Does the embryo vitrification procedure impact the skewed secondary sex ratio?

Skewed sex ratio and sex-dimorphic effects in offspring born after a vitrified embryo transfer procedure in a rabbit model

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Abstract

Increasing evidence indicates that assisted reproductive technologies (ART) are associated with skewed sex-ratio. However, ART procedures are diverse, being the relatively more invasive intervention the embryo vitrification procedure. Even though this procedure represents an essential advance for ART, a possible disadvantage in the skewed sex-ratio has been scarcely explored. This study aims to test the hypothesis that the vitrification procedure could induce a biased sex ratio and to determine its heritability. The current study using an F1 generation derived from 3-day vitrified embryos found a skewed secondary sex ratio (SSR) imbalanced towards male increased by 12%. Besides, using an F2 generation derived from 3-day vitrified embryos from F1 generation, we found an accumulative SSR imbalanced towards male by 25%. Finally, using an F2c generation derived from crossing F1 generation males with naturally conceived females, we found an SSR imbalanced towards male by 12%, indicating that SRR was heritable. Phenotypic evaluation of the F1, F2 and F2c generation bodyweight identified significant changes at birth, weaning and adulthood. Also, there was a statistically significant interaction between vitrified animals and sex in the F2 generation, demonstrated a plausible sex-dimorphic effect of vitrification procedure. At adulthood, body weight was significantly lower in male compared with female. Therefore, we demonstrate, for the first time, that vitrification procedure induced SSR and show sex-dimorphic patterns bodyweight in adulthood.

Keywords: cryopreservation; embryo transfer; assisted reproduction; sex; blastocyst
1. INTRODUCTION

In the last decades, assisted reproductive techniques have been consolidated as a combination of methods intended to exceed medical difficulties in humans and to enhance the genetic advancement in livestock [1,2]. So, predetermination of sex allows selecting sex-specific embryos for transfer, avoiding sex-linked diseases in humans and animal breeding programmes or animal products [3]. However, assisted reproductive techniques seems to change the sex ratio of offspring compared to natural reproduction [3–5]. The first evidence was published in bovine as early as 1991 [6], and this observation has been confirmed repeatedly in several species including hamster, mouse, porcine, bovine and humans [7–18]. It is well known that the environment seems to modify embryo sex-ratio of offspring (reviewed in Gardner et al. [19]). This fact is of high interest for the reproductive field, where the embryos are mandatory exposed to non-standardized in vitro condition, such as media, media supplements, temperature, CO₂ concentration, pH, osmolarity, etc. [20]. Interestingly, epidemiologic studies associate some assisted reproductive techniques, such as in vitro fertilisation and embryo transfer with higher proportions of the male sex, while others such as intracytoplasmic sperm injection has been proposed as a significant predictor of the female sex [5,9,18,21].

The underlying hypothesis is based on the fact that the lack of proper culture conditions that mimic the in vivo embryonic environment [22,23]. These suboptimal conditions are believed to be manifested in the embryo reprogramming, developing adaptive responses (developmental plasticity), as a cause of disturbances in the dynamic epigenetic remodelling that take place during preimplantation development [23,24]. Consequently, one would expect differences in the embryo reprogramming depending on the nature of the assisted reproductive techniques used [1,23,25–29]. Nowadays, cryopreservation of embryos has become a routine procedure in both veterinary and human medicine [30]. This technique requires embryos exposition to an
environment with toxic chemical agents and shallow non-physiological temperatures, in which they have no intrinsic ability to survive [31]. In this scenario, several studies point out that assisted reproductive techniques significantly skewed sex-ratio [1,15,32–34]. It appears that the embryo reprogramming may have an epigenetic basis, which may have some relevance to the subsequent offspring [35,36].

Despite all those mentioned above, the effect of vitrification procedure and its cumulative impact on skewed sex-ratio has not yet been studied. This study aims to test the hypothesis that the vitrification procedure could induce a biased sex ratio and to determine its heritability.

2. MATERIALS AND METHODS

All chemicals, unless otherwise stated, were reagent-grade and purchased from Sigma-Aldrich Química S.A. (Alcobendas, Madrid, Spain).

2.1. Animals and Ethics

New Zealand rabbits belonging to the Universitat Politècnica de València were used throughout the experiment. The animal study protocol was reviewed and approved by the “Universitat Politècnica de València” Ethical Committee prior to initiation of the study (research code: 2018/VSC/PEA/0116). All experiments were performed in accordance with relevant guidelines and regulations set forth by Directive 2010/63/EU EEC. Animal experiments were conducted in an accredited animal care facility (code: ES462500001091).

2.2. Experimental design
Figure 1 illustrates the experimental design. Firstly, F1 generation was derived as follows. Ten donor females were superovulated and inseminated three days after, being ovulation induced by one µg of buserelin acetate (Hoechst Marion Roussel, Madrid, Spain). Three days after insemination, 289 embryos were recovered and subjected to vitrification. After warming, only undamaged embryos (presenting homogenous cellular mass, mucin coat and spherical zona pellucida) were kept and transferred into foster mothers (14-16 embryos per female). At birth, offspring constituted the F1 generation progeny. Secondly, F1 generation animals reach adulthood, males and females were crossed, and twelve donor females were used to produce 310 embryos that were recovered, vitrified and transferred as above. At birth, offspring constituted the F2 generation progeny, which accumulate two vitrification procedure events. Also, skewed sex-ratio transmission through the male was tested by crossing F1 generation males with 18 natural conceived females. At birth, offspring constituted the F2c generation progeny. In all generations, an equal number of naturally conceived counterparts were generated without embryo manipulation. Therefore, analysing both vitrified and transferred and natural conceived progenies, direct effect of the vitrification procedure can be assessed using an F1 generation and those cumulative using an F2 generation, while using an F2c generation the sex-specific developmental outcomes could be partially attributed to an impact inherited from F1 generation males.

All derived animals, regardless of their experimental group and generation, were microchipped, sexed and weighed on the birthday. Then, the secondary sex ratio (SSR), defined as the proportion of live-born males out of all live births and birth weight were annotated. After that, all animals were weighed at weaning (4th week) and in adulthood (20th week).

2.3. Vitrification procedure
Embryos were vitrified and thawed according to the high efficient protocol developed previously to cryopreserve rabbit embryos by vitrification [37]. This protocol allows the survival of >80% of the thawed embryos, having generated thousands of descendants in our laboratory since its implementation [38]. Briefly, vitrification was achieved in two steps. In the first step, embryos were placed for 2 min in a solution consisting of 12.5% (v/v) dimethyl sulfoxide (DMSO) and 12.5% (v/v) ethylene glycol (EG). In the second step, embryos were suspended for 1 min in a solution of 20% DMSO and 20% EG. Then embryos were loaded into cryotop devices and directly plunged into liquid nitrogen to achieve vitrification. After thawing, embryos were successfully transferred into the oviduct of synchronous foster mothers by laparoscopy, following the protocol described by Besenfelder and Brem [39]. Briefly, foster mothers were anaesthetized and placed in Trendelenburg’s position. Then, embryos were loaded in a 16G epidural catheter, which was inserted through a 17G epidural needle into the inguinal region. Finally, meanwhile, the process was monitored by single-port laparoscopy; the catheter was introduced in the oviduct through the infundibulum to release the embryos. Vitrification procedure was described in detail in our recent report [38].

2.4. Statistical analyses

Differences in SSR were assessed using a generalized linear model (probit link) with binomial error distribution, including the experimental group (vitrification procedure vs natural conceived) and generation (F1, F2, F2c) as fixed effects, and considering their interaction (group*generation). Birth, weaning and adult weight were compared in each generation (F1, F2, F2c) using a generalized linear model (linear), including as fixed effects the experimental group (vitrification procedure vs natural conceived) and sex (male vs female), and considering their interaction (group*sex). Litter size was used as a covariate for data correction. A p-value of less than 0.05 was considered to indicate a statistically significant difference. Data are presented as least square mean ± standard error of the mean. All statistical analyses were carried out using a
commercially available software program (SPSS 21.0 software package; SPSS Inc., Chicago, IL, USA).

3. RESULTS

3.1. F1 generation: SRR

The overall SRR was imbalanced towards male sex in vitrified F1 generation animals compared to natural conceived F1 generation (0.59 ± 0.029 vs 0.42 ± 0.023, for the vitrified procedure and natural conceived, respectively; p<0.05). No effect of generation (0.48 ± 0.030 vs 0.54 ± 0.038 vs 0.50 ± 0.028, for F1, F2 and F2c, respectively; p>0.05) nor interaction between experimental group and generation were detected (Table 1). As showed in Table 1, vitrified F1 generation increased by 12% SSR (0.54 ± 0.052 vs 0.42 ± 0.030, for vitrified procedure and natural conceived, respectively, p>0.05). This effect was more pronounced in the F2 generation, which skew SSR increased by 25% (0.67 ± 0.057 vs 0.42 ± 0.043, for the vitrified procedure and natural conceived, respectively, p>0.05). Besides, we demonstrated that F1 generation males could transmit SSR imbalance by 12% SSR (0.56 ± 0.036 vs 0.44 ± 0.042, for the vitrified procedure and natural conceived, respectively; p<0.05). Therefore, the high SRR increment observed for F2 generation could be attributed to a synergic effect of those inherited from the F1 generation and those newly added by the second vitrification procedure. In this sense, vitrification procedure skew SSR in a direct, cumulative and transmissible manner.

3.2. F2 and F2c generations: Sex-dimorphic and heritable effects

The birth, weaning and adult weight was significantly affected by the experimental group in each generation, but not by sex (Table 2). However, there was a statistically significant interaction between experimental group and sex in the F2 generation. At adulthood, body weight was significantly lower in male compared with the female (Figure 2). Besides, males descended from
F2c generation showed also a deviant bodyweight. This effect would be explained in part the sex-dimorphism seemed in vitrified animals in the F2 generation.

4. DISCUSSION

Our results indicate that early embryo vitrification procedure induces male-biased sex ratio in rabbit, accumulated after two consecutive embryo vitrification procedures. Besides, male-biased sex ratios can be partially attributed to an inherited effect, and it is suggested a dimorphic sex effect on the bodyweight at adulthood after two consecutive embryo vitrification procedures.

The results obtained in this study clearly show that the vitrification procedure significantly skewed sex-ratio in the rabbit. These findings matched with previous studies using assisted reproductive techniques and conducted in other mammalian species, such as hamster, mouse, porcine, bovine and humans [1,15,32–34]. To our best knowledge, this is the first study evaluating how an embryo vitrification procedure could skew sex ratio in a randomised model, where criteria for embryo inclusion are based only on the presence of homogenous cellular mass, and spherical zona pellucida and mucin coat, not in their developmental degree. Our result agrees with Leme et al. [40], who observed deviations in the primary sex ratio in an in vitro bovine model. However, Martínez et al. [41] found no differences in SSR between vitrified and fresh bovine embryo transfers. Nevertheless, the limitations of this study are plentiful, and the risk of bias is high due to the sample size and the embryo criteria inclusion applied.

Sexual dimorphism of mammalian embryos has been observed through differences in development, genetics and epigenetics [42]. Also, early recognition of embryonic sex induces
changes in the properties and composition of uterine fluid [43]. Therefore, it is thought that female and male embryos differ in its specific needs during development and, thereby, the different preimplantation environment can affect the sex ratio at birth [19,44]. Thus, it has been proposed that different stressors during ART will generate different effects in the two sexes, which might be responsible for skewed sex ratios and sex-dimorphic patterns in the long-term offspring phenotype [45]. Therefore, we hypothesised that the radical strategy of vitrification method, that requires embryo exposition to extreme conditions in which they have no intrinsic ability to survive [31], could prove that embryo manipulation stimulates a biased sex ratio as the result of programmed variation within developmental systems. There is a great agreement in the literature on the effects of ARTs on male-biased sex ratio [21]. It is known that culture media may favour the selection of more male blastocysts for transfer. A plausible explanation for this effect is that blastocysts with the highest degree of expansion at the time of transfer are selected, and it is thought that male embryos have higher preimplantation developmental rates, seeming more viable [17,18,21,46]. Besides, in vitro conditions have been associated with abnormal inactivation in one of the two X chromosomes in females, which lead to higher female embryo mortality at early post-implantation stages [15,47,48]. Furthermore, IVF female embryos showed significantly higher incidence in morphological abnormalities than their male counterparts, accompanied by a higher frequency of abnormal extraembryonic tissues [16,47]. Then, proper placentation events have been observed for IVF male embryos over those female and, accordingly, higher survival of IVF male embryos has been detected at mid- and late-gestation [15]. Our findings reinforce the idea that sex ratio is biased towards males offspring after embryo manipulation during vitrification procedure. However, data on SSR after embryo thawing are still sparse and with differing results. Thus, while some authors reported that cryopreserved embryos could increase SSR, others showed that embryo vitrification decreased the SSR [49,50]. A plausible explanation for this difference is that the faster-developing blastocysts are generally considered more viable and more competent for surviving embryo
transfer and cryopreservation [51,52]. Thereby, as male embryos develop faster, depending on the policy of the medical centre in prioritising the transfer of high-quality embryos in thawed or fresh cycles, the results reflected an increase or decrease in the SRR, respectively. Therefore, the selection criteria for embryos has been proposed as the underlying reason for skewed SSR after embryo thawing in humans [9,49]. Therefore, and matching with the hypothesis proposed by Carvalho et al. [53] decades ago, our data evidence that embryo manipulation during vitrification procedure skewed sex ratio, avoiding any biased as occur in the human clinic.

One of the most exciting findings of our study is that the vitrification procedure induced skewed sex-ratio, being cumulative across generations. This suggests that the effects caused by the first vitrification procedure must be transmissible since otherwise the outcome in the F2 generation was doubled. We develop a crossbred model that confirmed our hypothesis, as the male-biased sex ratio was found in the crossbred animals. There is evidence that developmental alterations induced by ART can be inherited across generations in mammals based on epigenetic mechanisms [35,36]. In light of these results, we suggest that sex-dependent developmental reshapings induced by vitrification procedure might have an epigenetic basis, but further studies are needed to confirm it.

During preimplantation development, male and female embryos can display phenotypic differences that can only be attributed to the transcriptional differences resulting from their different sex chromosome complements (for review see Bermejo-Alvarez et al. [45]). This sex transcriptional differences can affect several molecular pathways, which may have developmental consequences, including sex-selective embryo loss and sex-specific epigenetic responses to environmental hazards, leading to sex-dimorphic long-term effects. The results obtained in this study indicated that although vitrification procedure induced long-term phenotypic changes in the F1 generation, it was not sex-dimorphic. It suggests that those male
and female vitrified embryos that implant, and ultimately complete gestation, have the same ability to sustain developmental reshapes induced by the vitrification procedure. However, animals subjected to two following vitrification procedures showed a sex-dimorphic effect on adulthood, being more deviant in males than in females. This finding match with previous studies conducted in mice and humans [34,54]. Other studies have also shown IVF-induced significant sexual dimorphic patterns [15,26,32,33]. However, it is interesting to note that the effects of a first vitrification procedure seemed to be inherited only by male offspring, based on crossbred progeny information yielded (F2c generation). Therefore, it is possible that synergy might explain sex-dimorphic pattern observed in animals subjected to two following vitrification procedures among male-to-male inherited effect. A possible explanation for the male-biased growth defects is the gender-specific disruption of epigenetic events, such as gene imprinting and DNA methylation [15,55–58]. In addition, it is thought that both oocyte and reproductive tract may preferentially select the sex-specific population of sperm [19,45]. Future studies should be designed to crossbreed naturally conceived females and vitrified males to decipher if sex-specific developmental reshapes could also be transmitted by the female.

5. CONCLUSION

In summary, this work has demonstrated, for the first time, that embryo vitrification procedure, including vitrification and embryo transfer, skewed offspring sex ratio and induce long-term effects that can be sex-dimorphic in rabbit. Moreover, our data support that preimplantation embryo development is a particularly sensitive environmental period. Besides, the vitrification procedure could determine adult phenotype, which may vary between both genders. Future studies should be conducted at understanding the epigenetic mechanisms holding the reported findings.
6. REFERENCES


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**CRediT authorship contribution statement**

Francisco Marco-Jiménez: Conceptualization, Methodology, Investigation, Formal analysis, Resources, Funding acquisition, Supervision. Ximo Garcia-Dominguez; Methodology,
Investigation, Writing - original draft. Jose Salvador Vicente: Conceptualization, Methodology, Investigation, Formal analysis, Resources, Funding acquisition, Supervision.

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Table 1. Impact of embryo vitrification procedure on the secondary sex ratio in rabbit.

<table>
<thead>
<tr>
<th>Generation</th>
<th>Vitrified-transferred</th>
<th>Naturally-conceived</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>352</td>
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<tr>
<td>F1</td>
<td>368</td>
<td>0.54 ± 0.052&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>F2</td>
<td>203</td>
<td>0.67 ± 0.057&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>F2c</td>
<td>323</td>
<td>0.56 ± 0.036&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

n: number of rabbits. Data are expressed as least square means ± standard error of means. The F1 generations derived from 3-day vitrified-transferred embryos and naturally mating. The F2 generations derived from 3-day vitrified-transferred embryos from vitrified F1 generation and naturally mating from F1 generation. The F2c generation derived from mating vitrified F1 generation males and naturally conceived F1 generation females. <sup>a,b</sup> Values within a row with different superscripts differ (p<0.05).
Table 2. Significance of factors and interactions at birth, weaning and adult weight.

<table>
<thead>
<tr>
<th>TRAIT</th>
<th>Body weight</th>
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<tbody>
<tr>
<td></td>
<td>Birth</td>
<td>Weaning*</td>
<td>Adult weight†</td>
<td></td>
</tr>
<tr>
<td>GENERATION</td>
<td>F1</td>
<td>F2</td>
<td>F2c</td>
<td>F1</td>
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<tr>
<td>Group</td>
<td>*</td>
<td>*</td>
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<td>Sex</td>
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<td>ns</td>
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<tr>
<td>INTERACTION</td>
<td>Group*Sex</td>
<td>ns</td>
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<td>ns</td>
</tr>
<tr>
<td>COVARIATES</td>
<td>Litter size</td>
<td>*</td>
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</tbody>
</table>

*Weaned at 4 weeks. †Considering the adult weight at 20 weeks. The F1 generation derived from 3-day vitrified-transferred embryos and naturally mating. The F2 generation derived from 3-day vitrified-transferred embryos from vitrified F1 generation and naturally mating from F1 generation. The F2c generation derived from mating vitrified F1 generation males and naturally conceived F1 generation females. *Significant at p<0.05. ns: nonsignificant.
**Figure 1.** Experimental design. The F1 generation derived from 3-day vitrified-transferred embryos and naturally mating. The F2 generation derived from 3-day vitrified-transferred embryos from vitrified F1 generation and naturally mating from F1 generation. The F2 crossbred generation derived from mating vitrified F1 generation males and naturally conceived F1 generation females.
Figure 2. Sex-dimorphic effect of vitrification procedure on the body weight. Weaned at 4 weeks. Adulthood was consider at 20 weeks. NC: natural conceived animals. VT: animals derived from vitrification procedure. The F1 generation derived from 3-day vitrified-transferred embryos and naturally mating. The F2 generation derived from 3-day vitrified-transferred embryos from vitrified F1 generation and naturally mating from F1 generation. The F2c generation derived from mating vitrified F1 generation males and naturally conceived F1 generation females. Values between both sexes differ within each experimental group (p<0.05). *Asterisk denote significant differences of VT animals compared with its NC counterpart (p<0.05).