogen dewar, allowing fast temperature scans. Each oven contains an independent heater and a thermal sensor, and both ovens are surrounded by gaseous helium purged with dry nitrogen while inserting or removing sample, that allows a more homogenious cooling [375]. The sample is sealed in an aluminum pan. The reference can be an inert material such as alumina, or an empty aluminum pan (Fig 9).

The power compensation unit provides equivalent heat to both pans, at a constant and defined rate, increasing their temperature. During a phase transition, the sample will release (exorthermic reaction, eg. crystallization) or absorbe (endothermic reaction, eg. melting) heat, and a lag will temporarly exist between both pans. The power compensation unit will detect this lag, and compensate it adding or substracting energy to the sample, in order to keep both

sample and reference furnaces at the same temperature [376]. This difference of energy provided to both pans is recorded, as a function of temperature or time, and a thermogram is obtained.

Fig. 9. Principle of power compensation calorimetry.



Several informations can be obtained from the thermogram, some of the most important ones are:

- **Crystallization temperature (Tc):** this parameter is measured at the onset of the crystallization peak. A significant T_c variability can be observed between repetitions, as solution impurities can act as a nucleation site [32]. Consequently, this parameter is not commonly used to compare complexe solutions.

- **Enthalpy of crystallization (ΔH):** this parameter represents the quantity of liberated energy by the system during crystallization; it is calculated in J/g and represents the area of the exothermic peak (between the baseline and the thermogram curve), measured on cooling. ΔH can be used to calculate the **heat of ice crystallisation (Q):**

$$Q(\%) = \frac{\Delta H}{\Delta H_{water}} \text{ X } 100$$

Q is close to the quantity of ice crystallized in the sample (*w/w*), if there is only ice formation, during a cooling, and is instrinsically related with the solution composition, and expressed in percentage [377]. The maximal value of Q is obtained for a cooling rate of -2,5 °C/min. The corresponding value, Q_{max}, is suitable for comparison between cryopreservation media.

When analyzing cryopreservation media designed to be rapidly cooled, particularly interesting parameters can be obtained on warming, after a rapid cooling allowing to start reheating in a complete amorphous state. These parameters are:

- **Glass transition temperature (T_g)** which characterizes the range of temperatures over which a glassy state is transformed to a viscous state on warming. Although it is a temperature range, it is reported as a single temperature defined as the midpoint of the temperature slope of shifting.
- **Devitrification temperature (T_d)**, measured at the minimum of the crystallization peak (called “devitrification peak”);
- **Melting temperature (T_m)**, which represents the temperature at which the last ice crystal melts, during ice melting.

The knowing of these values and the use of the semi-empirical Boutron’s model allows the calcul of the **critical cooling and warming rates** [378]. The **critical cooling rate** of a medium is the lowest rate that allows this medium to vitrify during rapid cooling, without detectable ice crystals formation. Classicaly, it increases with the decrease of cryoprotectants concentration and **critical warming rates values** are higher than those required during cooling the same sample [379–381]. If warming rate is inferior to the estimated critical value, devitrification may occur during warming, meaning ice formation between T_g and T_m.

The aim of this experiment was to determine the critical cooling rates and the corresponding critical warming rates required to avoid crystallization in the vitrified samples of three cryopreservation media: a medium containing animal products, and two potential synthetic substitutes.

1.1 Cryopreservation media

The three cryopreservation media contained the same CPAs composition: 20 % (*v/v*) DMSO (Sigma-Aldrich, Saint Quentin Fallavier, France) and 20 % (*v/v*) EG (Sigma-Aldrich, Saint Quentin Fallavier, France), and the following base media:

- **BSA:** IMV Embryo holding medium® (IMV Technologies, L'Aigle, France), containing 0.4 % (w/v) BSA;
- **D-PBS + 20 % CRYO3:** D-PBS (Sigma-Aldrich, Saint Quentin Fallavier, France) supplemented with 20 % of CRYO3;
- **CRYO3:** 100 % CRYO3 medium.

The three cryopreservation solutions were prepared simultaneously to ensure greater homogeneity in their production.

1.2 Thermodynamic evaluation using DSC

The phase transitions of the cryopreservation solutions were characterized using a power compensation DSC (Diamond DSC, Perkin-Elmer, Waltham, Massachusetts, USA) equipped with a liquid nitrogen cooling accessory (Cryofill) and the Pyris software (R 11.1.1 version). The DSC was calibrated for temperature and energy at +2,5 °C/min with standards: the ice melting of pure bi-osmosed water (0.00 °C; ΔH_{water} = 333.4 J/g), the crystallographic transition of cyclohexane in its solid state (-87.06 °C; 79.58 J/g) or the melting of pure indium (156,60 °C; 28,45 J/g), for a high data range of 720 mW. After the calibration, additional tests were systematically performed on reverse-osmosis purified water and sometimes on cyclohexane to verify the accuracy of the DSC under our experimental conditions. The maximal error of reproducibility obtained with these pure standards was ± 0,4 °C (temperature) and ± 1,2 % (energy). The validity of the calibration was verified regularly using tests on pure bi-osmosed water. Experiments were conducted using standard hermetically sealed aluminium pans (Ref. 0219–0062, Perkin-Elmer, Waltham, Massachusetts, USA) designed for volatile samples.

Cryopreservation media were placed in clean eppendorfs, and the takings for a given cryoprotective solution were all made in the same eppendorf tube. Two replicate measurements were taken for each cryoprotective solution. During each test, each eppendorf tube was placed at room temperature for approximately 30 min, and the solution was gently miwed through a serie of 20 capsizes. The solution was then loaded to cleaned aluminum pans (20µL) (procedure provided by Perkin-Elmer) using a micropipette to limit the variations in

weight between the samples. The aluminum pans were first weighed without cryopreservation solution on a high sensibility scale balance (XS105 DualRange, Mettler Toledo, France) and were then weighed after the loading of the cryopreservation solution in order to measure the sample mass. The weights were determined with a resolution of 10^{-5} g. To ensure the sealed pans' insulation, sealed pan weights were measured again at the end of the experiments and were compared with the weight obtained before the DSC measurements.

For each cryopreservation medium, two protocols were applied: the first protocol aimed to measure the tendency of the sample to form a glass during cooling. Both pans underwent 7 cooling/warming cycles. In each cycle, distinct cooling rates were applied from 10 °C to -150 °C (-160 °C/min, -80 °C/min, -40 °C/min, -20 °C/min, -10 °C/min, -5 °C/min, -2.5 °C/min). The pans were then maintained at -150 °C for 2 min, and a warming rate of 20 °C/min was applied from -150 °C to 10 °C. The thermogram obtained with this protocol allowed the estimation of ΔH (the area of the exothermic curve) used to evaluate Q (%). Critical cooling rates were determined from the evolution of Q with the tested cooling rate [80].

The second protocol intended to mesure the stability of the amorphous state during warming. Both pans underwent 7 cooling/warming cycles from 10 °C to -150 °C, with a constant cooling rate (-300 °C/min), were holded at -150 °C for 2 min, and distinct warming rates were applied from -150 °C to 10°C (2.5 °C/min, 5 °C/min, 10 °C/min, 20 °C/min, 40 °C/min, 80 °C/min and 160 °C/min). This protocol allowed the evaluation of the following additional parameters: T_d (determined at the minimum of the crystallization peak) and T_m (determined at the top of the main melting peak). These parameters allowed the estimation of the critical warming rates [84].

The semi-empirical Boutron's model was used to calculate critical cooling and warming rates [378,379].

1.3 Results

The obtained Q_{max}, critical cooling rate, T_m and critical warming rate, for each cryopreservation medium are summarized in table I.

Table I. Q_{max}, critical cooling rate, Tm and critical warming rate estimated for the three cryopreservation media from DSC thermograms obtained during cooling and warming (n = 2, except if indicated).

Base medium	Q_{max} (%)	Critical cooling rate (°C/min)	T_m (K)	Critical warming rate (°C/min)
BSA	15.55±0.25	168.0±1.0	243.24±0.11	1 530 711.5±23 819.5
D-PBS + 20 % CRYO3	15.46±1.47*	73.6±42.2*	242.71±0.05	400 098.5±49 732.5
CRYO3	13.70±0.56	29.5±3.5	238.89±0.23	6 114.0±23.0

*(n=4)
The results were presented as mean ± standard deviation.

1.4 Discussion and conclusion

In this study, we evaluated specific thermodynamic properties of three cryopreservation media containing the same cryoprotectant composition (20 % (*v/v*) DMSO and 20 % (*v/v*) EG) and different base media: a base-medium containing animal derived-products (BSA), a base-medium composed of D-PBS supplemented with 20 % CRYO3 and a synthetic base-medium only composed of CRYO3.

Taking into consideration that only two replicate measurements were performed per protocol for each cryopreservation solution, we estimate that Q_{max}, T_m and critical cooling rates's reproducibility are satisfactory, with the exception of the medium D-PBS + 20 % CRYO3. A conservation problem between the first and the second measurement of this solution didn't allow us to perform a second measurement. For this reason, the same solution was prepared, and two measurements were done. We observed that the results obtained with the first measurement of the second preparation of the DPBS + 20 % CRYO3 solution were similar to those obtained with the first preparation. However, the second and third measurements of this solution showed similar results between them, but different form the previous measurements. This suggests a possible rapid evolution of the solution composition (between the first and second measurement), that may be due to the interaction of components of D-PBS and CRYO3.

The medium containing CRYO3 showed a mean Q_{max} value lower than the BSA-based media and than the D-PBS + 20 % CRYO3-based medium. This means that the maximal quantity of crystallized ice, in the case of ice formation, would be lower on the CRYO3

medium. A possible justification would be that one (or more) component(s) of CRYO3 would have a greater capacity to bound with water molecules, and the quantity of water available to crystallize decreases. The mean melting temperatures of the three media were close, but the one with CRYO3 is the lowest, that strengthens the idea that CRYO3 contains molecules that bind to water molecules.

With reference to critical rates, BSA-based medium showed a mean critical cooling rate more than twice higher than the D-PBS + 20 % CRYO3-based medium and more than five times higher than the CRYO3 based medium. However, common rapid cooling techniques, used to attain vitrification, usually overcome our higher (obtained with BSA-based medium) critical cooling rate [382]. For instance, the cooling of a 0.25 mL straw in LN_2 vapor allowed a cooling rate of 187 °C/min [147], higher than the obtained critical value for BSA-based medium in our experiment. The use of devices especially designed for vitrification may allow cooling rates to over 20 000 °C/min [134–136].

As expected (and discussed on the "Review of literature section"), critical warming rates (to avoid devitrification during warming) were much higher than critical cooling rates, as ice forms much more rapidly on warming [146]. This shows that overcoming critical rates is more challenging during warming than during cooling. The estimated critical warming rate for the BSA-based medium was 3.8 times higher than the D-PBS + 20 % CRYO3-based medium and 250 times higher than the CRYO3-based medium. Several factors seem to reduce the critical warming rate of a medium, such as the presence of physiologic support solutes [383], polymers [381,384] some specific solutes [385] or solute mixtures [386,387], and high hydrostatic pressure [11,117,384,388]. CRYO3 contains HA of high molecular weight ($> 10^6$ Da). We hypothesize that it can play a major role in the reduction of critical warming rate. The addition of HA to artificial gels seemed to interrupt the growth of ice cystals during freezing and to alter the ice pattern to smaller ice units [389], showing this molecule is a good candidate for embryo cryopreservation.

As the complete and exact composition of CRYO3 is not published, it remains difficult to evaluate which components contribute to the decrease of the critical warming rate, and in which way and magnitude.

The cryopreservation medium containing D-PBS and 20 % CRYO3 showed critical cooling and warming rate values between the two other media, which is not surprising, since it contains a smaller proportion of CRYO3. However, this medium showed a more important variability for both critical rates. A possible explanation is a possible evolution of the solution

composition between measurements. More replicates would be of value to confirm this hypothesis.

The obtained results show that the medium containing CRYO3 seems to have a composition that allows this medium to more easily vitrify during cooling, and go through warming process with a greater stability.

Even if the three cryopreservation media contained the same penetrating cryoprotectant (EG and DMSO) composition, some differences were observed regarding the evaluated thermodynamic properties, showing that the composition of the base medium can play a major role on the avoidance of ice formation and, consequently, on the survival of cells during cryopreservation.

These results show that, among the three evaluated cryopreservation media, the solution containing a base-media composed of CRYO3 seems to show the greatest tendency to form a stable glass and the greastest amorphous state stability.

2. Mitochondrial activity evaluation: JC-1 dye

The aim of this experiment was to propose a JC-1 staining method for rabbit embryo in order to evaluate the mitochondrial activity of fresh and cryopreserved embryos.

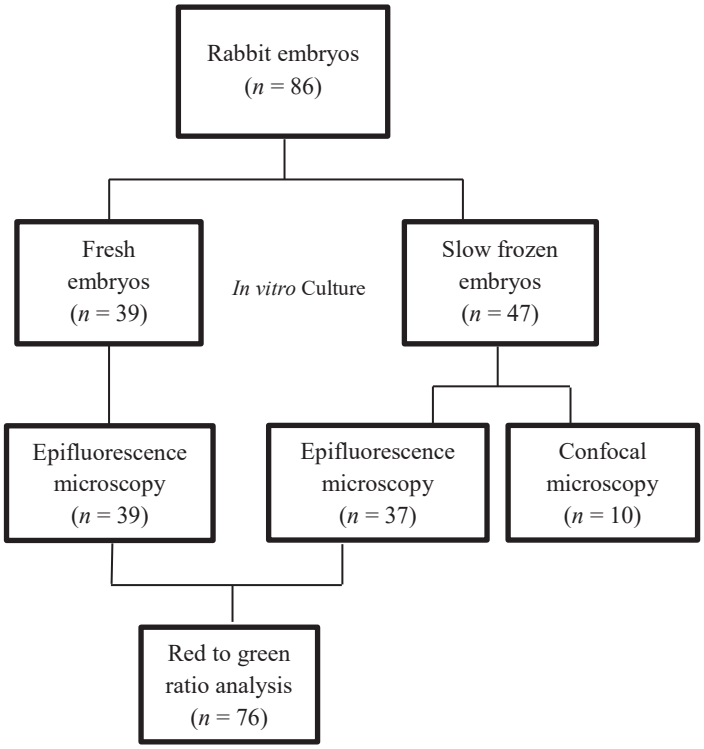
2.1 JC-1 staining method adaptation to rabbit embryos

This study was approved by the Ethical and Animal Welfare Committee of VetAgro Sup (Permit Number: 05/26). All animals were handled according to the EU Directive 2010/63/EU for animal experiment guidelines.

Embryo production and recovery

A total of 10 multiparous rabbit New Zealand does (SARL HYCOLE, Marcoing, France), aged between 38 and 50 weeks, were housed in groups of five and fed with a commercial diet. Does received a twice-daily subcutaneous administration of five doses of a pFSH : LH (ratio 5 : 1) preparation, (31.5 µg total, Stimufol, Reprobiol, Belgium). Eight hours after the last injection, does were inseminated with sperm from multiple males (pooled ejaculates), and a subcutaneous injection of 1.6 µg buserelin (Receptal®) was administered. Rabbit does were euthanized by cervical dislocation, 65 to 68 h after the buserelin administration. The oviducts and uteri were flushed using Euroflush® medium at room temperature. Embryos were recovered at the morula stage and classified according to International Embryo Transfer Society (IETS) manual [42]. Good quality embryos were pooled and a part of these embryos was used in this study ($n = 86$). Embryos were divided into two groups: one fresh control group ($n = 39$); and a slow freezing group ($n = 47$), as described in Fig. 10.

Fig. 10. Schematic representation of the experimental design.



Embryo cryopreservation

Embryos from the cryopreservation group ($n = 47$) were transferred into a freezing solution composed of 1.5 M DMSO in IMV Embryo holding medium® at room temperature for 10 min. The embryos were subsequently loaded into 0.25 mL straws between two drops of freezing medium separated by air bubbles and placed in a programmable freezer (Cryocell 1200, IMV Technologies, L'Aigle, France). The straws were cooled from room temperature to -7 °C at a rate of 5 °C/min before seeding was manually induced by application of a cold spot on the first column (not containing embryos) of the straws. Next, the straws were held for 5 min at –7 °C before being cooled to -30 °C at a rate of 0.5 °C/min. Finally, they were plunged and stored in LN_2.

Warming was performed by holding the straws in air (15 s) before plunging them into a 20 °C water bath (60 s). The frozen-thawed embryos were incubated in three successive baths in order to remove DMSO (1.0 M, 0.5 M and 0.0 M DMSO respectively, in IMV Embryo holding medium®). Embryos were then placed at the incubator for *in vitro* culture before staining.

Embryo culture and morphology assessment

Embryos analysed by epifluorescence microscopy: embryos (n = 37) were cultured (38.5 °C, 5 % CO_2) to the blastocyst stage, in M199 medium (without glutamine; Dutscher, Brumath, France) supplemented with 10 % foetal calf serum and antibiotics (67 UI/mL penicillin and 67 µg/mL streptomycin, Dutscher, Brumath, France).

In vitro development was assessed after 32 hours of culture. Embryos were classified according to the IETS grading system [42] and good quality embryos (not delayed, uniform mass, ≥ 85 % cellular material intact, smooth ZP) were considered "non-damaged", and embryos with < 85 % intact cellular mass, presenting ZP irregularities or delayed were considered "damaged".

*Embryos analysed by confocal microscopy: e*mbryos (n = 10) were cultured under the same conditions, to the compacted morula stage (n = 5), after 12 h of culture and to the blastocyst stage (n = 5), after 32 h of culture.

2.1.1 Staining protocol and fluorescence intensity assessment (JC-1 staining)

The JC-1 (Thermofisher scientific, Illkirch, France) loading protocol was previously validated in our laboratory, with different stock concentrations of JC-1, dye loading time, with or without pretreatment with pronase (data not shown).

The mucin coat of living embryos was dissolved at 38.5 °C by a pronase pretreatment (protease, from *Streptomyces griseus*, 5 mg/mL, Sigma-Aldrich, Saint Quentin Fallavier, France), in Dulbecco's Phosphate-Buffered Saline medium (D-PBS) supplemented with D-glucose (5.56 mM), sodium pyruvate (0.33 mM) and bovine serum albumin (3 mg/mL). Embryos were monitored intermittently for two to three minutes, until the mucin coat started to dissolve. Next, embryos were washed in six drops of modified D-PBS.

Embryos (n = 76) from both groups were incubated with JC-1 for 75 min (1.5 µM, 38.5 °C, 5 % CO_2). Embryos were observed using an Olympus IX71 epifluorescence microscope, with an excitation wavelength of 488 nm. JC-1 aggregates were detected with a red filter (590 nm wavelength) whereas JC-1 monomers were detected with a green filter (530 nm wavelength). To evaluate the mitochondrial activity, the staining intensity (by pixel) was determined in two randomly defined areas on each embryo, from both channels, and the red to green ratio was measured using the Fiji package of ImageJ software [390].

A MMP disruptor (CCCP, carbonyl cyanide 3-chlorophenylhydrazone; Thermofisher scientific, Illkirch, France) was used as a control to ensure that directional changes in the dye signal were appropriately interpreted.

To validate the protocol obtained with the epifluorescence microscope, stained cryopreserved embryos ($n = 10$) at the (not delayed) morula and blastocyst stage were observed under a confocal microscope (Yokogawa CSU22 Confocal Spinning Disc).

2.1.2 Statistical analysis

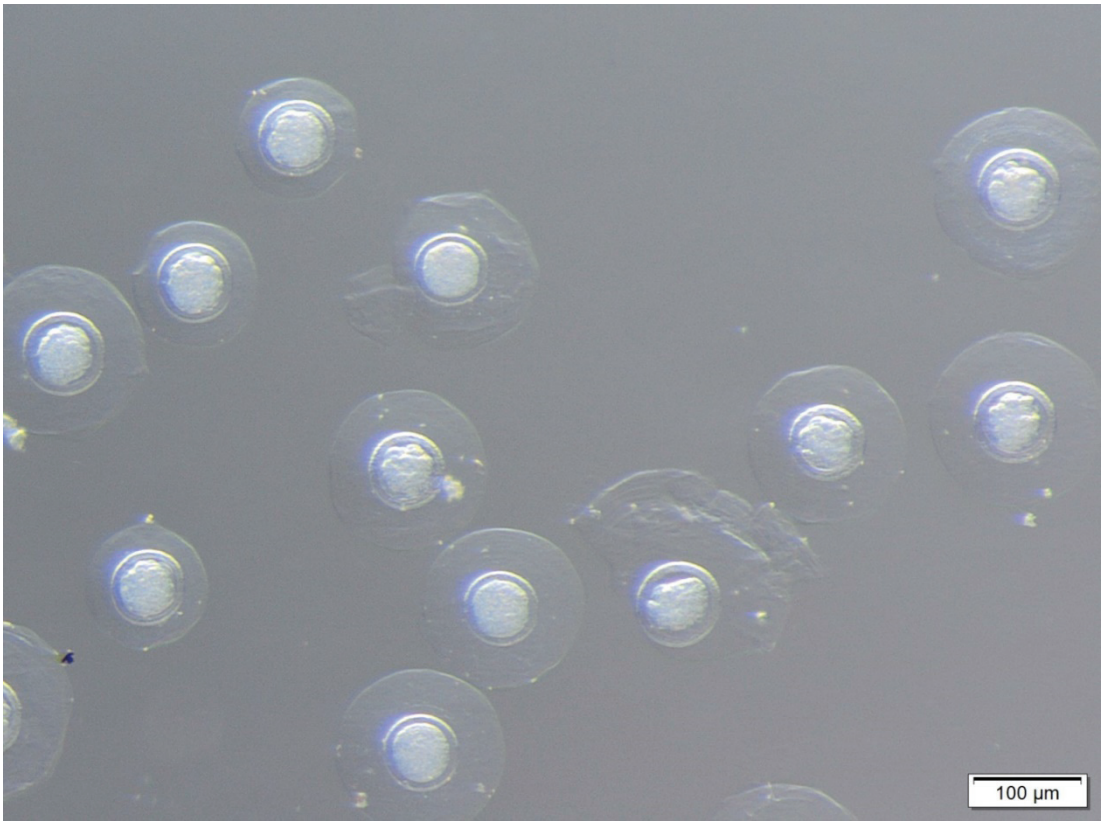
JC-1 red/green ratios were analysed by one-way analysis of variance, with R-Studio software (R Core Team, Vienna, Austria) [391]. Groups were considered to be significantly different at $P < 0.05$.

2.1.3 Results: assessment of mitochondrial activity

In vitro development after cryopreservation

Frozen-thawed embryos are presented in Fig. 11. *In vitro* morphology assessment at 32 h of culture (damaged and non-damaged embryos) is presented in Table II. No damaged embryos were observed in the fresh embryo group after 32 h of culture whereas 29.7 % of the cultured frozen-thawed embryos were classified as damaged.

Fig. 11. Morphology of frozen embryos after thawing.



Fractured mucous layers may be noted.

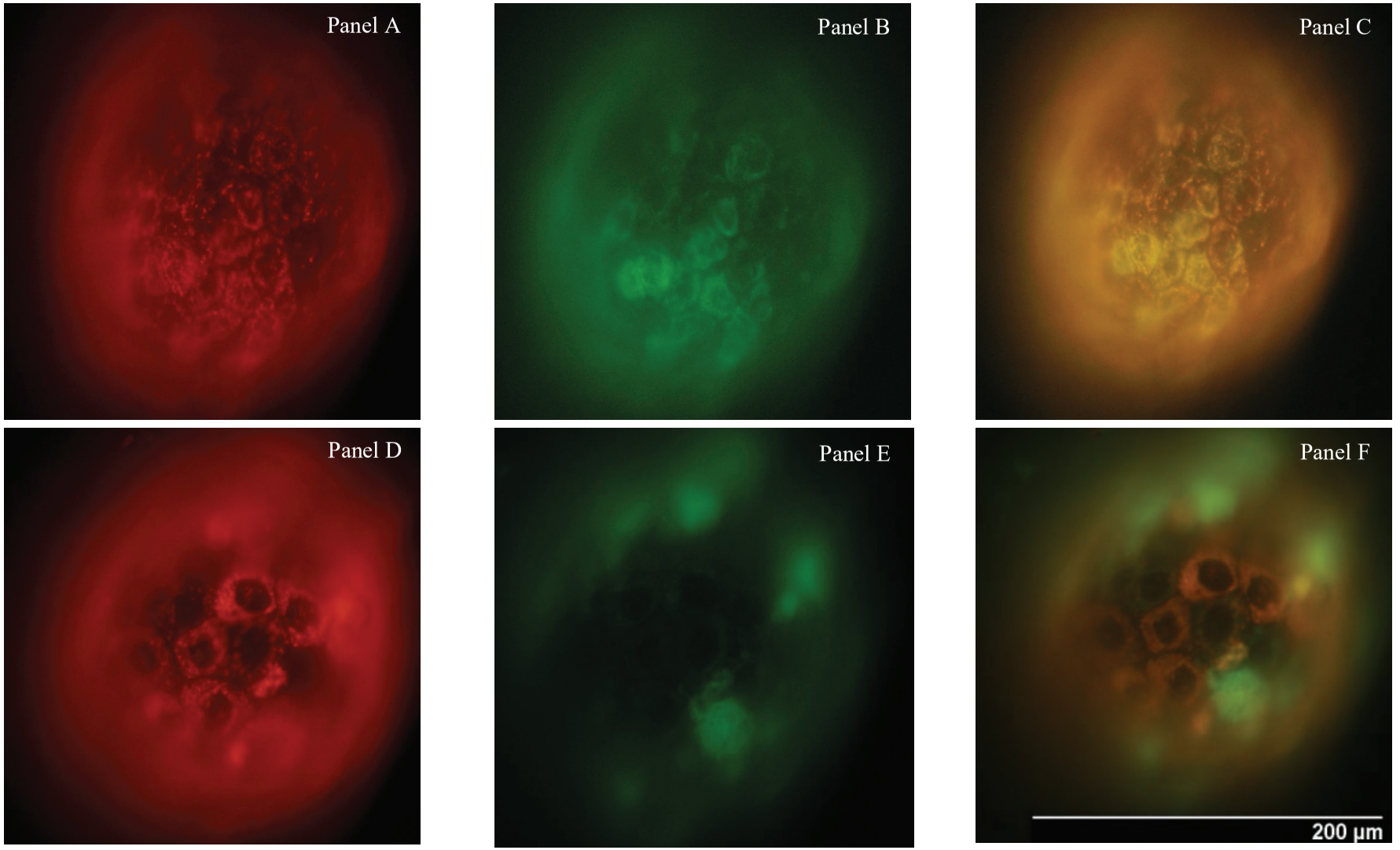
Table II. *In vitro* morphology assessment at 32 h of culture.

Group	**Damaged embryos (%)**	**Non-damaged embryos (%)**
Fresh embryos	0 % (0/39)	100 % (39/39)
Cryopreserved embryos	29.7 % (11/37)	70.3 % (26/37)

JC-1 staining

Representative images, obtained with epifluorescence microscopy, of embryos stained with JC-1 are presented in Fig. 12.

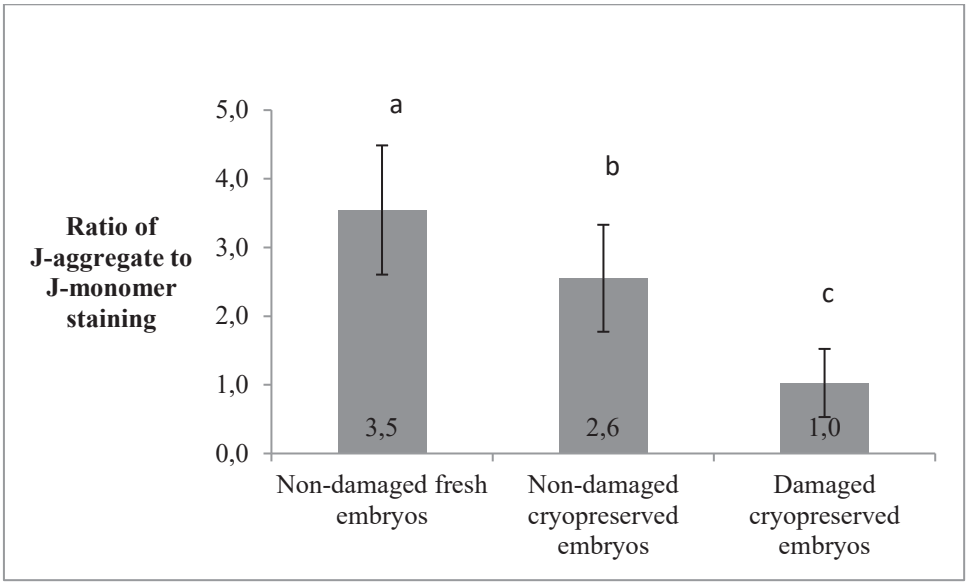
Fig. 12. Representative epifluorescence photomicrographs of two rabbit embryos stained with JC-1.



A, D) Regions of high MMP are indicated by red fluorescence (emission ~590 nm).
B, E) Depolarized regions are indicated by green fluorescence (emission ~529 nm).
C, F) Merged images.

Ratios of J-aggregate to J-monomer of fresh and cryopreserved embryos, from images obtained using epifluorescence microscopy, are presented in Fig. 13.

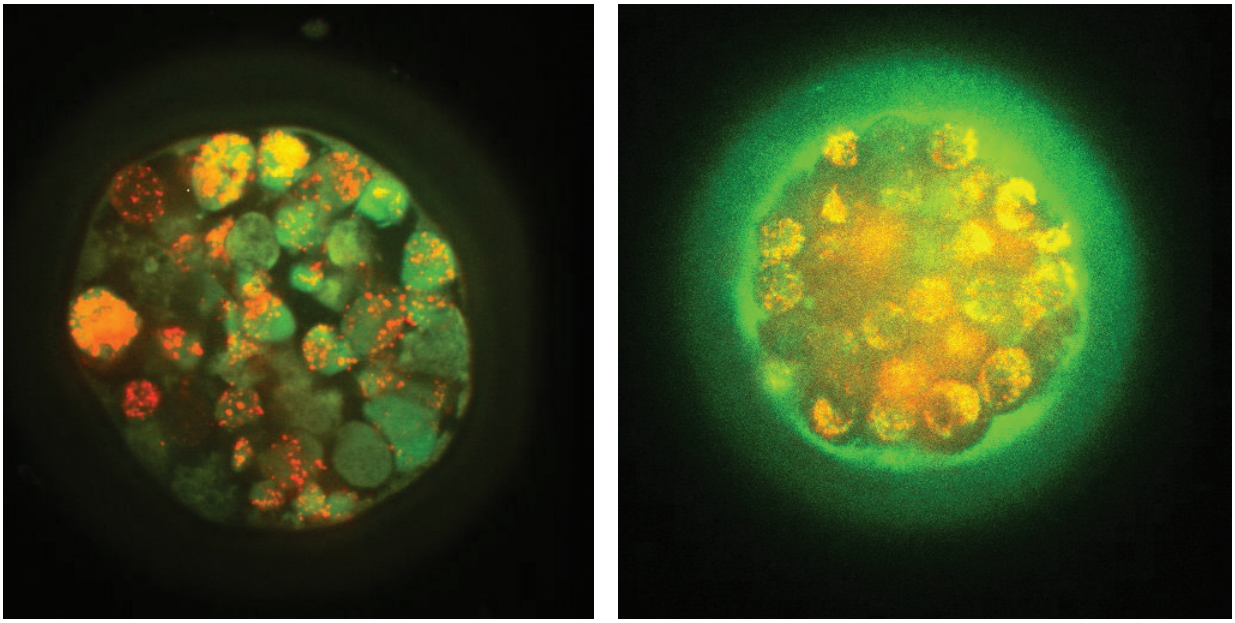
Fig. 13. JC-1 staining ratio of fresh (n = 39), cryopreserved non-damaged (n = 26) and cryopreserved damaged (n = 12) embryos obtained with epifluorescence microscopy.



Bars represent the mean ± SEM. Different letters indicate a statistically significant difference. Significant differences were found between non-damaged fresh and non-damaged cryopreserved embryos, as well as between non-damaged and damaged cryopreserved embryos ($p < 0.05$).
Significant differences were found between non-damaged fresh embryos and non-damaged cryopreserved embryos, as well as between non-damaged and damaged cryopreserved embryos.

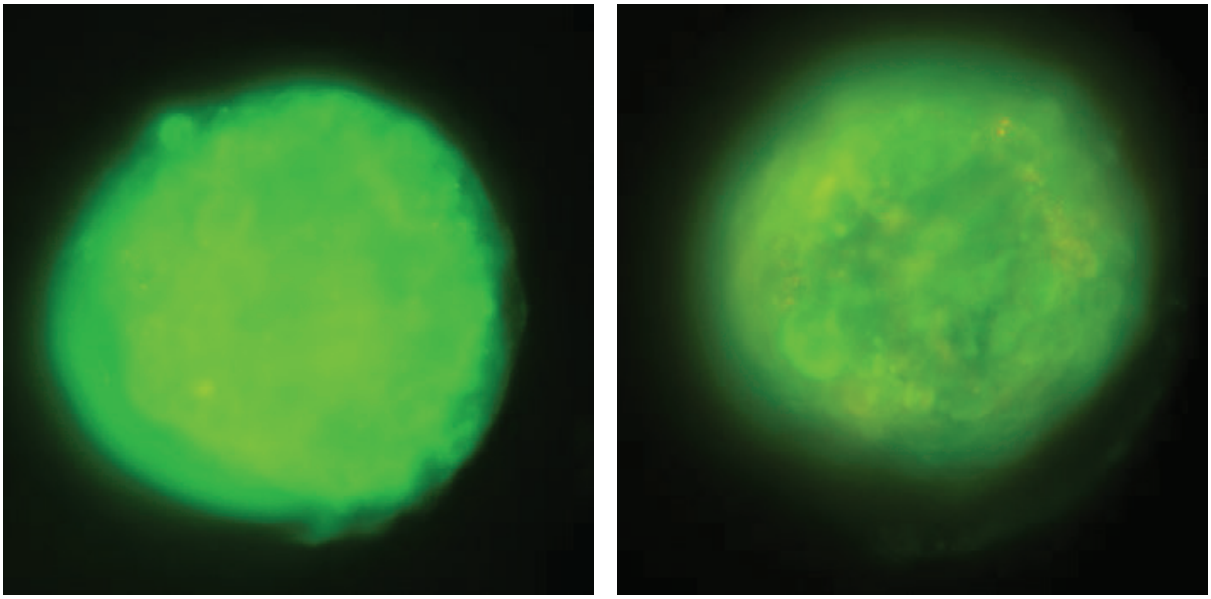
Confocal microscopy observation confirmed JC-1 dye penetration through the entire thickness of the embryos (Fig. 14), and the observation after incubation with a CCCP control showed no red fluorescence (Fig. 15).

Fig. 14. Confocal fluorescence photomicrographs of two rabbit embryos stained with JC-1 (still video).



Merged images: regions of high MMP are indicated by red fluorescence (emission ~590 nm), regions with JC-1 monomers (low MMP) are indicated by green fluorescence (emission ~529 nm).

Fig. 15. Representative epifluorescence photomicrographs of two rabbit embryos stained with CCCP (merged images).



No regions of high MMP are visible.

2.1.4 Discussion and conclusion

The aim of our study was to adapt a JC-1 staining method to rabbit embryos. Current JC-1 staining protocols for embryos and oocytes [344,347,349,355,359–361] do not allow a JC-1 penetration through the embryo (*non-published data*). We believe this is the reason why no studies on rabbit embryo JC-1 staining had been published yet.

In contrast to other mammalian species, rabbit morula contains a mucoprotein layer (mucin coat) surrounding the ZP, that derives from the tubal secretions [392]. In the absence of pretreatment with pronase, the mucin coat prevents JC-1 dye penetration. Pronase treatment does not impair embryo developmental capacity of pig embryos [393]. Incubation time with JC-1 dye (75 min) was considerably longer than in mice and human embryos (25 - 30 min) [347,359]. These two particularities should be taken into account when validating this protocol in the presence of different laboratory conditions. Observation of stained embryos using confocal microscopy confirms appropriate penetration of the dye throughout the embryo.

In our study, frozen damaged embryos presented with lower MMP than frozen non-damaged embryos, which tends to confirm that damaged embryos have less active mitochondria. In contrast, Acton et *al.* studied JC-1 activity in fragmented 8-cell human embryos according to the degree of fragmentation, and observed a trend towards increased MMP with increased levels of fragmentation [347]. These findings can be explained by a degradation of low polarized mitochondria, due to apoptosis, especially because these embryos were stained in a later stage of fragmentation (stained 4 to 10 days after retrieval) [347]. We hypothesize that since embryos were stained earlier (1.5 days after thawing) in our study, inactive mitochondria from fragmented embryos did not have time to degrade.

Non-damaged fresh embryos presented higher MMP than non-damaged cryopreserved embryos, indicating that the freezing process tends to impair mitochondrial activity, even in the absence of morphological alterations. Zhao et *al.* analysed the effect of vitrification on mouse embryo MMP and observed fresh embryos presented with significantly more JC-1 aggregates than vitrified embryos [349].We cannot exclude the possibility of interference between the cryopreservation process and the dye uptake. However, since JC-1 dye has a double emission spectrum, we hypothesize that this interference would have an equivalent impact on both spectra, maintaining an equivalent red to green ratio. Nevertheless, in regards to interference, JC-1 remains, in our point of view, more interesting than other monocolour dyes, since their intensity would be affected in only one direction, in constrast to JC-1.

Further investigations could be carried out to evaluate whether there is a difference in high MMP mitochondria localization between fresh and cryopreserved embryos.

Acton et *al.* observed differences in JC-1 red/green ratio between 2-cell mouse embryos derived from *in vitro* fertilization and *in vivo* fertilization, as well as between embryos cultured in different culture media [347]. These results suggest that the comparison of embryos derived from different production conditions or different environments (such as culture medium) should be made with caution. Indeed, these conditions may affect embryo gene expression and regulation [394–396], including perturbations in mtDNA regulation [397]. In the same study, the observation of an augmentation of the J-aggregate to J-monomer ratio, from the 4-cell stage to the blastocyst stage, may indicate that embryo development requires an increase in MMP and even a recruitment of some quiescent mitochondria [347]. These interesting observations indicate that mitochondrial activity comparisons should be made between embryos at the same developmental stage. It has been observed that lower MMP 1- to 2-cell mice embryos tended to be arrested after the 2-cell stage and presented with inferior developmental competence than higher MMP embryos [344,360]. These results confirm the usefulness of MMP analysis to assess embryo developmental competence.

The development of a reliable method for rabbit embryo mitochondrial activity assessment allows the improvement of *in vitro* rabbit embryo evaluation studies. It allows a better assessment of embryo health, for better prediction of the developmental and implantation ability. This technique can be a valuable tool to evaluate embryos *in vitro*, especially as a complement to morphological evaluation or other methods. This method can be used to compare morphologically identical embryos or embryos derived from different cryopreservation processes.

Our results show that a pretreatment with pronase allows a good JC-1 penetration through rabbit embryo. The proposed protocol allowed a good staining on fresh and frozen embryos, and detects MMP alterations.

Morphological defects of cryopreserved embryos are associated with MMP disruption, and cryopreservation seems to impair mitochondrial metabolism, even in the absence of morphological alterations of the embryo.

This study was the first to describe a JC-1 staining protocol for rabbit embryo evaluation, suggesting that it can be used as an indicator of embryo health and functionality for this species.

3. Ice nucleation agents

Whether one's goal is to simplify and better standardize embryo slow freezing in programmable freezers or to simulate manual seeding in equipment where access to the samples is not possible, an alternative to slow freezing is missing.

The aim of this third study was to validate the use of Snomax® as a manual seeding substitute in embryo slow freezing by evaluating the physiological characteristics of the slow freezing medium and the impact of this ice nucleating agent on the development of fresh and frozen-thawed embryos.

3.1 Evaluation of the use of Snomax® as "Manual seeding substitute" in mouse embryo slow freezing

The Ethical and Animal Welfare Committee of VetAgro Sup approved this study (agreement number 1307). All animals were handled according to the EU Directive 2010/63/EU for animal experiment guidelines.

3.1.1 Embryo production and recovery

A total of 42 outbred RjOrl: Swiss mature mice were used in this study: 32 females and 10 males. Females were housed in groups of up to 6 and males were housed individually, under a controlled 14 h light / 10 h dark photoperiod and fed with a commercial diet. Female mice were submitted to a superovulation treatment by administrating 10 IU of pregnant mare's serum gonadotrophin (PMSG, Chronogest PMSG®, MSD Animal Health, France) and, 48 h later, 10 IU of human chorionic gonadotrophin (hCG, Chorulon®, MSD Animal Health, France) subcutaneously. Four hours after the last administration, each female was placed with one male and mating was confirmed by the presence of a vaginal plug the next morning. After euthanazia by cervical dislocation, the oviducts and uteri of mated females were flushed (room temperature) 68 to 72 h after the hCG administration. The flushing medium was composed of Dulbecco's Phosphate-Buffered Saline medium (DPBS) supplemented with D-glucose, sodium pyruvate and bovine serum albumin (BSA). Embryos (8-cell to early compacting morula stages) were classified according to the International Embryo Transfer Society manual [42]. Fair and good quality embryos were pooled and randomly divided into different study groups.

3.1.2 Embryo cryopreservation media composition

Two slow freezing media were used. The slow freezing medium of the "manual seeding group" and of the "no-seeding group" contained 1.5 M DMSO in IMV Embryo holding medium® and the slow freezing medium of the "Snomax group" contained 1.5 M DMSO in IMV Embryo holding medium® supplemented with Snomax® (10 mg/L). Snomax® concentration was selected after performing studies with thermocouples, where the authors observed a reproducible T_c of approximately -7 °C (table III). Other studies in literature using Snomax® as oocyte nucleation inductor describe the same Snomax® concentration [48,49,105,371].

Table III. Temperature of crystallization (T_c) of freezing medium without (BM) and with Snomax® (BM + Snomax®).

	T_c (°C)
BM	-14.79 ±2.83
BM + Snomax®	-6.89 ±0.25

Basic medium (BM) was 1.5 M DMSO in IMV Embryo holding medium®. Snomax was used at the final concentration of 10 mg/L (n = 4 experiments).

3.1.3 Evaluation of osmolality, pH and Snomax's deleterious effect

Evaluation of osmolality and pH

The osmolality and pH of the two cryopreservation media were measured at room temperature, using a Vapro 5600 vapour pressure osmometer (Logan, USA) and a Mettler Toledo FE20 pH Meter (Schwerzenbach, Switzerland), respectively.

Snomax's effect on development

Snomax's effects on development were evaluated by assessing the embryo development potential after incubating fresh embryos in a solution containing Snomax®. In this experiment, none of the groups contained CPAs.

Briefly, a total of 52 embryos were divided into 3 groups and incubated in the corresponding incubation medium for 45 min. Two control groups were incubated: one in the "culture medium" composed of M16 (37 °C, 5 % CO_2), and one in a "freezing based medium" composed of IMV Embryo holding medium® (37 °C, atmospheric air). The third

group was incubated in a "Snomax medium" (37 °C, atmospheric air) composed of IMV Embryo holding medium® supplemented with Snomax® (10 mg/L). After incubation, the embryos were washed in culture medium and cultured to the hatching stage.

Embryo culture and morphology assessment

Embryos were cultured (37 °C, 5 % CO_2) in M16 medium supplemented with antibiotics (penicillin 67 µg/mL and streptomycin 67 UI/mL), under paraffin oil (Ovoil, Vitrolife, Göteborg, Sweden).

In vitro development was assessed at day 1, 2 and 3 of culture. Delayed embryos and embryos classified as poor or degenerating embryos were eliminated.

3.1.4 Slow freezing and thawing protocols and embryo culture

Embryo cryopreservation

Embryos (n = 312) were collected in four sessions and divided into four groups. One fresh control group, cultured without cryopreservation, and three slow freezing groups: one manual seeding group, one group without any seeding (no manual seeding and no Snomax®) and one Snomax® group.

Embryos from the three freezing groups were transferred in one step to the freezing solution (room temperature, 10 min). They were then loaded into 0.25 mL straws, between two drops of freezing medium separated by air bubbles, and placed in a programmable freezer (Freezal, Cryopal, Bussy-Saint-Georges, France). The straws were cooled from room temperature to –7 °C at a rate of -2 °C/min, then held for 7 min at – 7 °C before manual seeding by application of a cold spot (a swab cooled in LN_2) on the first column of the straws (without embryos). Then, the straws were held for 10 min at – 7 °C, at the end of the holding time the programmable freezer was quickly opened to confirm if the straws contained ice). The straws were then cooled to -36 °C at a rate of - 0.3 °C/min. Finally, they were directly plunged into LN_2 before storage.

To submit both groups to similar freezing conditions, the three groups were frozen in the same freezer at the same time. During the manual seeding step, the cover of the freezer was opened and the cold spot was applied only to the manual seeding group.

Warming was performed by holding the straws in air (40 s) before plunging them into a 30 °C water bath (40 s) [398]. The frozen-thawed embryos were incubated (room temperature) for five minutes in three successive baths to remove DMSO (1.0 M, 0.5 M and 0.0 M DMSO,

respectively, in IMV Embryo holding medium®; supplemented with 0.2 M sucrose). Embryos were then cultured to the hatching stage. The survival rate after cryopreservation (non-damaged embryos / per frozen embryo), blastocyst formation rate and hatching rate (per frozen embryo) were evaluated.

3.1.5 Ratiometric semiquantitative assessment of mitochondrial activity

Ratiometric semiquantitative assessment of mitochondrial activity:

The cationic dye JC-1 accumulates within mitochondria according to their mitochondrial membrane potentials (MMP), exhibiting different fluorescent properties, based on its accumulation. High MMP mitochondria accumulate more cationic dye (J-aggregates) and exhibit red fluorescence, while low MMP mitochondria accumulate J monomers, showing green fluorescence [358]. Therefore, embryos with higher MMP present a higher red to green ratio than less active embryos.

Living embryos at the hatching stage from both freezing groups were incubated with JC-1 for 30 min (3 µM, 37 °C, 5 % CO_2). Embryos were observed under an Olympus IX71 epifluorescence microscope with an excitation wavelength of λ = 488 nm. JC-1 aggregates were detected with a red filter (λ = 590 nm wavelength) whereas JC-1 monomers were detected with a green filter (λ = 530 nm wavelength). The JC-1 loading protocol was previously validated in our laboratory, with different stock concentrations of JC-1 and dye loading times.

To evaluate the mitochondrial activity, the staining intensity (number of pixels) was determined in two randomly defined areas on each embryo, from both channels, and the red to green ratio was measured using the Fiji package in the ImageJ software [390].

3.1.6 Statistical analysis

Embryo survival rate, blastocyst formation rate and embryo hatching rate were analysed with the chi-square test, whereas JC-1 red/green ratios were analysed by one-way analysis of variance. All tests were performed with R-Studio software [391]. Groups were considered significantly different at $p < 0.05$.

3.1.7 Results: assessment of survival rate, *in vitro* development rate and mitochondrial activity

Influence of Snomax® on osmolality and pH

The osmolality and pH of both groups' cryopreservation media are presented in Table IV. Adding Snomax® to the freezing medium did not modify the solution's osmolality or pH.

Table IV. pH and osmolality of cryopreservation media.

Seeding	Medium	pH	Osmolality (mmol/kg)
Manual	BM	7.25	1902
Nucleating agent	BM + Snomax®	7.20	1936

BM: basic medium is 1.5 M DMSO in IMV Embryo holding medium®.
Snomax®: 10 mg/L

Snomax® deleterious effect

The blastocyst formation and hatching rates of the two control groups and of the group exposed to Snomax® are summarized in Table V. No difference was observed between the three groups regarding *in vitro* development parameters.

Table V. *In vitro* development rates after 45 min incubation of fresh embryos.

Incubation medium	**% Blastocyst**	**% Hatching**
M16	100.0 % (16/16)	93.8 % (15/16)
IMV Embryo holding medium®	100.0 % (16/16)	87.5 % (14/16)
IMV Embryo holding medium® + Snomax®	100.0 % (20/20)	88.9 % (16/18)

Snomax®: 10mg/L

In vitro development after cryopreservation

During the freezing protocol, at the end of the holding time, all the conventional seeding and all the Snomax® straws contained ice. The non-seeding straws didn't contain ice. The *in vitro* development rates after freezing are summarized in Table VI. No difference was observed between the conventional seeding group and the Snomax® group regarding the survival rate, the blastocyst rate or the hatching rate after *in vitro* culture. The blastocyst formation rates and hatching rates were significantly higher in the control group compared to both frozen groups. All the embryos from the no-seeding group were morphologically damaged, presenting small mass, debris, irregular shape and thin or broken ZP.

Table VI. *In vitro* development rates after embryo cryopreservation.

Group	**% Survival**	**% Blastocyst**	**% Hatching**
Fresh embryos (Control)	-	100.0 % (30/30) [a]	93.3 % (28/30) [a]
Non-seeding group *	0.0 % (0/40) ** [a]	0.0 % (0/40) [b]	0.0 % (0/40) [b]
Conventional * (Manual seeding)	93.3 % (112/120) [b]	76.7 % (92/120) [c]	56.7 % (68/120) [c]
Snomax® * (Nucleating agent)	97.5 % (119/122) [b]	75.4 % (92/122) [c]	59.8 % (73/122) [c]

***** Conventional and No-seeding group: 1.5 M DMSO in IMV Embryo holding medium®; Snomax: Snomax® (10 mg/L) +1.5 M DMSO in IMV Embryo holding medium®.

[a, b, c] Different letters indicate significant differences ($p < 0.05$), for each column.

% Survival: the percentage of morphologically intact recovered embryos after freezing.

****** 37 damaged embryos found

Assessment of mitochondrial activity with JC-1 staining

The mitochondrial activity ratios of hatching blastocysts from both freezing groups are illustrated in Fig. 16a and summarized in Fig. 16b. No significant difference regarding the red to green ratio was observed between embryos frozen using manual seeding and embryos frozen with the ice nucleating agent Snomax®.

Fig 16. Mitochondrial activity assessment of embryos seeded by conventional or nucleating agent.

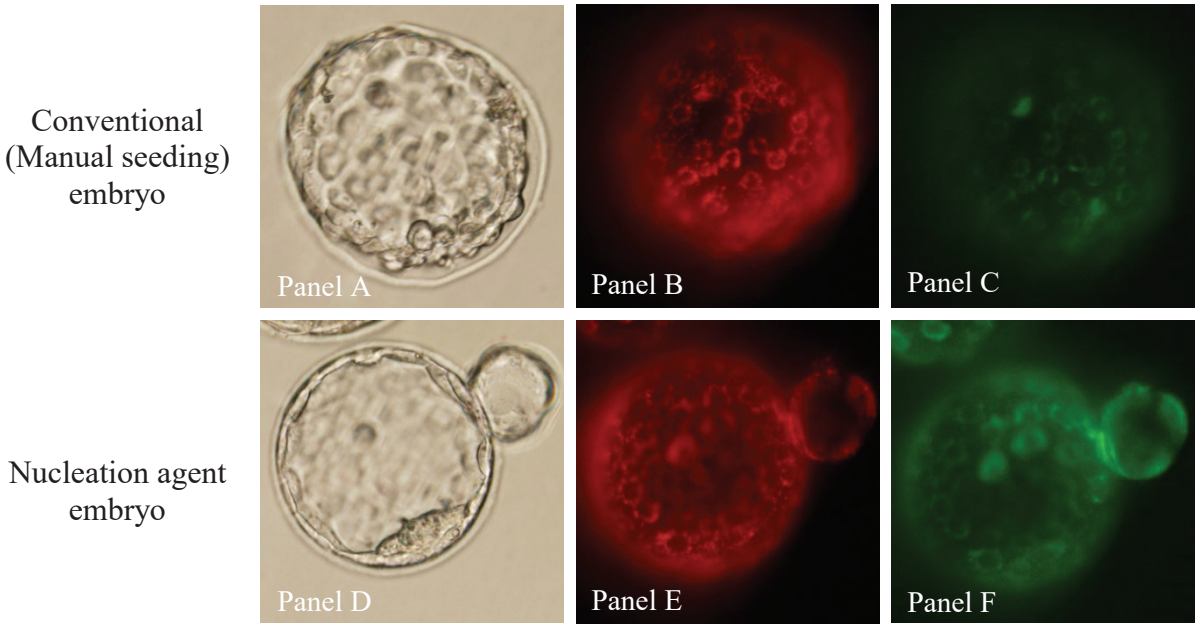


Fig 16a. JC-1 mitochondrial staining of frozen thawed embryos after IVC. Light microscopy images (Panels A and D). JC-1 fluorescence in the red channel images: J monomers (Panels B and E). JC-1 fluorescence in the green channel images: J-aggregates (Panels C and F).

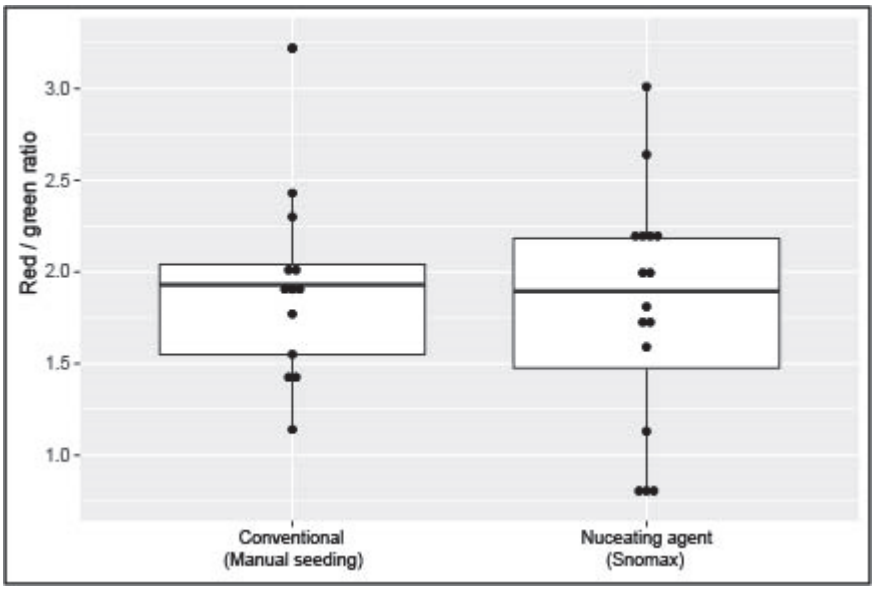


Fig 16b. Mitochondrial activity assessment with JC-1 staining (Mean ± SD).

3.1.8 Discussion and conclusion

In recent decades, several parameters have been studied to improve embryo slow freezing protocols: CPAs composition [399,400], cooling rates, holding time and plunging temperature [105,401]. The substitution of animal products in freezing media was investigated as well, since their composition is variable and not completely known [402].Despite numerous advances in this field, there are few studies on the substitution of manual seeding in embryo slow freezing protocols. In the 1980s, silver iodide alginate-gel droplets were used satisfactorily to induce crystallization during rabbit and bovine slow-freezing [97,98]. In 2005, Snomax® was used to induce nucleation in media containing mouse embryos and oocytes in cryomicroscopy experiments [6,17]. Nevertheless, to our knowledge, its influence on the embryo's post-freezing survival and functionality have not previously been reported.

Our study demonstrated that adding Snomax® to the freezing medium did not modify the solution's pH or osmolality to non-physiological values.

The exposure of fresh embryos to Snomax® did not alter their *in vitro* development rates. Moreover, our development rates were equivalent to other development rates found in the literature [25,403–405]. Subsequently, the addition of Snomax® to the embryo medium did not seem to have a negative impact on embryo cryopreservation nor embryo survival after cryopreservation. The development rates obtained after thawing were equivalent to other studies found in the literature [25,114,400,406–408]. However, some studies with 8-cell frozen embryos obtained superior post-thaw development rates [409,410]. This may be due to the mice strain [410,411], or to the use of a culture medium better adapted to 8-cell to morula stages [409,412].

In this study, we observed that the absence of seeding didn't allow post-thaw survival or development. Indeed, it demonstrates that controlled induction of ice crystallization is essential to embryo survival. Whittingham demonstrated when ice nucleation occurs under -7 °C, survival rates drop drastically [408].

In vitro development rates after cryopreservation indicated that Snomax® did not have deleterious effects on embryo development. JC-1 ratios showed that mitochondrial activity was equivalent between both freezing groups' embryos. These results indicate that the addition of Snomax® for cryopreservation does not alter embryo morphology, embryo development, or embryo metabolism.

This preliminary study indicates Snomax® seems to be a valuable alternative to the manual seeding step during embryo cryopreservation, in physical studies. It can be used as a nucleation agent in experiments with a programmable freezer, if a better standardization is

required, or if no experimented manipulator can perform the manual seeding. However, since Snomax® has a bacteriological origin, it should be used with caution, especially if *in vivo* transfers are planned. Studies on the biological safety of Snomax® should be conducted and *in vivo* transfers should be made.

In thermodynamic experiments involving DSC or cryomicroscopy, Snomax® can be a valuable tool to induce manual seeding. Since the nucleation temperature can be efficiently controlled by the Snomax® concentration [104], it can be used as a nucleation agent in cryopreservation protocols with different seeding temperatures, or even with different cellular types.

Even if the addition of Snomax® to the freezing media does not seem to affect embryos during the cryopreservation process, further studies could be conducted to investigate the influence of Snomax® on the ice crystal growth, on the ice crystal organization and even on the ice front displacement. Supplementary researches, with a larger number of embryos, and with *in vitro* produced embryos, to increase sensivity to cryopreservation, would also be of value.

To the best of the authors' knowledge, this is the first study on the influence of Snomax® on embryo quality during cryopreservation.

The use of Snomax® as an ice nucleating agent is an effective method to control the induction of ice nucleation during mouse embryo slow freezing, with no effect on subsequent *in vitro* development of embryos or their mitochondrial activity. This study validates, therefore, the use of Snomax® on embryo slow freezing physical studies, as manual seeding substitute.

In a programmable freezer, the use of Snomax® allows parallel freezing of as much embryo as the freezer can allow in a single run, under identical conditions. The presence of the manipulator is no longer required during this step. In addition, the uncontrolled increase of the temperature inside the programmable freezer, due to opening the freezer door, is avoided, improving the procedure standardization. In equipment where access to the sample is not possible, Snomax® can be efficiently used as an alternative to the manual seeding step. These results will help the development of strategies to simulate nucleation in cryobiological experiments.

A scientifical article, entitled “Ice nucleating agents allow embryo freezing without manual seeding”, was published in the journal “Theriogenology”, based on this experiment (DOI:10.1016/j.theriogenology.2017.08.012).

4. Ewe superovulation

4.1 State of the art

Superovulation and embryo collection represent the most efficient techniques to cryopreserve or disseminate high genetic value ovine embryos.

In these species, ovulation rate and litter size may depend on intrinsic factors, such as genotype [413,414], and on environmental factors [415].Most ovine breeds ovulate between one and two oocytes at a time, but some prolific breeds, such as Boorola Merino [416], Dahman [417], Finnish Landrace [418], Romanov [419] and French Grivette [420], may physiologically ovulate three or four oocytes per estrous cycle. Diverse genes were identified as having a major impact on ovulation rate [413], such as *BMPR1b* – (known as Booroola gene), *BMP15* and *GDF9* [421] or as having a minor impact on ovulation rate [414]. French Grivette sheep carry a mutation in the *FecXGr* allele of *BMP15* gene [420], which explains their high prolificacy. In physiological conditions, litter size in homozygous carriers was reported as averaging 2.5 lambs, and in heterozygotes as averaging 1.9 lambs [420]. A reason that can explain greatest prolificacy in ewes with *BMP-15* mutation (such as Grivette ewes) is that granulosa cells of antral follicles exhibit lower sensitivity to FSH. Consequently, when FSH concentration decreases, smaller antral follicles can escape atresia and continue development [422]. It was also hypothesized that granulosa cells in heterozygous ewes with mutations in *BMP15* may develop an earlier sensitivity to LH, increasing ovulation rate in these ewes [423].

Environmental factors that influence sheep ovulation include nutrition [424,425], season and photoperiod (being autumn the most favorable period for reproduction) [426], social factors such as the “ram effect” [427], age and parity [428] and superovulation hormonal treatments [429,430]. Superovulation can increase ovulation rate from to 19.5 oocytes in particularly prolific breeds such as Romanov [431].

Follicular waves and dominance

Antral follicular growth development in sheep occurs in a wave-like pattern, with a predominance of two to three follicular waves per estrous cycle [432–434], whose length is 15 to 17 days [433]. Briefly, a transient increase in FSH concentration precedes the recruitment of a cohort of follicles (2 to 3 mm in diameter) [433], which are FSH-dependents. Approximately one (in non-prolific breeds) to three (in prolific breeds) follicles are selected to become dominant and to develop further (≥ 5 mm). The functionally dominant follicles

produce high levels of estradiol [432,435] and inhibin, which appears to cause a decrease in FSH concentrations [436,437]. Additionally, these follicles acquire LH receptors, being capable of shifting their dependency from FSH to LH [438], whilst smaller follicles (2 to 4 mm) undergo atresia [433,437]. In animals exhibiting estrus cycles, the increase of estradiol induces a LH surge, followed by maturation of the dominant follicles and ovulation [439]. If a *corpus luteum* from a precedent cycle is present (luteal phase), its progesterone production prevents LH secretion, and ovulation doesn't occur. In this situation, dominant follicles will undergo atresia [440], giving rise to a new FSH rise and to a new follicular wave. Although the mechanisms of dominance in sheep are not fully elucidated, they seem to be attenuated during luteal phase [438,440].

Estrous control

Traditional superovulation protocols in sheep include synchronization of estrus with a progestogen-releasing intra vaginal device for 11 - 14 days [433,441,442]. The exogenous administration of progestogen prevents ovulation, mimicking the effect of the progesterone produced by the *corpus luteum* (CL) [442]. It is followed by six to eight injections of decreasing concentrations of a commercial pFSH, beginning 48 – 60h before device withdrawal; to allow development of smaller follicles that would normally go through atresia [443,444]. The administration of a GnRH analogue or of PMSG, in the second half of the FSH administration protocol, is often realized, to stimulate a LH pic. However, the administration of PMSG seems to induce anti-PMSG antibody production, which will have a detrimental effect on response after the next treatments with PMSG [445].

In 2009, Menchaca observed that the administration of buserelin acetate (a GnRH analogue) 24 hours after the removal of progestogen sponges improved the ovulation rate and the number of transferable embryo [430].

In the ovine species, as in mammals in general, a variable response to superovulation treatments is observed, concerning the number of ewes that superovulate, with 20 to 30 % of ewes not responding to treatment, and concerning the number of ovulated oocytes and of viable oocytes [443]. Several factors have been associated with this important variability, such as the ovarian follicular status of the donors, or the preparations used in the protocol [443]. For instance, some commercial FSH preparations may present inconstant FSH : LH ratios, which may affect ovarian response [446], with higher LH activities being responsible for reduced ovulation rates [431].

The lack of response to superovulation treatment may be related to the absence of a LH surge [447]. Based on the physiological variations of FSH and LH preceding ovulation, Cognié suggested that the administration of LH at the end of the FSH treatment could enhance ovulation rate and the number of recovered embryos [429]. However, the use of commercial FSH : LH mixtures (such as Folltropin® or Stimufol®) limits the opportunity to modulate the FSH : LH ratio.

The objective of this experiment was to to compare two superovulation protocols on ewes: one based on a constant FSH : LH ratio followed by the administration of a GnRH analogue and the other one using a LH supplementation at the end of the FSH treatment.

Stimufol® (Reprobiol, Ouffet, Belgium) is a commercial mixture of FSH and LH (ratio 5 : 1). Non-published reports from fieldwork seem to have satisfactory results using a protocol that comprises decreasing doses of this FSH : LH mixture, with a final administration of higher dose of Stimufol®. We hypothesize that this last administration would provide the necessary concentration of LH, before ovulation, and avoid GnRH administration.

4.2 Comparison of superovulation protocols

4.2.1 Superovulation and embryo collection protocols

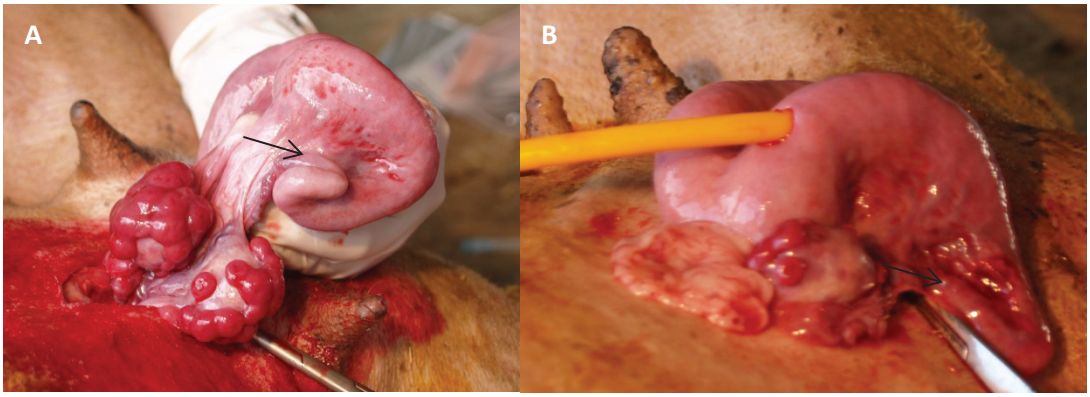
For this experiment, two ovine breeds (Grivette and Blanche du Massif Central (BMC)) have been used. Different superovulation protocols were initially tested, and two protocols were retained for this experiment.

Grivette multiparous ewes (*n* = 50), aged between 3 and 9 years, were synchronized with an intra-vaginal sponge impregnated with flugestone acetate (Chronogest CR®, MSD Animal Health, France) over 14 days. In group 1 (*n* =25), ewes received a twice-daily administration of 6 decreasing doses (50 µg, 50 µg, 30 µg, 30 µg, 20 µg, 20 µg) of a FSH : LH (ratio 5 : 1) preparation, (Stimufol®), starting on day 12, followed by an injection of 7.6 µg buserelin acetate (Receptal®, MSD Animal Health, France) 24 hours after the sponge removal. In group 2 (n=25), the treatment consisted of twice-daily administration of 6 decreasing doses (5 UA, 5 UA, 3 UA, 3 UA, 2 UA, 2 UA) of purified pFSH (Reprobiol, Belgique), starting on day 12, followed by two increasing doses of purified LH (Reprobiol, Belgique) given on day 14. Responding ewes were naturally mated by fertile rams (each female was in contact with several rams) and 7 days later ewes were subjected to a laparotomy. Briefly, the flushing media - Euroflush® (IMV Technologies, L'Aigle, France) was injected via a Tomcat urethral catheter inserted at the oviduct and recovered with a Foley catheter that had been inserted next to the bifurcation of each uterine horn.

CL on both ovaries were identified, oviducts and uterine horns were flushed and recovered embryos were counted and evaluated at the morula and blastocyst stages.

A total of 11 multiparous Blanche du Massif Central ewes (group 1: *n* = 6; group 2: *n* = 5), aged between 3 and 6 years, were subjected to the same superovulation protocol, and inseminated by cervical insemination with sperm from multiple males (pooled ejaculates). Ewes were then subjected to a laparotomy (technique described above), CL were evaluated, oviducts and uterine horns were flushed and recovered embryos were counted and evaluated at the morula and blastocyst stages.

Fig. 17. Anatomical points of insertion of both catheters, during embryo flush.



Panels A and B)Insertion point of the Tomcat urethral catheter (arrows).
Panel B) Insertion of the Folley catheter.

4.2.2 Statistical analysis

The rate of responding ewes to superovulation protocols was analysed with χ^2. The mean number of CL, recovered oocytes and embryos and good quality embryos per treated ewe between the two superovulation protocoles were analysed with Mann-Whitney U test. A comparison between the two breeds was not done.

4.2.3 Results: ovulation and embryo quality evaluation

Results concerning the rate of responding ewes to superovulation, the mean number of CL, recovered oocytes and embryos and good quality embryos per treated ewe for each superovulation protocol are presented in Table VII.

No significant difference was observed between the two superovulation protocols, for each ewe breed.

Table VII. Response to two superovulation protocols in Grivette and BMC ewe breeds.

Grivette	**Donors with *CL* / total donors (%)**	**No. *CL** (total)**	**No. ova and embryos* (total)**	**Collection rate %** (total)**	**reezable embryos (q1 + q2) responding donors (total)**	**Q3 embryos %*** (total)**	**Oocytes %*** (total)**
Group 1	20 / 25 (80.0 %)	12.15 ± 6.27 (243)	6.80 ± 4.94 (136)	56.0 %	3.55 ± 4.07 (71)	9.6 % (13)	37.5 % (51)
Group 2	22 / 25 (88.0 %)	14.86 ± 10.36 (327)	6.50 ± 5.75 (143)	43.7 %	3.23 ± 3.34 (71)	12.6 % (18)	44.1 % (63)
Blanche du Massif Central	**Donors with *CL* / total donors (%)**	**No. *CL** (total)**	**No. ova and embryos* (total)**	**Collection rate %** (total)**	**Freezable embryos (q1 + q2)/ r / responding donorsespondi ng donors/ re (total)**	**Q3 embryos %*** (total)**	**Oocytes %*** (total)**
Group 1	6 / 6 (100.0 %)	8.5 ± 3.78 (51)	5.83 ± 3.76 (35)	68.6 %	4.50 ± 4.18 (27)	20.0 % (7)	2.9 % (1)
Group 2	5 / 5 (100.0 %)	13.40 ± 12.10 (67)	7.40 ± 1.82 (37)	55.2 %	4.40 ± 3.21 (22)	8.1 % (3)	27.0 % (10)

Unless indicated otherwise, data are the mean ± s.e.m.
* per responding donor
**No. of collected ova and embryos per *CL*
*** per collected
Group 1: control, intra-vaginal sponge impregnated with flugestone acetate + decreasing doses of commercial FSH : LH mixture + GnRH analogue
Group 2: intra-vaginal sponge impregnated with flugestone acetate + decreasing doses of pFSH + LH

4.2.4 Discussion and conclusion

Several distinct ovine superovulation protocols exist in the literature. Most of these protocols are complex and include an important number of administrations (sponge application / withdrawal, different molecules injections), which can be impeditive and compromise the adequate realization of the protocol in farms, in large-scale programs.

An important variability is often observed in ewes superovulation protocols and compromises the success of this programs [437]. The variable number of ovulated oocytes and of viable embryos may be influenced by several factors. Vivanco observed that the breed factor seems to account for approximately 30 % of the variability in the embryo yields [448]. Differences in follicle growth, ovulation rate and fluctuations of FSH and LH between different breeds are responsible for distinct responses to exogenous gonadotrophins [449,450]. One copy of the Inverdale allele (FecXI) increases ovulation rate by 0.8 and enhance both the number and LH sensitivity of antral follicles in ovaries [451].

Based on this breed variability and as Grivette and BMC experiments were not performed at the same time, under the same conditions, we didn't pool (or compare) the results obtained between the two studied breeds.Superovulation treatment stimulates the growth of a higher number of follicles. Some of these follicles could be, at the time of stimulation, in the initial phase of atresia. Even though these follicles may escape atresia and continue their growth, this development can be inadequate, resulting in ovulation failure [444].

Another frequently found obstacle after ewe superovulation is a low number of viable oocytes. In literature, several anomalies were observed in oocytes of stimulated cattle, associated with an inconsistent number of viable oocytes: an oocyte maturation asynchrony between nucleus and cytoplasm, as well as atypical steroidogenic profiles of preovulatory follicles [452]. Premature activation of the oocytes or premature ovulations were also reported, and associated with the exogenous LH action [452,453].

Sperm survival and passage through the uterine cervix of superovulated ewes seems to be impaired [454], explaining why better fertilization rates are obtained after intrauterine insemination [455,456].

Due to logistic issues, in our protocol, intrauterine deposition of sperm wasn't possible. Consequently, the rate of fertilized embryos could also be influenced by the capacity of the sperm to survive and to go across the cervix and not only the hormonal protocol. In the same way, the manipulators experience seems to have an important impact on the collection

rate. The manipulation of the oviduct and the insertion of the catheter need delicate and practiced handling. The swelling of the Folley catheter in the uterus is also of major delicacy, since too much air would damage the uterine mucosa, with a leakage of flushing liquid, and an insufficient swelling would allow the flushing liquid to pass around the balloon, with an incomplete recovery of the flush. An important difference between the number of counted CL and the number of structures found in the flush confirms a low collection rate. It can be explained by inadequate flushing, if done by unexperienced manipulators, or by the existence of numerous unfertilized oocytes, especially if the sperm wasn't deposed in the uterus. Even if unfertilized oocytes are commonly found in the flush, they can degenerate and be eliminated by the female reproductive tract, before the flushing.

For these reasons, we estimate that the evaluation of the number of CL is possibly the most objective method to evaluate our superovulation protocols, over the collection rate or the freezable embryos rate.

In our experiment, no significant difference was observed between both protocols, suggesting that the administration of LH or GnRH analogue at the end of a superovulation protocol have similar effects on the ovulation rate and embryo yield.

Menchaca (2009) designed and evaluated a protocol to synchronize ovulation and initiate superovulation on Day 0 of the cycle at the time of emergence of the first follicular wave, to avoid the presence of a dominant follicle at the time of FSH injections (day 0 protocol). He observed a tendency to have better results with the day 0 protocol than with a classic protocol, an increase of approximately two embryos per treated ewe using the Day 0 protocol [430]. This protocol seems to be a good choice to improve embryo yield. However it requires more injections than usual protocols, which is less practical for breeders and increases the error possibility.

Oliveira evaluated the impact of adding LH at the end of the superovulation protocol in Santa Inês ewes, concluding that it didn't affect the ovulation rate or the embryo yield. Nevertheless, the LH treatment increased the frequency of ewes with an important superovulation response ($\geq$ 11 CL) [457]. Picazo didn't observe a positive effect of LH in other breeds of sheep (Manchega, Churra and Merino) [458]. However, both studies' authors used preparations containing a mixture of FSH and LH for the FSH injections [457,458].

We can observe, when comparing both species' ovulation rates, that Grivette obtained, roughly, similar ovulation rate (based on CL number) than BMC ewes, whereas in natural conditions Grivette ewes present a greater prolificacy. The lower sensibility of ewes with BMP-15 mutation (as Grivette ewes) to FSH hormone, explained by a down-regulation of

FSH-receptors [422], can possibly explain a lower proportional response to superovulation treatments based on FSH injections. Another possible explanation is the age of the ewes, as more old ewes were used in the Grivette group.

Overall, the ewes' response to the superovulation treatment was satisfactory in both protocols. Due to the important variability in response to superovulation treatments, a larger study with a greater sample and intrauterine sperm deposition is required to obtain a significant difference regarding the number of ovulations or of viable embryos.

We conclude both superovulation treatments can be used to produce ovine embryos *in vivo,* with a superovulation rate higher than 70 %.

II.II Main studies: Evaluation of a chemically defined substitute (CRYO3) for animal-based products for rabbit and ovine cryopreservation media

Two main studies were conducted and aimed to evaluate the substitution of animal products in rabbit and ovine embryo cryopreservation media, and constituted the biological approach of this work.

The first study evaluated the use of a chemically defined substitute (CRYO3) to replace BSA in rabbit embryo slow-freezing (*in vitro*) and vitrification (*in vitro* and *in vivo*) media. A mitochondrial activity evaluation complemented the *in vitro* embryo evaluation.

The second study evaluated the use of the same synthetic product (CRYO3), to replace BSA during ewe embryo slow-freezing (*in vitro*) and vitrification (*in vitro* and *in vivo*) media.

1. Rabbit embryo cryopreservation

The Ethical and Animal Welfare Committee of VetAgro Sup approved this study (Permit Number: 05/26). All animals were handled according to the EU Directive 2010/63/EU for 1.

Our objective was to evaluate the effect of replacing "IMV Embryo Holding Medium®" – a commercial medium containing animal derived products (0.4 % (*w/v*) BSA) with a chemically defined medium with no animal-derived products - CRYO3- in rabbit embryo slow-freezing and thawing media (Experiment 1.1) and in rabbit embryo vitrification and warming media (Experiment 1.2).

1.1 Rabbit embryo slow freezing (assessment of *in vitro* development)

1.1.1 Embryo production and recovery

A total of 20 multiparous rabbit New Zealand does (SARL HYCOLE, Marcoing, France), aged between 38 and 50 weeks, were housed in groups of five and fed with a commercial diet. Does received five doses of a pFSH : LH (ratio 5 : 1, 31.5 µg total, Stimufol, Reprobiol, Belgium) preparation (administered twice-daily, subcutaneously). Eight hours after the last injection, does were inseminated with sperm from multiple males (pooled ejaculates), and an intramuscular injection of buserelin (2.0 µg Receptal®) was administered.

Rabbit does were euthanized 65 to 68 h after the buserelin administration by cervical dislocation. The oviducts and uteri were flushed using Euroflush® medium at room temperature. Embryos were recovered at the morula stage and classified according to the International Embryo Transfer Society (IETS) manual, and quality 1 embryos [42] were pooled. Embryos (*n* = 549) were randomly divided into three slow freezing groups (SF1, SF2, SF3).

1.1.2 Embryo slow freezing and thawing

Embryos from the slow freezing group (*n* =549) were randomly divided into three groups. All the media contained the same CPAs composition, and the following base media:
SF1: IMV Embryo holding medium® (containing 0.4 % (*w/v*) BSA) (*n* = 186);
SF2: D-PBS supplemented with 20 % of CRYO3 (*n* = 188);
SF3:CRYO3 medium (*n* = 175).

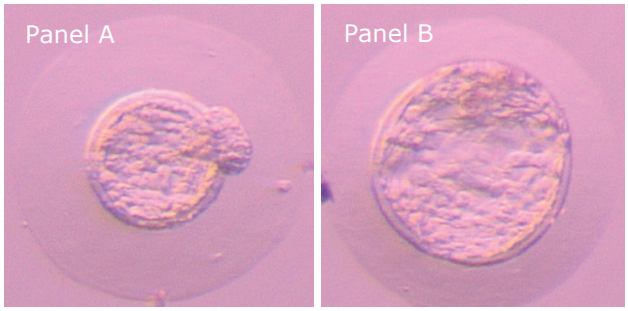
Embryos were transferred into a freezing solution composed of 1.5 M DMSO in SF1 / SF2 / SF3 medium at room temperature for 10 min. Embryos were subsequently loaded into 0.25 mL straws between two drops of freezing medium separated by air bubbles and placed in a programmable freezer (Cryocell 1200). The straws were cooled from room temperature to -7 °C at a rate of 5 °C/min, seeding was then manually induced by application of a cold spot on the straws. The straws were held for 5 min at –7 °C and cooled at a rate of 0.5 °C/min to -30 °C. Finally, the straws were plunged and stored in LN_2.

Thawing was performed by holding the straws in air (15 s) before plunging them into a water bath (20 °C, 60 s). The frozen-thawed embryos were incubated in three successive baths in order to remove DMSO (1.0 M, 0.5 M and 0.0 M DMSO respectively, in SF1 / SF2 / SF3 medium).

1.1.3 *In vitro* embryo culture and morphology assessment

Embryos (n = 549) were cultured (38.5 °C, 5 % CO_2) to the expanded blastocyst stage in M199 medium (without glutamine) supplemented with 10 % foetal calf serum and antibiotics (67 UI/mL penicillin and 67 µg/mL streptomycin). *In vitro* development was assessed after 24 h and 48 h of culture and classified according to their development stage as morula, blastocyst, expanded and hatching embryos. Slightly expanded blastocysts with herniation of embryonic cells (Fig. 18) were included in the expanded / hatching embryo group.

Fig. 18. Stereoscopic pictures of rabbit embryos.



Panel A) A slightly expanded blastocyst with embryonic cell herniation.
Panel B) An expanded blastocyst.

1.1.4 Mitochondrial activity assessment with JC-1

At the end of embryo culture, living embryos (n = 93) at the expanded / hatching stage from the three slow freezing groups were subjected to a pretreatment of pronase (a protease,

from *Streptomyces griseus*, 5 mg/mL) in Dulbecco's Phosphate-Buffered Saline medium (D-PBS) supplemented with D-glucose (5.56 mM), sodium pyruvate (0.33 mM) and bovine serum albumin (3 mg/mL), at 38.5 °C, until the mucin coat began to dissolve. Embryos were then washed in six drops of modified D-PBS. Embryos were incubated with JC-1 for 75 min (1.5 µM, 38.5 °C, 5 % CO_2) and observed using an Olympus IX71 epifluorescence microscope, with an excitation wavelength of 488 nm. JC-1 aggregates were detected with a red filter (590 nm wavelength), whereas JC-1 monomers were detected with a green filter (530 nm wavelength). To evaluate embryo mitochondrial activity, the staining intensity (by pixel) was measured, from both channels, in two randomly defined areas on each embryo, using the Fiji package [390] of ImageJ software (National Institute of Health, Bethesda, Maryland, USA), and the red to green ratio was quantified.

An MMP disruptor (CCCP) was used as a control to confirm that directional changes in the dye signal were correctly interpreted.

1.1.5 Statistical analysis

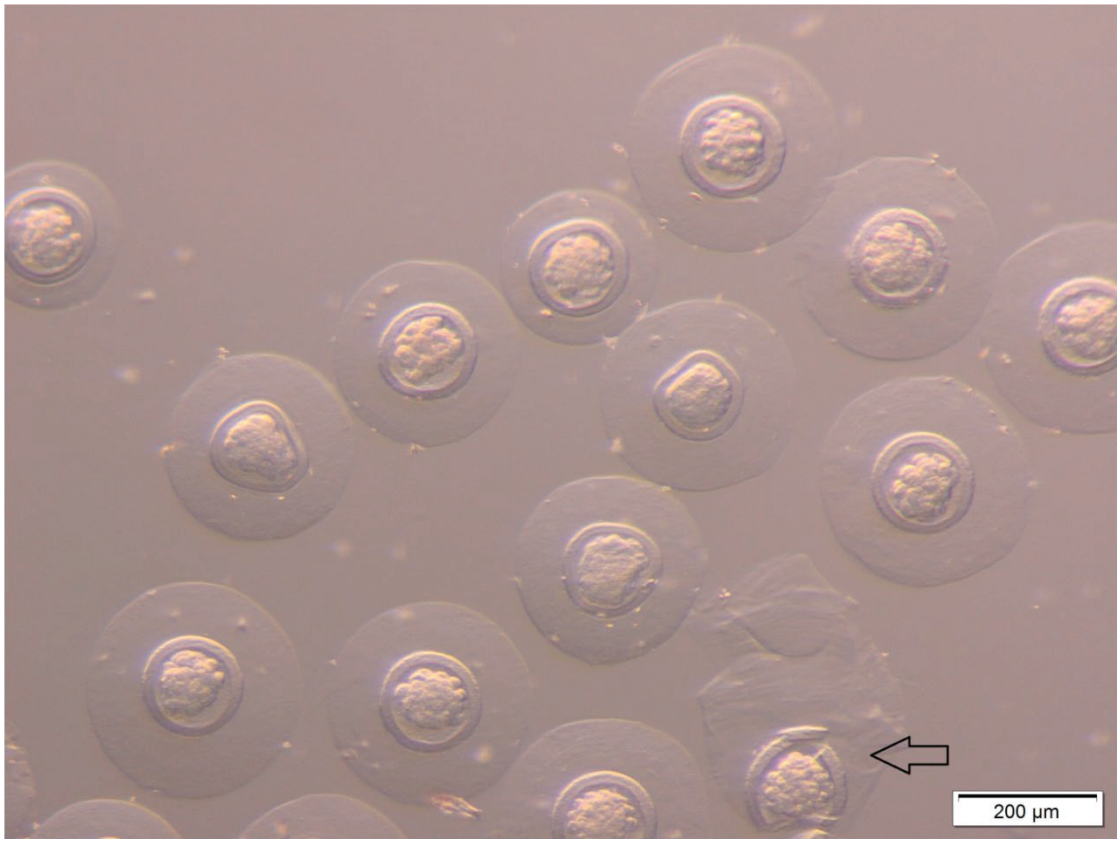
In vitro and *in vivo* development rates were analysed with the chi-square test, whereas JC-1 red/green ratios were analysed by one-way analysis of variance. All tests were performed with R-Studio software [391]. Groups were considered significantly different at $p < 0.05$.

1.1.6 Results: assessment of *in vitro* development rate and mitochondrial activity

In vitro development after slow freezing

Morphological aspect of embryos during thawing is represented on Fig. 19. The *in vitro* blastocyst formation and expansion/hatching rates after slow freezing with media containing animal products (SF1) or chemically defined products (SF2 and SF3) are summarized in Table VIII. No statistical difference was found regarding survival rate. The SF3 group appeared significantly superior in *in vitro* blastocyst formation rate than SF1 and SF2, and in expansion or hatching rate than SF1.

Fig. 19. Slow frozen embryos (SF3) during thawing, on the last bath.



Note the presence of a morphologically damaged embryo's ZP and mucin coat (arrow).

Tab VIII. *In vitro* and *in vivo* rabbit embryos development rates after slow freezing with media containing animal products (SF1) or chemically defined products (SF2 and SF3).

		Slow freezing media (+1.5 M DMSO)		
		SF1 (0.4 % BSA)	**SF2 (20 % CRYO3)**	**SF3 (CRYO3)**
In vitro development	**% Survival**	166/186 (89.2)	165/188 (87.8)	164/175 (93.7)
	% Blastocyst	75/166 (45.2)[a]	84/165 (50.9)[a]	101/164 (61.6)[b]
	% Expansion or Hatching	29/166 (17.5)[c]	40/165 (24.2)	59/164 (36.6)[d]

For each rate, different letters in the same row indicate a statistically significant difference ($p < 0.05$).

% Survival: the percentage of embryos that were not morphologically damaged immediately after cryopreservation (per vitrified embryo).

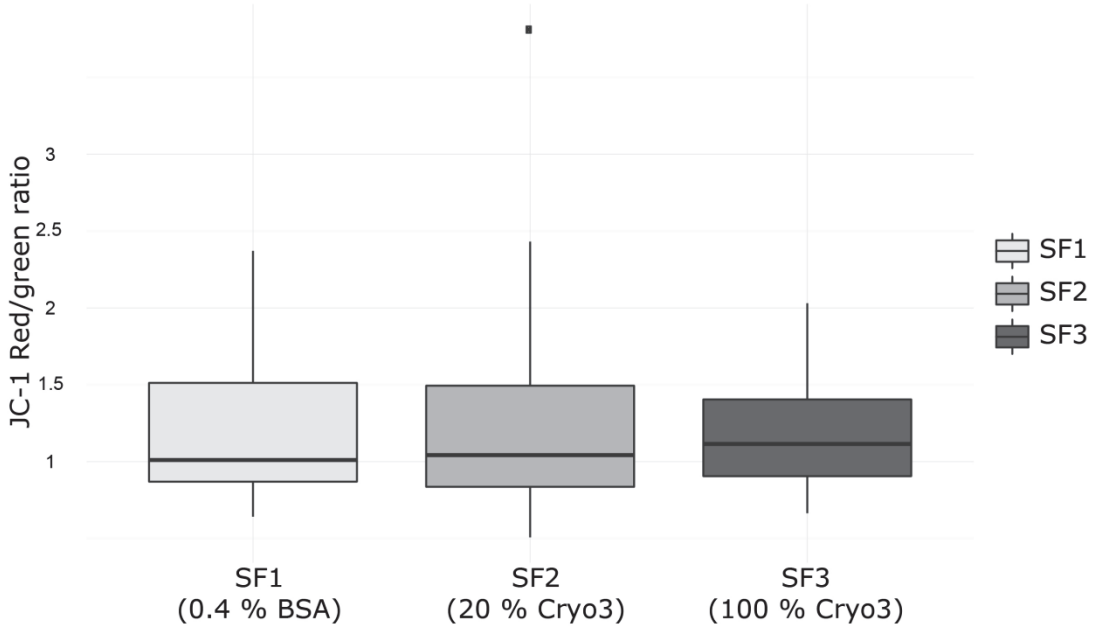
% Blastocyst: the percentage of embryos that attained the blastocyst stage, determined by the presence of well-developed blastocoel cavity and a distinguishable embryoblast (per cultured embryo).

% Expansion or hatching: the percentage of embryos that attained the expanded blastocyst stage or that developed a trophectoderm herniating through ZP (per cultured embryo). Slightly expanded blastocysts with herniation of embryonic cells comprised in this group.

Mitochondrial activity assessment with JC-1

Ratios of J-aggregate to J-monomer of cryopreserved expanded or hatching embryos, slow frozen with media containing animal products (SF1) or chemically defined products (SF2 and SF3) are summarized in Fig. 20. No significant differeazznces were observed between the three groups. After incubation with the CCCP control, images showed no red fluorescence.

Fig. 20. JC-1 staining: red/green ratio of cryopreserved expanded or hatching blastocysts slow frozen with media containing animal products (SF1) or chemically defined products (SF2 and SF3).



Red/green ratio of embryos frozen with SF1 (n = 27), SF2 (n = 30), or SF3 (n = 36), obtained with epifluorescence microscopy. No significant difference was observed between groups.

1.2 Rabbit embryo vitrification (assessment of *in vitro* and *in vivo* development)

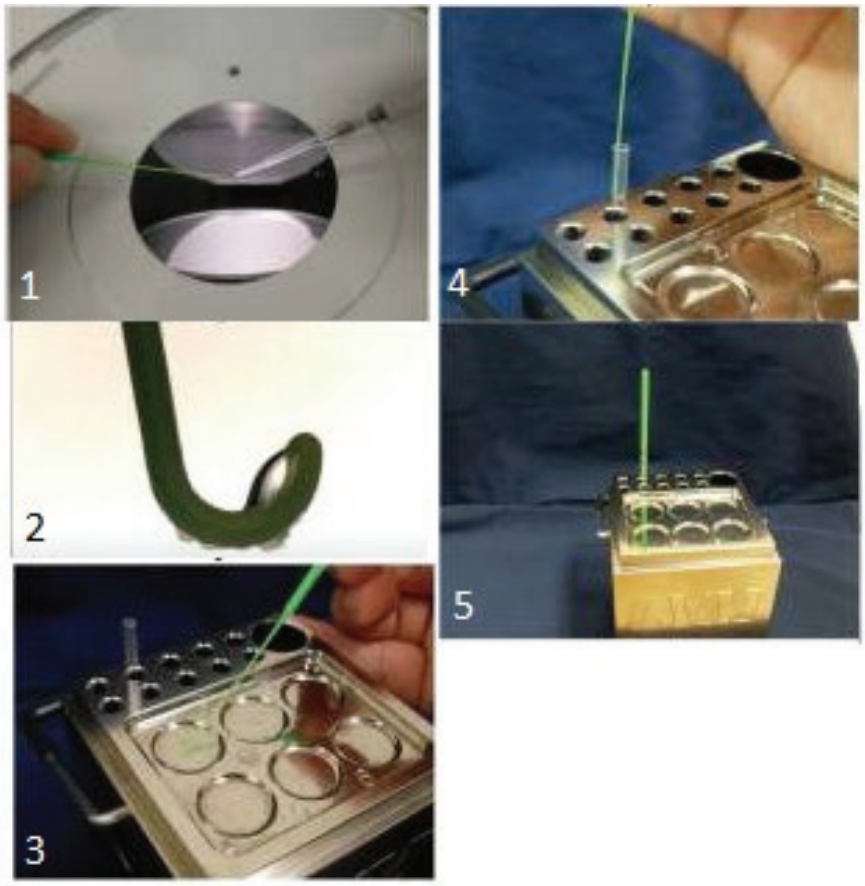
1.2.1 Embryo production and recovery

A total of 62 rabbit New Zealand does (SARL HYCOLE, Marcoing, France), aged between 38 and 50 weeks, were housed in groups of five and fed with a commercial diet. Does received a twice-daily subcutaneous administration of five doses of a pFSH : LH (ratio 5 : 1) preparation, (31.5 µg total, Stimufol, Reprobiol, Belgium). Eight hours after the last injection, does were inseminated with sperm from multiple males (pooled ejaculates), and a subcutaneous injection of 1.6 µg buserelin (Receptal®) was administered. Rabbit does were euthanized 65 to 68 h after the buserelin administration by cervical dislocation. The oviducts and uteri were flushed using Euroflush® medium at room temperature. Embryos were recovered at the morula stage and classified according to the International Embryo Transfer Society (IETS) manual, and quality 1 embryos [42] were pooled. Embryos (n = 1540) were randomly divided into three cryopreservation groups and two control groups: a group of embryos (n = 40) was cultured without cryopreservation (*in vitro* fresh control), and a group of embryos (n = 59) was transferred without cryopreservation (*in vivo* fresh control).

1.2.2 Embryo vitrification and warming

Prior to vitrification, embryos (n = 1441) were randomly divided into three vitrification groups. All media contained the same CPAs composition and the following base media: VS1: IMV Embryo holding medium®, containing 0.4 % (*w/v*) BSA (n = 543); VS2: D-PBS supplemented with 20 % of CRYO3 (n = 423); VS3: 100 % CRYO3 medium (n = 475). Embryos were transferred into a first equilibration solution composed of 5 % (*v/v*) DMSO and 5 % (*v/v*) EG (5 min), and a second equilibration solution composed of 10 % (*v/v*) DMSO and 10 % (*v/v*) EG (2 to 3 min). Embryos were then exposed to a vitrification solution of VS1, VS2, or VS3 base medium (30 s) containing 20 % (*v/v*) DMSO (~ 2.8 M) and 20 % (*v/v*) EG (~ 3.6 M), before being loaded to a Fibreplug (CVM kit, Cryologic) and vitrified by solid surface vitrification (three to four embryos per Fibreplug, 1 to 2 µL of volume), as represented in Fig. 21. Warming was performed by immersing the end of the Fibreplug directly into a thawing solution (0.5 M sucrose in VS1, VS2, or VS3 base medium, respectively) at 38.5 °C for 5 min, followed by three successive dilution baths (0.3 M, 0.1 M and 0.0 M sucrose).

Fig. 21. Vitrification procedure using the CVM Kit and the Fibreplug dispositive.



1) Embryo loading to the Fibreplug; 2) droplet containing the embryo; 3) cooling procedure; 4) Moving to the cap (that is plunged into LN_2); 5) Ready to plunge the Fibreplug and the cap into LN_2. Adapted from [459].

1.2.3 *In vitro* embryo culture and morphology assessment

Embryos (n = 40) from the *in vitro* culture group were cultured (38.5 °C, 5 % CO_2) to the expanded blastocyst stage in M199 medium (without glutamine) supplemented with 10 % foetal calf serum and antibiotics (67 UI/mL penicillin and 67 µg/mL streptomycin). *In vitro* development was assessed after 24 h and 48 h of culture and classified according to their development stage as morula, blastocyst, expanded and hatching embryos. Slightly expanded blastocysts with herniation of embryonic cells were included in the expanded / hatching embryo group.

1.2.4 Mitochondrial activity assessment with JC-1

At the end of embryo culture, living embryos (n = 89) at the expanded / hatching stage from the three vitrification groups were subjected to a pretreatment of pronase, incubated with

JC-1 for 75 min (1.5 µM, 38.5 °C, 5 % CO2), and observed according to the technique described in the section 2.1 of Chapter I ("JC-1 staining method adaptation to rabbit embryos").

To evaluate embryo mitochondrial activity, the staining intensity (by pixel) was measured, from both channels, in two randomly defined areas on each embryo, using the Fiji package [390] of ImageJ software (National Institute of Health, Bethesda, Maryland, USA), and the red to green ratio was quantified.

An MMP disruptor (CCCP) was used as a control to confirm that directional changes in the dye signal were correctly interpreted.

1.2.5 Embryo transfer

Fresh embryos (n = 59) and warmed vitrified (total = 905; group 1 n = 358, group 2 n = 270, group 3 n = 277) embryos were transferred to synchronized New Zealand recipient does (n = 84, adult nulliparous, aged 22 weeks), according to the protocol described by Salvetti [460]. Briefly, recipient does were synchronized with a buserelin injection (0.8 µg, intramuscular, Receptal), 50 to 60 h before transfer. After anaesthesia, a midventral laparotomy was performed, and 4 to 7 embryos (mea n = 5.4) were transferred to each uterine horn. Pregnancy diagnosis was realized by palpation 20 days after embryo transfer.

1.2.6 Statistical analysis

Development rates were analysed with the chi-square test, whereas JC-1 red/green ratios were analysed by one-way analysis of variance. All tests were performed with R-Studio software [391]. Groups were considered significantly different at $p < 0.05$.

1.2.7 Results: assessment of *in vitro* and *in vivo* development rate and mitochondrial activity

In vitro and *in vivo* embryo development after vitrification

The *in vitro* blastocyst formation and expansion/hatching rates and *in vivo* development rates (pregnancy rate, implantation live-birth rates) after rapid cooling with media containing animal products (VS1) or chemically defined products (VS2 and VS3) are summarized in Table IX. *In vitro* fresh control embryos expressed significantly superior blastocyst and expansion / hatching rates. VS3 medium appeared significantly superior in *in*

vitro post-warm survival rates than VS2 and VS3 media. No significant difference was observed regarding the other *in vitro* and *in vivo* development rates.

Table IX. *In vitro* and *in vivo* rabbit embryo development rates after rapid cooling with media containing animal products (VS1) or chemically defined products (VS2 and VS3).

		Vitrification media (+ 20% DMSO and 20% EG)			
		VS1 (0.4 % BSA)	**VS2 (20 % CRYO3)**	**VS3 (CRYO3)**	**Control (Fresh)**
In vitro development	**% Survival**	90.8 %[a] (168/185)	85.6 %[a] (131/153)	94.9 %[b] (188/198)	
	% Blastocyst	85.1 %[a] (143/168)	77.9 %[a] (102/131)	83.3 %[a] (156/188)	97.5 %[b] (39/40)
	% Expansion*, or Hatching	63.1 %[a] (106/168)	63.4 %[a] (83/131)	58.0%[a] (109/188)	97.5 %[b] (39/40)
In vivo development	**% Pregnancy**	80.0 %[NS] (24/31)	68.0 %[NS] (17/24)	95.2 %[NS] (20/23)	83.3 %[NS] (5/6)
	% Implantation	40.5 %[NS] (117/303)	45.9 %[NS] (84/183)	44.8%[NS] (94/234)	46.9 %[NS] (23/49)
	% Live birth	35.6 %[NS] (103/303)	35.5 %[NS] (65/183)	38.1 %[NS] (80/234)	40.8%[NS] (20/49)

Different letters in the same row indicate a statistically significant difference ($p < 0.05$). NS indicates no statistically significant difference was observed.

% Survival: the percentage of embryos that were not morphologically damaged immediately after cryopreservation (per vitrified embryo).

% Blastocyst: the percentage of embryos that attained the blastocyst stage, determined by the presence of well-developed blastocoel cavity and a distinguishable embryoblast (per cultured embryo).

% Expansion or hatching: the percentage of embryos that attained the expanded blastocyst stage or that developed a trophectoderm herniating through ZP (per cultured embryo). Slightly expanded blastocysts with herniation of embryonic cells comprised in this group.

% Pregnancy: the percentage of females that delivered per transferred fermale

% Implantation: the percentage of born kits (alive and dead) per transferred embryos on pregnant females

% Live birth: the percentage of live-born kits per transferred embryos on pregnant females

Mitochondrial activity assessment with JC-1

Ratios of J-aggregate to J-monomer of cryopreserved expanded or hatching embryos, vitrified with media containing animal products (VS1) or chemically defined products (VS2 and VS3) are represented in Fig. 22 and summarized in Fig. 23. No significant differences were observed between the three groups. After incubation with the CCCP control, images showed no red fluorescence.

Fig. 22. Ratios of J-aggregate to J-monomer of vitrified rabbit embryos; cryopreserved with media containing animal products (VS1) or chemically defined products (VS2 and VS3).

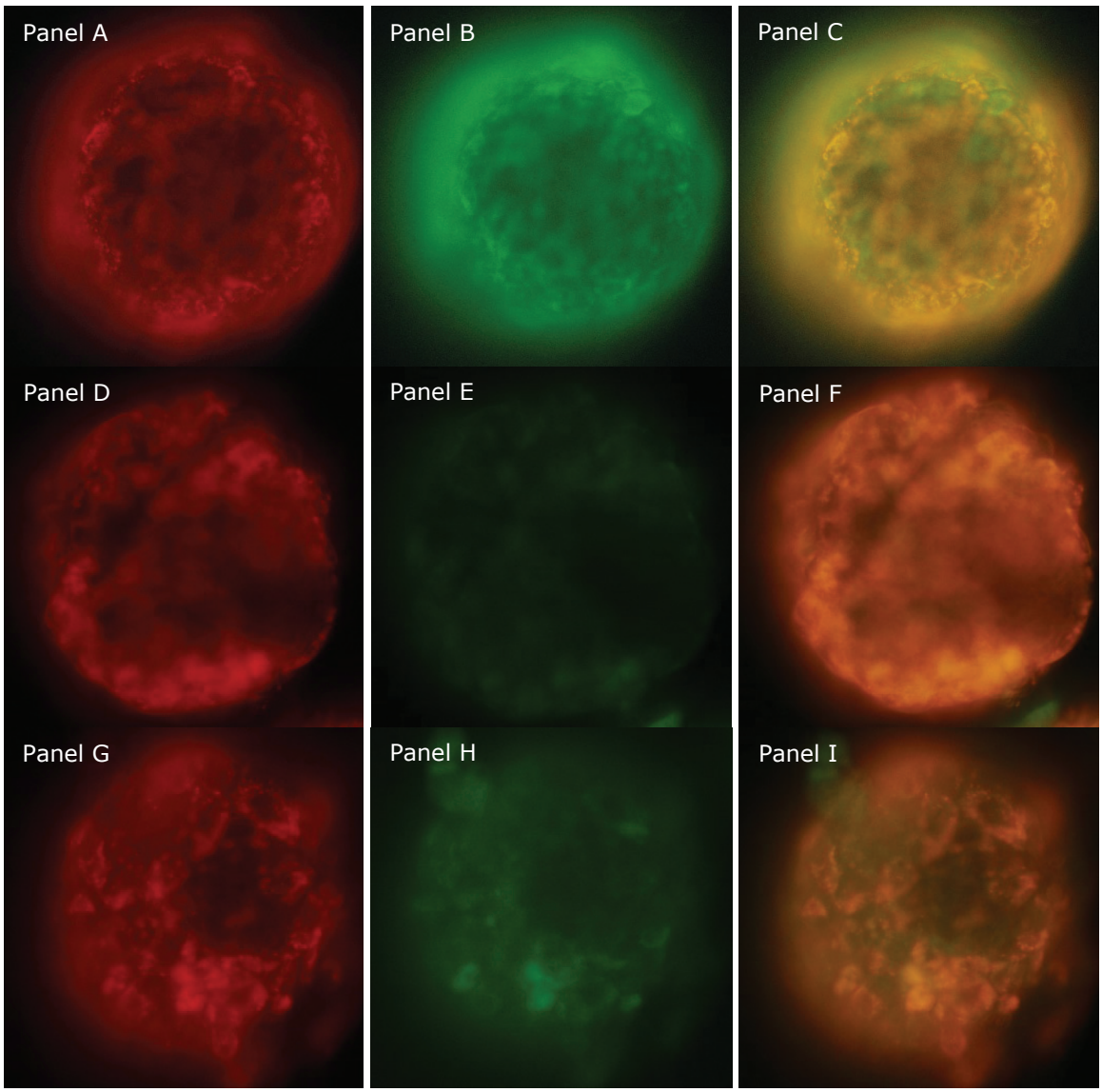


Fig. 22a.

A, D, G) Regions of high MMP are indicated by red fluorescence (emission ~590 nm).

B, E, H) Depolarized regions are indicated by green fluorescence (emission ~529 nm).

C, F, I) Merged images.

A, B, C) Embryo vitrified with a medium containing 0.4 % BSA.

D, E, F) Embryo vitrified with a medium containing 20 % CRYO3.

G, H, I) Embryo vitrified with a medium containing 100 % CRYO3.

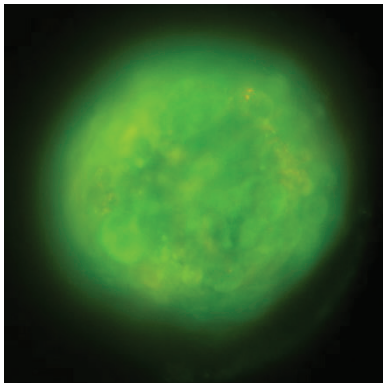
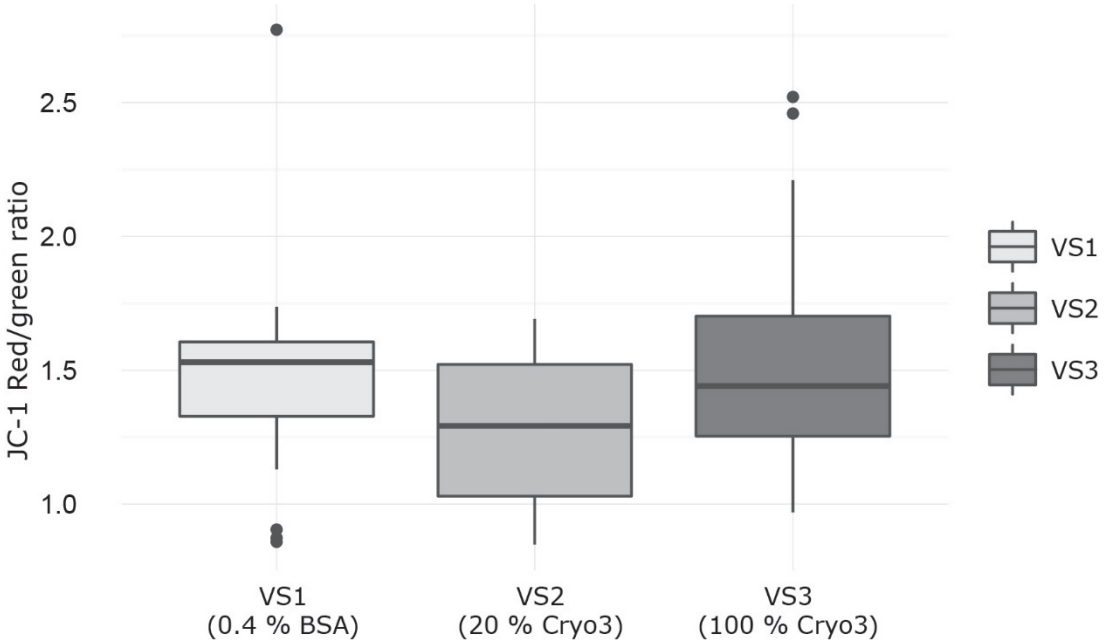


Fig. 22b. After CCCP control (merged images). No regions of high MMP are visible.

Fig. 23. JC-1 staining: red/green ratio of cryopreserved expanded or hatching blastocysts vitrified with media containing animal products (VS1) or chemically defined products (VS2 and VS3).



Red/green ratio of embryos vitrified with VS1 (n = 31), VS2 (n = 27), or VS3 (n = 31), obtained with epifluorescence microscopy. No significant difference was observed between groups.

2. Ovine embryo cryopreservation

Our objective was to compare the "IMV Embryo Holding Medium®" – a commercial medium containing animal derived products (0.4 % (*w/v*) BSA) and a synthetic medium with no animal products (CRYO3) for the slow freezing (Experiment 2.1) and the vitrification (Experiment 2.2) of ovine embryos.

2.1 Ovine embryo slow freezing (assessment of *in vitro* development)

2.1.1 Embryo production and recovery

Grivette multiparous ewes (n = 15), aged between 3 and 9 years, were synchronized and superovulated according to protocols described in section 4.2 of Chapter I ("Comparison of superovulation protocols"). Responding ewes were mated by fertile rams (each female was in contact with several rams) and 7 days later ewes were subjected to a laparotomy. Briefly, the flushing media (Euroflush®) was injected via a Tomcat urethral catheter inserted at the oviduct and recovered with a Foley catheter that had been inserted next to the bifurcation of each uterine horn. Oviducts and uterine horns were flushed and recovered embryos were counted and evaluated at the morula and blastocyst stages. Freezable embryos (n = 26) were mixed and randomly divided into two slow freezing groups (SF1 and SF2).

2.1.2 Embryo slow freezing and thawing

Embryos were transferred into an equilibration medium containing 5 % (*v/v*) EG (~ 0.9 M), added to a base medium composed of IMV Embryo holding medium® (0.4 % (*w/v*) BSA) – SF1, or synthetic medium (D-PBS + 20 % CRYO3) – SF2, at room temperature for 10 min.

After equilibration, embryos (n = 46) were exposed to a slow freezing medium composed of the same base medium (SF1 – 0.4 % (*w/v*) BSA and SF2 – 20 % CRYO3), supplemented with 10 % (*v/v*) EG (~ 1.8 M), before being loaded to a straw and slow frozen in a programmable freezer.

Thawing was performed by immersing the straw in a water bath at 32 °C for 30 s, and washing the embryos in four successive dilution baths: 0.5 M sucrose during 1 min, and 0.25 M, 0.15 M and 0.0 M of sucrose, during 5 min each, in the respective base medium.

2.1.3 *In vitro* embryo culture

Thawed embryos (n = 26) were cultured under paraffin oil (38.5 °C, 5 % CO2), to the hatching stage, in M199 medium supplemented with L-glutamine (Dutscher, Brumath, France), 10 % fetal calf serum and antibiotics (67 UI/mL penicillin and 67 µg/mL streptomycin). *In vitro* development was assessed and embryos were classified according to their development stage as morula, blastocyst, expanded or hatching embryos.

2.1.4 Statistical analysis

Development rates were analysed with the Pearson's chi-squared test. All tests were performed with R-Studio software [391]. No significative difference was observed between the two media ($p < 0.05$).

2.1.5 Results: assessment of *in vitro* development rate

The *in vitro* development rates after slow freezing with media containing animal products (SF1) or chemically defined products (SF2) are summarized in Table X. No statistical difference was found regarding survival rate.

Table X. *In vitro* ovine embryos development rates after slow freezing with media containing animal products (SF1) or chemically defined products (SF2).

	Slow freezing media (+10 % EG)	
	SF1 **(0.4 % BSA)**	**SF2** **(20 % CRYO3)**
% Survival	95.7 % (22/23)	87.0 % (20/23)
% Expansion	40.9 % (9/22)	50.0 % (10/20)
% Hatching	40.9 % (9/22)	45.0 % (9/20)

No statistically significant difference was observed ($p < 0.05$).

% survival: the percentage of embryos that were not morphologically damaged immediately after cryopreservation (per cryopreserved embryo).

% expansion: the percentage of embryos that attained the expanded blastocyst stage, determined by the presence of well-developed blastocoel cavity and a thin ZP, with an important increase of embryo volume (per cultured embryo).

% hatching: the percentage of blastocysts with a trophectoderm herniating through ZP (per cultured embryo).

2.2 Ovine embryo vitrification (assessment of *in vitro* and *in vivo* development)

2.2.1 Embryo production and recovery

BMC multiparous ewes (*n* = 21), aged between 3 and 6 years, were synchronized and superovulated according to protocols described in in section 4.2 of Chapter I ("Comparison of superovulation protocols"). Responding ewes were inseminated by cervical insemination and 7 days later ewes were subjected to a laparotomy. Briefly, the flushing media (Euroflush®) was injected via a Tomcat urethral catheter inserted at the oviduct and recovered with a Foley catheter that had been inserted next to the bifurcation of each uterine horn. Oviducts and uterine horns were flushed and recovered embryos were counted and evaluated at the morula and blastocyst stages. Freezable embryos (*n* = 109) were mixed and randomly divided into two vitrification groups (VS1, *n* = 55 and VS2, *n* = 54).

2.2.2 Vitrification and warming

Embryos were transferred into an equilibration medium containing 10 % (*v/v*) and 20 % (*v/v*) EG, for 5 and 3-4 min, respectively, and then exposed (30 s) to a vitrification solution composed of with 40 %(*v/v*) EG (~ 7.2 M), added to a base medium of IMV Embryo holding medium® (0.4 % (*w/v*) BSA) – VS1, or synthetic medium (100 % CRYO3) – VS2. Embryos were then loaded to a fibreplug (one to two embryos per dispositive, 1 to 2 µL) and vitrified by solid surface vitrification.

Warming was performed by immersing the fibreplug in a warming solution, composed of 0.5 M sucrose and equilibrated at 38.5 °C, during 1 min, before transferring the embryo to three dilutions baths: 0.25 M, 0.15 M and 0.0 M of sucrose, during 5 min each, in the respective base medium.

2.2.3 *In vitro* embryo culture

81 embryos (41 from the VS1 medium, and 40 from the VS2 medium) were thawed embryos were cultured under paraffin oil (38.5 °C, 5 % CO_2), to the hatching stage, in M199 medium supplemented with L-glutamine (Dutscher, Brumath, France), 10 % fetal calf serum and antibiotics (67 UI/mL penicillin and 67 µg/mL streptomycin). *In vitro* development was assessed and embryos were classified according to their development stage as morula, blastocyst, expanded or hatching embryos.

2.2.4 *In vivo* embryo transfer

Warmed vitrified embryos (total = 28, VS1 n = 14, VS2 n = 14) were transferred to synchronized BMC recipient multiparous ewes (n = 9), aged between 3 and 6 years. Briefly, 22 days before embryo transfer, recipient ewes were synchronized with an intra-vaginal sponge impregnated with flugestone acetate (Chronogest CR®), that was removed 14 days ater. At the time of removal, an intramuscular PMSG injection (600 UI, Chronogest PMSG®) was administered. After anaesthesia, a midventral laparotomy was performed, and 1 to 2 embryos were transferred to each uterine horn (with an average of 3 embryos per ewe). Pregnancy diagnosis was realized by ultrasound 40 to 45 days after embryo transfer.

2.2.5 Statistical analysis

Development rates were analysed with the Pearson's chi-squared test. All tests were performed with R-Studio software [391]. No significative difference was observed between the two media ($p < 0.05$).

2.2.6 Results: assessment of *in vitro* and *in vivo* development rate

The *in vitro* development rates after vitrification with media containing animal products (VS1) or chemically defined products (VS2) are summarized in Table XI. No statistical difference was found regarding survival rate between both media.

Table XI. *In vitro* ovine embryos development rates after vitrification with media containing animal products (VS1) or chemically defined products (VS2).

		Vitrification media (+40 % EG)	
		VS1 (0.4 % BSA)	**VS2 (CRYO3)**
In vitro development	**% Survival**	87.8 % (36/41)	92.5 % (37/40)
	% Expansion	61.1 % (22/36)	75.7 % (28/37)
	% Hatching	52.8 % (19/36)	70.3 % (26/37)
In vivo development	**% Pregnancy**	100.0 % (4/4)	80.0 % (4/5)
	% Live birth	8/14 (51.1 %)	6/11 (54.5 %)

No statistically significant difference was observed ($p < 0.05$).

% survival: the percentage of embryos that were not morphologically damaged immediately after cryopreservation (per cryopreserved embryo).

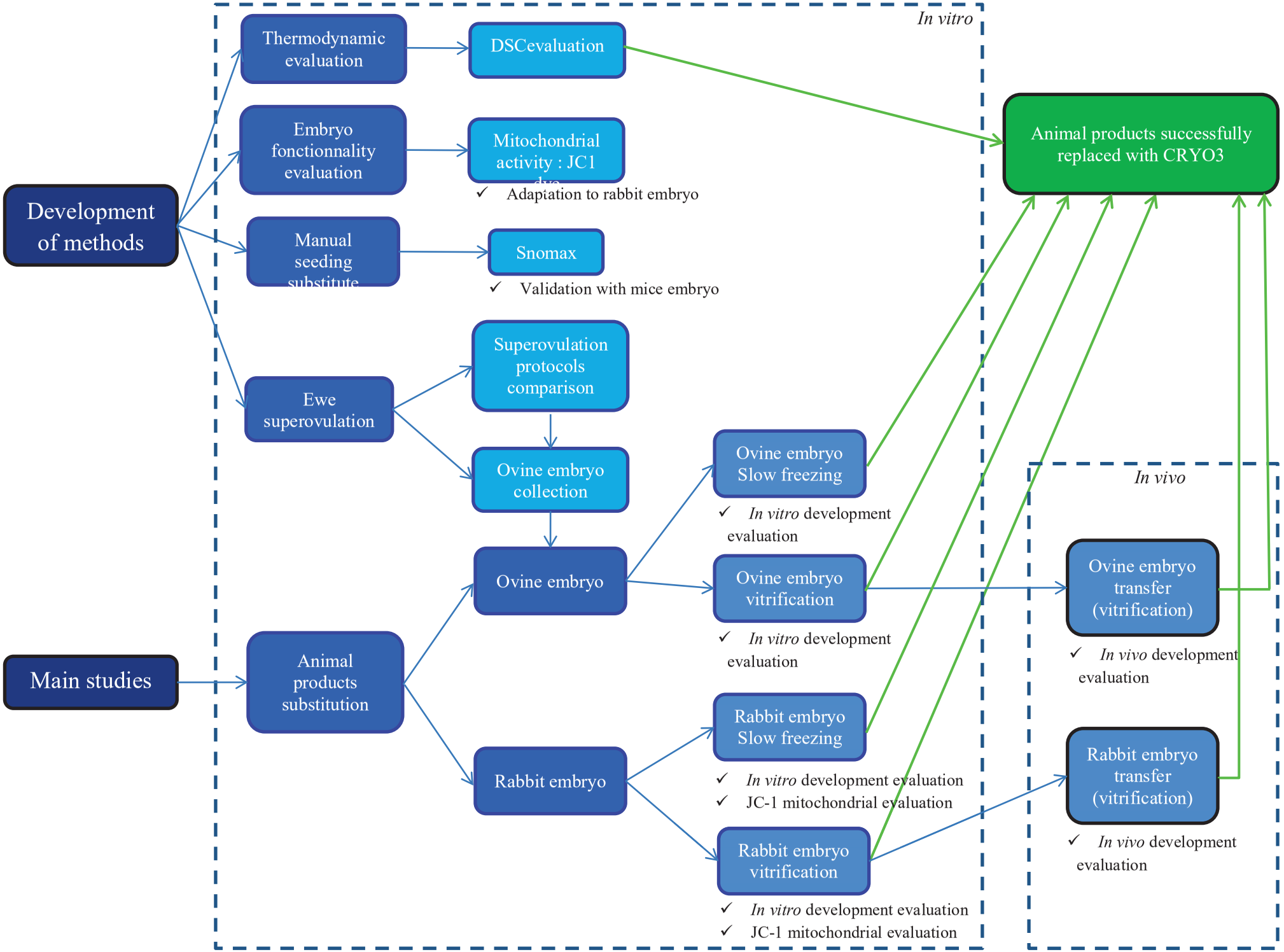
% expansion: the percentage of embryos that attained the expanded blastocyst stage, determined by the presence of well-developed blastocoel cavity and a thin ZP, with an increase of embryo volume (per cultured embryo).

% hatching: the percentage of blastocysts with a trophectoderm herniating through ZP (per cultured embryo).

% pregnancy: the percentage of ewes positive to pregnancy diagnosis per recipient

% Live birth - the percentage of live-born lambs per transferred embryos on pregnant females

Fig. 24. Schematic representation of the experiments conducted during this thesis.



Chapter III - Discussion

During this work, we aimed to replace animal products in ovine and rabbit slow freezing and vitrification media. In order to do so, experiments were divided in two main phases (Fig. 24). The first phase included preliminary experiments, with:

- the evaluation of the thermodynamic properties of the animal-based solution and of the potential substitutes;

- the adaptation of a method to evaluate embryo functionality (JC-1 staining) to rabbit species;

- the set up of superovulation protocols adapted to our field conditions, and the evaluation of these protocols, to produce viable ovine embryos;

- the substitution of manual seeding by an ice nucleation agent (Snomax®). This experiment was performed as an independent work and not as a tool for the main studies. This thesis was conducted in parallel with a physicist PhD thesis, whose main objective was the study of thermodynamic properties of embryo slow-freezing media. The development of a manual seeding alternative, with a biological validation, allowed the pursuit of this parallel work.

The second phase included the biological evaluation of the potential substitute – CRYO3, with:

- ovine embryo slow-freezing, followed by *in vitro* development evaluation;

- ovine embryo vitrification, followed by *in vitro* and *in vivo* development evaluation;

- rabbit embryo slow-freezing, followed by *in vitro* development evaluation and embryo functionality evaluation;

- rabbit embryo vitrification, followed by *in vitro* development evaluation, embryo functionality evaluation and *in vivo* development evaluation.

Over the last few decades, there have been important efforts to replace animal products with animal-free synthetic products for embryo cryopreservation. Numerous natural or synthetic molecules have been used in slow freezing, as in vitrification media, to replace the biological and physical properties of animal albumin [1,232,234,238].

Studies demonstrate that animal products can be successfully replaced with products such as the silk protein sericin [232] and vegetal peptones [234] during bovine embryo slow-freezing.

The non-organic macromolecule PVA has been used to slow-freeze and vitrify embryos from different species, obtaining equivalent post-thaw development rates for murine [236–238] and porcine [243] embryos. However, inferior development rates were also obtained for murine [239], bovine [238,240,241] and ovine [242] embryos. Studies using polyvinylpyrrolidone tend to demonstrate a negative effect on cryopreservation media [240], as well as inferior surfactant properties [236,240] and toxic properties to embryos [126,245].

In 2000, Kuleshova vitrified mice embryos, using a medium with no animal products, containing 35 % polymers (dextran or Ficoll) and 25 % EG, obtaining *in vitro* development rates of 100 % blastocyst expansion and *in vivo* foetuses rates of 76 %[247]. One year later, the same author obtained development rates (96 - 100 % blastocyst expansion and 62 - 76 % live foetuses) after vitrifying mouse embryos with Ficoll and dextran macromolecules, in protein-free media. However, authors did not compare these medium with a medium containing animal products [126]. Another author evaluated the substitution of foetal calf serum with Ficoll, on mouse embryo quick freezing, obtaining equivalent development rates [236]. These studies suggest that these two molecules may be good candidates for replacement of animal products.

Some authors successfully replaced animal derived products with HA. Palasz investigated the substitution of NCS with 0,1 % HA (1 mg/mL), or PVA during murine embryo slow-freezing (n = 309), and observed no difference regarding *in vitro* development [256]. This author did the same media comparison using bovine embryos (n = 178), and obtained higher *in vitro* development rates in NCS and HA groups, than in PVA group [238]. When comparing distinct HA molecular weights, the same author observed that higher HA molecular weights (> 5-7.5 x 10^5 Da) allowed a greater survival rate after embryo freezing [238]. Joly successfully slow-froze murine (n = 443) and ovine (n = 120) embryos with BSA (4 mg/mL) or HA (1 mg / mL), obtaining approximatively 80 % of *in vitro* development for both groups murine embryos, and 75 % of *in vitro* development for both groups ovine embryos [257].

In our previous work, we showed that animal products could be successfully replaced with the synthetic chemically defined medium - CRYO3 (that contains HA), in bovine [3] and rabbit embryo slow-freezing [220]. In this last work, better *in vivo* development rates were obtained with 20 % CRYO3, compared to foetal calf serum [220]. In these studies, the indicated percentage of CRYO3 is 18 %, since it was the final proportion of CRYO3 (including CPAs), but the base-medium was composed of D-PBS + 20 % CRYO3.

These previous studies aimed to evaluate the effect of replacing animal products with CRYO3 only in slow freezing media, and not in the entire process of cryopreservation (slow freezing and thawing). Therefore, thawing media was the same for both groups, and contained the animal product BSA (IMV embryo holding medium®). The promising results obtained by our collegue led us to extend our research to the whole slow freezing process - embryo slow freezing and thawing media, to embryo vitrification, and to the ovine species.

During our collegues work, it was observed that a slow-freezing medium with a base medium composed of 100 % CRYO3 showed equivalent thermodynamic properties to a medium containing BSA, and led to similar *in vitro* development rates (unpublished data). Based on these findings, we added a 100 % CRYO3 group to our slow-freezing studies.

In the same way, an evaluation of the thermodynamic properties of three vitrification media, containing similar base-media (BSA, 20 % CRYO3 and 100 % CRYO3), was conducted, using DSC. This experiment confirmed the interest of the synthetic product CRYO3 during vitrification. Critical cooling and warming rates for the three vitrification media were evaluated, and BSA-based medium presented a mean critical cooling rate (168.0 °C/min) more than twice higher than the D-PBS + 20 % CRYO3-based medium (73.6 °C/min) and more than five times higher than the CRYO3 based medium (29.5 °C/min).

Critical warming rates were considerably higher than critical cooling rates for the three media (superior to 200 times). Estimated critical warming rate for the BSA-based medium (1 530 711.5 °C/min) was almost four times higher than the D-PBS + 20 % CRYO3-based medium (400 098.5 °C/min) and 250 times higher than the CRYO3-based medium (6 114.0 °C/min). Besides confirming that attaining higher rates is more important during warming than during cooling, this experiment showed that both synthetic-base media allowed vitrification and warming with inferior cooling and warming rates than the medium containing animal products. Between these two synthetic media, the vitrification medium containing 100 % CRYO3 presented lower cooling and warming rates, showing it can potentially go through these processes with a greater stability.

If the cooling rates estimated in our experiment are commonly attained (as discussed in the section II.I- 1 of Chapter II (“Thermodynamic evaluation of the cryopreservation media: differential scanning calorimetry” discussion), the attainment of these warming rates can be of greater difficulty.

Due to the minimal volume of the droplets used in vitrification (1 to 2 µL), to the possibility of modification of the droplet shape and energy conduction after the insertion of a thermocouple, and to the need of ultra thin and extremely reactive thermocouples, an accurate measurement of cooling and warming rates during vitrification is particularly difficult. Without confirming that the rates attained during the procedures are superior to calculated critical rates with DSC, we cannot be sure that there is no ice crystal formation, especially during warming. A more correct term in this case would be rapid-cooling freezing, instead of vitrification (since vitrification implies no ice crystal formation) [461]. However, most embryo "vitrification" studies use the term "vitrification" without estimating critical rates and confirming that their rates are effectively superior to these critical rates, as the macroscopic visualization of the droplet may not be sufficient [241,462,364,463,464].

In 2005 Kuwayama evaluated the cooling and warming rates obtained during a vitrification procedure with two dispositives (Cryotip and Cryotop), using a medium containing 15% EG and 15% DMSO and 0.5 mol/l sucrose dissolved in TCM199 supplemented with 20% synthetic serum substitute. The average values of the cooling and warming rates were 12 000 and 24 000 °C/min (respectively) with the CryoTip, and 23 000 and 42 000 °C/min (respectively) with the Cryotop method [134]. Even if we didn't use the same dispositive, nor the same solution or conditions, when comparing to the critical rates obtained in our DSC experiments, only the 100 % CRYO3-based medium would avoid the formation of ice crystal during the entire process.

Jin achieved ultra high rates (10 000 000 °C/min) using a laser pulse [116]. We can hypothesize that the use of this warming method would allow vitrification with no ice formation, using our three media. Indeed, Jin obtained approximately 100% survival, with slower cooling rates, and with a medium containing 1/3 of the standard cryoprotectants concentration [116].

In conclusion, the medium containing 100 % CRYO3 presented better properties to undergo rapid-cooling and warming processes without forming ice crystals, with a greater stability. The effectiveness of vitrification (with no ice formation) will depend on the vitrification dispositive and technique methods, that would allow (or not) the achievement of rates superior to the estimated critical rates. Further studies are needed to accurately measure the cooling and warming rates achieved during our experiments, taking into consideration the combination of our dispositive technique and media. A possibility would be the use of ultra thin and extremely reactive thermocouples, even if its placement on the droplet would possibly modify its shape, and cooling and warming kinetics.

The experiment “JC-1 staining method adaptation to rabbit embryos” was the first to describe a JC-1 staining protocol for rabbit embryo evaluation. This mitochondrial activity staining can be used as an indicator of embryo health and functionality, in complement of *in vitro* development evaluation. This protocol can be used to compare rabbit embryo functionality, even in the absence of morphological alterations of the embryo. During our main experiments, this protocol allowed us to complete rabbit embryo *in vitro* development evaluation with functionality evaluation.

The adaptation of JC-1 staining to ovine embryo was not possible, due to the important quantity of embryos needed to test distinct conditions and to the small quantity of obtained embryos.

In our experiment “Ewe superovulation - Comparison of superovulation protocols”, the ewes’ response to the superovulation treatment was, generally, satisfactory with the two protocols. Due to the important variability in response to superovulation treatments, a study with a greater number of ewes and intrauterine sperm deposition would be of value to increase statistical power. Nonetheless, both superovulation treatments can be used to produce ovine embryos *in vivo*, with a satisfactory superovulation rate.

In our experiment “Rabbit embryo slow freezing (assessment of *in vitro* development)” we aimed to evaluate the effect of replacing “IMV Embryo Holding Medium®” – a commercial medium containing animal derived products (0.4 % BSA) with a chemically defined medium with no animal-derived products - CRYO3- in rabbit embryo slow-freezing and thawing media.

Rabbit embryos were not subjected to a pronase treatment to remove the mucin coat prior to culture. Kasai compared the *in vitro* development with and without mucin coat digestion and observed that approximately half of the non-treated embryos did not expand to a diameter more than twice that of the morula (46 % non-treated vs 92 % treated embryos) [246]. Fischer observed that uterine components are vital in the transformation of the extracellular coverings in the rabbit embryo. In rabbit culture media lacking uterine components, the ZP does not dissolve and loses elasticity, leading to herniation of embryonic cells into the mucin coat, instead of expansion and hatching [465]. Indeed, we observed slightly expanded embryos with embryonic cell herniation in cryopreserved and in non-

cryopreserved groups. Considering these findings, we pooled slightly expanded herniated blastocysts with expanded and hatching blastocysts.

No significative difference was observed regarding *in vitro* survival rate after slow-freezing between the three media (wich was close to 90 %). These results are superior to those obtained by Bruyère, after slow-freezing rabbit embryos (73,3 to 77,6 % survival) [465].

Regarding the percentage of embryos that attained the blastocyst stage, the medium containing CRYO3 allowed a statistically superior rate (SF3 = 61.6 %) than the media containing BSA (SF1 = 45.2 %) or 20% CRYO3 (SF2 = 50.9 %). Our blastocyst development rates are not far from blastocyst rates obtained by Naik, after slow-freezing (55 %) [466].

Altough the rate of embryos that attained expansion or hatching stage was superior in the group CRYO3 (SF3 = 36.6 %) than in the groups BSA or 20 % CRYO3 (SF 1 = 17.5 % and SF2 = 24.2 %), the difference was statistically significative only for the groups CRYO3 and BSA.

To evaluate mitochondrial activity between slow-freezing groups, we only used developed embryos. If we had randomly picked embryos from all developmental stages, the development rates would have influenced the total mitochondrial activity and, therefore, confound our results. The evaluation of mitochondrial activity of slowly-frozen embryos showed no significant differences between the three groups. This result suggests that developed embryos from the three groups presented equivalent energetic capacity of continuing further development. The obtained JC-1 ratios are equivalent to ratios found in the literature for fresh mouse blastocysts (~ 1.35) [347]. JC-1 analysis of the CCCP control demonstrated that the JC-1 ratio is dependent on mitochondrial potential variations, confirming the interest of our test.

We can conclude that, although there was no difference regarding energetic capacity between developed embryos from the three groups, *in vitro* development rate of slowly-frozen rabbit embryos was, in general, better for the group containing 100 % CRYO3 as base-medium.

In our experiment “Rabbit embryo vitrification (assessment of *in vitro* and *in vivo* development)” we aimed to evaluate the effect of replacing “IMV Embryo Holding Medium®” – a commercial medium containing animal derived products (0.4 % BSA) with a chemically defined medium with no animal-derived products - CRYO3- in rabbit embryo vitrification and warming media.

As during rabbit embryo slow-freezing experiment, embryos were not subjected to a pronase treatment to remove the mucin coat prior to culture and slightly expanded herniated blastocysts were pooled with expanded and hatching blastocysts.

During this experiment, significantly superior survival rates were observed in the 100 % CRYO3 group (VS3 = 94.9 %).) compared to BSA (VS1 = 90.8 %) or 20 % CRYO3 (VS2 = 85.6 %) groups. No differences were found regarding other *in vitro* development rates between vitrified embryos (blastocyst formation VS1 = 85.1, VS2 = 77.9, VS3 = 83.3 %; and blastocyst expansion or hatching VS1 = 63.1, VS2 = 63.4, VS3 = 58.0 %). In the literature, quite variable post-warm *in vitro* development can be found (survival: 95.3 - 95.6 %, blastocyst formation: 56 – 91.7 %, hatching or expansion: 45 - 91.7 %) [467–470]. This variability may depend on several factors, such as donor genetics, the housing conditions of the animals and the embryo culture medium. Our post-warm development rates were in the range of values found in the literature.

Regarding mitochondrial evaluation, we obtained equivalent mitochondrial activity between the three groups, suggesting that all the developed embryos had the same energetic capacity of continuing further development. Images obtained with the CCCP control demonstrated the JC-1 ratio is dependent on mitochondrial potential variations.

In our *in vivo* experiments, no statistically significant difference was observed between fresh and vitrified embryos. No difference was found regarding pregnancy rates of the three vitrification groups, even if VS3 rates tended to be superior (VS1 = 80.0 %, VS2 = 68.0 %, VS3 = 95.2 %). Equivalent implantation rates (VS1 = 40.5 %, VS2 = 45.9 %, VS3 = 44.8 %) and live-birth rates (VS1 = 35.6 %, VS2 = 35.5 %, VS3 = 38.1 %) were obtained with the three vitrification media groups. As in *in vitro* development studies, post-transfer *in vivo* development rates found in the literature can considerably vary (pregnancy: 56 - 100 %, implantation: 8.7 – 60.4 %, live foetuses: 6.4 – 57.1 %) [113,470–472]. *In vivo* development rates may depend on the stage of development [205], cryopreservation medium [143], vitrification device and technique recipient genotype [473], or transfer technique [474,475]. The *in vivo* development rates obtained in our study were in the range and close to the superior limit for pregnancy and implantation rates.

Regarding cryopreservation effects on embryos, a difference was found between fresh (expansion or hatching rate 97.5 %) and vitrified embryos during *in vitro* development, but this difference was no longer observed after *in vivo* transfers. A possible explanation would be that cryopreservation partially impairs embryos, and this damage can be reversible if embryos return to physiological conditions after cryopreservation.

Altough our slow-freezing and vitrification experiments were not done at the same time, and aren't, therefore, directly comparable, *in vitro* development rates seem to be better after rabbit embryo vitrification than after slow-freezing.

Both *in vitro* and *in vivo* experiments in this study indicate that animal products can be replaced by both concentrations (20 % and 100 %) of CRYO3. An evaluation of the next generations would be of value, to evaluate the impact of this substitution on the growth, health and reproduction capacity of the next generations.

A scientifical article, entitled "Rapid cooling of rabbit embryos in a synthetic medium", was published in the journal "Cryobiology", based on rabbit embryo vitrification experiment (https://doi.org/10.1016/j.cryobiol.2018.07.006).

In our experiment "Ovine embryo slow freezing (assessment of *in vitro* development)" we aimed to evaluate the effect of replacing "IMV Embryo Holding Medium®" – a commercial medium containing animal derived products (0.4 % BSA) with a chemically defined medium with no animal-derived products, composed of D-PBS + 20% CRYO3 in ovine embryo slow-freezing and thawing media.

While in rabbit embryo experiments, we were able to compare three cryopreservation media at each experiment, the reduced number of embryos obtained collects forced us to reduce the number of experimental groups.

No difference was observed regarding survival after freezing, or *in vitro* development after culture. Our survival rates after freezing (95,7 % in the BSA group and 87,0% in the 20 % CRYO3 group) were superior to those obtained by Garcia-Garcia, after morula (46,3 %) and blastocyst slow-freezing (83,7 %), with 1.5M EG [205]

In both slow-freezing groups, almost a half of the embryos developed to the expanded blastocyst stage (40,9 % in BSA group and 50,0 % in 20% CRYO3 group), and from these embryos, only one embryo in the 20 % CRYO3 group didn't hatched. Hatching rates were superior to those obtained by Bhat with the same cryoprotectant composition (30 % hatching) [476].

In our experiment "Ovine embryo vitrification (assessment of *in vitro* and *in vivo* development)" we aimed to evaluate the effect of replacing "IMV Embryo Holding

Medium®" – a commercial medium containing animal derived products (0.4 % BSA) with a chemically defined medium with no animal-derived products, composed of CRYO3 in ovine embryo vitrification and warming media.

As we also faced the need of reducing the number of experimental groups in this experiment, due to the reduced number of collected embryos, we selected the synthetic medium 100 % CRYO3 as base-medium. Our choice was based on the promising results obtained in the rabbit embryo experiment, with the 100 % CRYO3 group.

Regarding *in vitro* development after vitrification, although no significant difference was observed, probably due to the reduced number of embryos, the group containing a base medium composed of 100 % CRYO3 presented superior development rates that the animal-product group (92,5 % survival, 75,7 % expansion, 70,3 % hatching for the 100 % CRYO3 group versus 87,8 % survival, 61.1 % expansion and 52,8 % hatching for the BSA group).
The obtained survival rates are equivalent to the survival rates obtained by Gibbons, after vitrifying ovine morula and blastocysts with 25 % glycerol and 25 % EG (89,5 % for morula and 85,7 % for blastocysts) [364].

Our blastocyst development rates are superior to those obtained by Shirazi (27,7 % to 53,3 %) after ovine morula vitrification [206]. Bhat obtained inferior re-expansion and hatching rates, after vitrifying ovine blastocysts using 33% EG (30,02 %), but superior rates after using 40 % EG (92,16 %) [476]. A possible explanation if that we used morula and blastocyst embryos while Bhat only use embryos at the blastocyst stage, that seem to present better tolerance to cryopreservation [206,477].

After *in vivo* transfer, only one recipient ewe wasn't pregnant. Nonetheless, similar rates of live birth were obtained for both groups (51,1 % in BSA group and 54,5 % in 100 % CRYO3 group). Gibbons obtained 41 % (morula) to 75 % (blastocyst) of development after *in vivo* transfer of vitrified embryos [364]. Although our results seem to follow this tendency, a larger number of embryos should be used to do a direct comparison.

Altough our slow-freezing and vitrification experiments were not done at the same time, and aren't, therefore, directly comparable, *in vitro* development rates seem to be better after vitrification. This was also observed by Green [478], and by Dalcin [479] when comparing OPS vitrification method and slow-freezing.

These results seem to indicate that animal products (such as BSA) can be replaced by synthetic well-defined products (such as CRYO3), during ovine embryo slow-freezing and vitrification. However, further studies are needed, with a more important number of donor ewes, and, consequently, of embryos, to increase statistical power.

Cryoprotectants play a major role in embryo cryopreservation [477]. Nonetheless, proteins seem to have an important role during cryopreservation, even if the nature of their benefits, and the interactions with the embryo or the cryoprotectants are not precisely defined [238]. High molecular proteins seem to supress or to inactivate ice nuclei in extracellular space, diminishing ice crystal formation [480].

Ménézo and Khatchadourian observed that non-defined peptides could bond to albumin, with subsequent deleterious effects on embryo post-thaw survival [481]. When using cryopreservation media entirely composed of synthetic chemically defined products, such as CRYO3, these interactions are avoided. Moreover, the use of a commercial synthetic medium for embryo cryopreservation, prepared industrially under rigorous quality control instead of laboratory-made media, avoids preparation variability, and increases reproducibility and standardization of the cryopreservation process.

The results obtained after rabbit and ovine embryo slow freezing and vitrification with the synthetic defined product CRYO3 seem very promising, as the substitution of animal products would allow to avoid all the concerns related to animal products. The advantages of using a synthetic defined medium, without animal serum, are widely recognized as providing more defined, more consistent and more reproducible conditions. Furthermore, the use of products with no animal origin decreases animal welfare and ethical concerns.

Chapter IV - Conclusions and perspectives

These results seem to demonstrate that a chemically defined substitute CRYO3 can successfully replace BSA during rabbit embryo and ovine embryo cryopreservation (slow-freezing and vitrification), through the entire process. Indeed, we have replaced animal products with CRYO3 in the flushing medium, in the freezing / thawing (or vitrification / warming) medium, and in the transfer medium, for these species.

The elimination of animal products from embryo cryopreservation media may improve procedure standardization, by avoiding variability in media composition and, consequently, more variable results. Additionally, it would avoid sanitary concerns inherent to animal-derived products and animal welfare and ethical concerns linked to the production of animal sera products.

Our results allowed the incorporation of these methods and media into the quality management system procedures of the biobank CryAnim, a biobank located at VetAgro Sup, Lyon (France), one of the structures that integrate CRB Anim, according to the principle of continual improvement. Consequently, whether to do international (or national) exchanges of biological resources, or to store them in the long-term, the two major drawbacks related with the use of animal products (variability and risk of disease transmission) may be overcome.

Although this critical point is potentially solved and a first step to supress animal products from embryo cryopreservation media in this biobank can have been taken, similar procedures and media should be used enlarged to all the biobanks that may potentially exchange products with the biobank CryAnim. A possible solution would be to render the substitution of animal derived products mandatory to every Cryobank that may be led to do international exchanges. Additionally, the standardization of cryopreservation protocols, ensuring all manipulators applicate the same equilibration steps, cooling / thawing rates and use the same material, would allow to decrease variability and to enhance post-thawing /warming survival, improving all the process of cryobanking.

Chapter V - References

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Chapter VI – Publications

Published articles

1. **Teixeira M**, Buff S, Desnos H, Loiseau C, Bruyère P, Joly T, Commin L. Ice nucleating agents allow embryo freezing without manual seeding. (2017) Theriogenology 104: 173-178 doi: 10.1016/j.theriogenology.2017.08.012

2. **Teixeira M**, Commin L, Gavin-Plagne L, Bruyère P, Buff S, Joly T. Rapid cooling of rabbit embryos in a synthetic medium. (2018) Cryobiology. Available online 24 July 2018 . doi: 10.1016/j.cryobiol.2018.07.006

3. Desnos H, Baudot A, **Teixeira M**, Louis G, Commin L, Buff S, Bruyère P. Ice induction in DSC experiments with Snomax®. (2018) Thermochimica 667: 193-206

Oral presentation

1. **Teixeira M**, Commin L, Gavin-Plagne L, Bruyère P, Philibert A, Buff S, Joly T. A chemically defined medium without animal products for rabbit embryo vitrification. Society for Cryobiology (CRYO) 2016 (Ottawa)

Scientific posters

1. **Teixeira MG**, Commin L, Desnos H, Gavin-Plagne L, Casarini D, Loiseau C, Bruyère P, Beckers J, Joly T, Buff S. *In vivo* ovine embryo production: comparison of two superovulation treatments. International congress of animal reproduction (ICAR) 2015 (Tours)

2. **Teixeira M**, Commin L, Gavin-Plagne L, Bruyère P, Philibert A, Joly T, Buff S. JC-1: A new method to evaluate fresh and cryopreserved rabbit embryo functionality. Society for Cryobiology (CRYO) 2016 (Ottawa)

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