In vitro and in vivo viability of vitrified and non-vitrified embryos derived from eCG and FSH treatment in rabbit does

Gamal Mohamed Kamel Mehaisen a,c, María Pilar Viudes-de-Castro b, José Salvador Vicente a,*, Raquel Lavara a

a Laboratorio de Biotecnología de la Reproducción, Departamento de Ciencia Animal, Universidad Politécnica de Valencia, Camino de Vera 14, 46071 Valencia, Spain
b Centro de Investigación y Tecnología Animal, Instituto Valenciano de Investigaciones Agrarias, Apdo 187, Polígono La Esperanza 100, 12400 Segorbe, Castellón, Spain
c Department of Animal Production, Faculty of Agriculture, Cairo University, 9 Gamaa Street, 12111 Giza, Egypt

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Abstract

This study aimed to evaluate the in vitro and in vivo viability of vitrified and non-vitrified embryos derived from eCG and FSH treatments in rabbit does. Ninety-six nulliparous does were randomly subjected to consecutive superovulation treatments with eCG (20 IU/kg body weight intramuscularly (i.m.), eCG group), FSH (3 × 0.6 mg/doe at 24 h intervals i.m., FSH group), or without superovulation treatment (control group). Does were artificially inseminated 3 days later and ovulation was induced immediately by hCG (75 IU/doe intravenous). Seven experimental groups were differentiated: first FSH and eCG treatment, second FSH and eCG treatment, eCG-interchanged group (does with previous FSH treatment), FSH-interchanged group (does with previous eCG treatments) and control group. Embryos were collected in vivo by laparoscopy 76–80 h post-insemination in the first and second recovery cycles and post mortem in the third recovery cycles. The ovulation rate was significantly higher in does treated with the first-FSH than in those treated with eCG or in control does (25.2 ± 2.0 versus 19.2 ± 1.4 to 11.0 ± 1.5, and 12.2 ± 1.2, first-FSH, first-eCG to second-eCG and control groups, respectively, P < 0.05). Significant differences were observed in the total recovery

* Corresponding author. Tel.: +34 96 387 97 54; fax: +34 96 387 74 39.
E-mail address: jvicent@dca.upv.es (J.S. Vicente).
influenced by ovulation rate in each group (20.3 ± 2.2 to 9.4 ± 1.2, first-FSH to control groups). Embryo donor rate (donor with at least one normal embryo) was similar among groups with an overall of 75.1%. The number of normal embryos recovered per doe with at least one normal embryo increased significantly in relation to ovulation rate (17.7 ± 2.2 to 8.4 ± 3, first-FSH and control groups). The vitrification of embryos negatively affected their in vitro development to hatched blastocyst in all groups (88.1% versus 48%, \( P > 0.05 \)). However, after embryo transfer, this negative effect was only observed in superovulated vitrified embryos (16.8 and 12.8% versus 39.4% total born rate from eCG, FSH and control vitrified groups, \( P < 0.05 \)). Results indicated that the primary treatments with eCG or FSH increased the number of normal embryos recovered per donor doe, but these embryos are more sensitive to vitrification protocols.

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Keywords: Superovulation; Vitrification; Embryo recovery; Embryo transfer; Rabbit

1. Introduction

Embryo cryopreservation and transfer have become important techniques to enhance the reconstitution of populations and the distribution of high genetic merit in rabbits. Embryo transfer could be definitive evidence that any embryo-related manipulation, such as superovulation [1,2], in vitro culture [3,4] and cryopreservation procedures [5–9], is compatible with the normal development of embryos.

Vitrification is a simple and cheap technique that permits the rapid cooling of liquid medium in the absence of ice crystal formation by using high levels of cryoprotectants [10–13]. Many protocols have been used for vitrification of rabbit embryos with different in vitro and in vivo viabilities post-vitrification [2,4–7,14–18]. However, the exposure of embryos to high levels of cryoprotectant solutions may induce deleterious effects on the embryo development after de-vitrification [19–22], especially at the early foetal development [17].

Superovulation treatment with eCG or FSH is still used commonly in rabbit does in order to ensure the maximum number of normal embryos recovered per donor [2,18,23–28]. A single injection of eCG has been administered in the range of 50–200 IU/doe with a high variability in the production of oocytes and embryos [18,27,29–31], while FSH usually needs to be administered twice daily over 3–4 days to stimulate the same amount of follicular growth that would result from a single injection of eCG [1,2,24]. However, Schmidt et al. [30] obtained equivalent ovarian and embryo recovery by the simple once daily administration of FSH-p for three consecutive days. The high doses of both FSH and eCG resulted in the production of a higher number of embryos [18,26,30,32,33]. However, the high doses and repeated administration of eCG and FSH may cause various problems, such as the ovulation of a high number of abnormal haemorrhagic and cystic follicles [30,34,35] and the decrease of embryo recovery rates [30,32,36], as well as the cytogenesis defects and chromosomal alterations of recovered embryos [2,37]. Moreover, Parvex [38] found that superovulation treatment affected the development of embryos in vitro and in vivo and resulted in abnormality and death of embryos after transfer.

The purpose of this study was to evaluate the in vitro and in vivo viability of vitrified and non-vitrified embryos derived from eCG and FSH treatment in rabbit does selected for growth rate.
2. Materials and methods

2.1. Animals

Ninety-six nulliparous rabbit does were used from a synthetic breed (R line) established in the Department of Animal Science (UPV, Spain). This line has been selected since 1989 for growth rate from weaning to slaughter (28–70 days of age) [39]. The does were 4.5–5 months old and 4–5 kg body weight. All does were kept individually under the same environmental conditions.

2.2. Embryo recovery

Does were randomly distributed into three groups according to superovulation treatment and were subjected to three consecutive recovery cycles: eCG group \( (n = 35) \) induced to superovulation with 20 IU eCG/kg body weight intramuscularly (i.m.) (Gonaser, Hipra, S.A.), FSH group \( (n = 30) \) induced to superovulation with 0.6 mg FSH i.m. (Ovagen, ICPbio, UK) diluted in 1 ml of physiological saline serum supplemented with polyvinylpyrrolidone (30%, w/v, molecular weight 40,000, Sigma) administered three times at 24 h intervals, and Control group \( (n = 31) \) without superovulation treatment. Three days after the first injection, only receptive does (does with red or violet vulvar lips) were artificially inseminated with 0.5 ml of semen pool from, at least, three fertile bucks of the same line. Ejaculates used were selected according to motility criteria, more than 70% of progressive motility, and diluted 1:3 with a Tris-citric-glucose extender described by Vicente et al. [40]. More than 20 million sperms were used in each insemination. Immediately after insemination, ovulation was induced by 75 IU of hCG intravenously (i.v.) (Coriogan, Ovejero) in the marginal ear vein. Embryos were collected in vivo by ventral midline laparoscopy 76–80 h post-insemination [41]. First, anaesthesia was induced by an intramuscular injection of 16 mg xylazine (0.8 ml of Rompun; Bayer AG, Leverkusen, Germany), followed by an intravenous reinjection of ketamine chlorohydrate at the rate of 1.2 ml/kg body weight (Imalgene; Merial, S.A., Lyon, France) to maintain does under anaesthesia during laparoscopy. Embryos were recovered by separate perfusion of each oviduct with 5 ml of pre-warmed collection media [Dulbecco’s phosphate-buffered saline (DPBS; Sigma–Aldrich Quimica S.A., Alcobendas, Madrid, Spain), supplemented with antibiotics (penicillin and streptomycin; Penivet 1)], followed by perfusion of each uterine horn with 50 ml. Finally, the collection media was aspirated from the vagina by a Foley Catheter (Minitube, Tiefenbach, Germany) connected with a vacuum pump of 20–40 mmHg. After embryo collection, the reproductive tract of doe was washed with 0.1% ethylene diamine tetra acetic acid in saline solution (EDTA, Sigma) in order to prevent any abdominal adhesions post-laparoscopy and the doe was injected i.m. with 1 ml of antibiotics (penicillin and streptomycin; Penivet (1)) in order to prevent any bacterial infection. Twelve days post-insemination, does were treated with 0.2 ml cloprostenol i.m. (Planate; Schering-Plough, S.A., Frieseoythe, Germany) to prevent unwanted gestation. The same superovulation treatment was repeated in each group and does were subjected to the second in vivo recovery at intervals of 21 days. At the end of the experiment (third recovery cycle), the superovulation treatment was interchanged and does were subjected to the last
recovery post-mortem (PM recovery). Does were killed 72 h post-insemination and the reproductive tract was immediately removed. Embryos were recovered by perfusion of each oviduct with 5 ml followed by perfusion of each uterine horn with 15 ml of DPBS containing 0.2% of bovine serum albumin (BSA, Sigma). Presumptive embryos were scored by morphological criteria. Only embryos in morulae or young blastocyst stage without morphological abnormalities in mucin coat, zona pellucida and embryo cells were catalogued as normal embryos.

The ovulation rate, the number of haemorrhagic follicles, the number of oocytes, abnormal and normal embryos per doe, and the number of normal embryos recovered in morulae stage or blastocyst stage were recorded.

2.3. Embryo vitrification

Six hundred and twenty-two embryos were vitrified and de-vitrified using the methodology described by Vicente et al. [6]. Briefly, the vitrification procedure was carried out in two steps at 20 °C. In the first step, embryos were placed for 2 min in a vitrification solution consisting of 12.5% (v/v) dimethyl-sulphoxide (1.75 M DMSO, Sigma) and 12.5% (v/v) ethylene glycol (2.23 M EG, Sigma) in DPBS supplemented with 0.2% (w/v) of BSA. In the second step, embryos were suspended for 1 min in a solution of 20% (v/v) DMSO and 20% (v/v) EG in DPBS supplemented with 0.2% (w/v) of BSA. Then, embryos suspended in vitrification medium were loaded into 0.25 ml plastic straws (IMV, L’Aigle, France) between two drops of DPBS separated by air bubbles. Finally, the straws were sealed and plunged directly into liquid nitrogen.

De-vitrification was performed in a water bath at 20 °C for 10–15 s. The vitrification medium was removed in two steps. In the first step, the embryos were expelled with the medium into a solution of DPBS with 0.33 M sucrose for 5 min, and in the second step embryos were washed in a solution of DBPS for another 5 min.

2.4. Embryo culture

A total of 185 non-vitrified and 198 de-vitrified embryos from control donor group and from the first and interchanged recovery cycles of superovulated donor groups were cultured for 48 h in medium 199 + 20% FBS (v/v) (Sigma) at 38.5 °C, 5% CO2 and saturated humidity. The in vitro development ability of non-vitrified and de-vitrified embryos until hatched blastocyst stage was recorded for analysis.

2.5. Embryo transfer

A total of 48 transfers on recipient does from a maternal line selected for litter size at weaning were carried out. Ovulation was induced in receptive does 55–60 h before transfer with intramuscular dose of 1 µg of Buserelin Acetate (Hoechst, S.A.). The recipients were anaesthetized as previously described with donor does. Nine to thirteen intact embryos were transferred to each recipient doe. In total, 272 non-vitrified and 277 de-vitrified embryos were used from control group and from the first and interchanged recovery cycles of superovulated donor groups.
In vivo survival rate was assessed on the basis of pregnancy rate (number of pregnant recipient does at 10th day post-transfer/total recipient does), the total born rate (died + live pups) and the live-born rate on the transferred embryos.

2.6. Statistical analysis

Seven experimental groups were defined to analyse the recovery variables:

(a) Control: donor does without superovulatory treatment (a preliminary analysis was performed to evaluate the effect of recovery cycles on embryo recovery variables in control does, and showed that repeated recoveries of control does had similar results to that obtained by the initial recoveries).
(b) eCG-first treatment.
(c) eCG-second treatment.
(d) eCG-interchanged treatment: donor does with two previous FSH treatments receiving the first eCG treatment.
(e) FSH-first treatment.
(f) FSH-second treatment.
(g) FSH-interchanged treatment: donor does with two previous eCG treatments receiving the first FSH treatment.

A chi-square test with Yates’ correction (Statgraphics Plus 4.1, Statistical Graphics Corp., copyright 1994–1999, USA) was used to compare the effects of the seven groups defined previously on the receptivity rate and the embryo donor rate (percentage of donors with at least one normal embryo from ovulating does).

The effect of superovulatory treatment (control-without-, first-eCG, second-eCG, interchanged-eCG, first-FSH, second-FSH and interchanged-FSH treatments) on the ovulation rate, the number of haemorrhagic follicles, the total recovery (oocytes, abnormal and normal embryos per doe), abnormality rate (oocytes + abnormal embryos on total recovery per doe), the percentage of oocytes in total recovery, the number of normal embryos recovered in does with at least one normal embryo and the percentage of embryos in blastocyst stage per donor doe were analysed by a general linear model (Statgraphics Plus 4.1, Statistical Graphics Corp., copyright 1994–1999, USA). Abnormality rate, oocytes and blastocyst percentage were analyzed after angular transformation (untransformed data are shown in Tables 1 and 2). A covariance analysis including ovulation rate as covariate were used to study the total recovery and normal embryos.

The effects of the superovulation treatments (control, eCG and FSH), the effect of vitrification procedure and its combinations on the in vitro development to hatched blastocysts, pregnancy rate, the total born and the live-born rates were analysed by chi-square test with Yates’ correction.

3. Results

The overall receptivity rate of females was 76.3% (183/240), and a significant increase in receptivity was observed in does treated with eCG groups than does treated with first-
FSH, interchanged-FSH or control group (91.4–94.1% versus 53.3, 54.1 and 67.5%, respectively, \(P < 0.05\), Table 1).

The ovulation rate was significantly higher in does treated with first-FSH than in those treated with eCG (first, second, interchanged-eCG groups) or in control does group (25.2 ± 2.0 versus 19.2 ± 1.4, 11.0 ± 1.5, 16.6 ± 2.0 and 12.2 ± 1.1, respectively, Table 1).

<table>
<thead>
<tr>
<th>Does</th>
<th>Receptivity rate (%) (n)</th>
<th>Ovulation rate (LSM ± S.E.) (n)</th>
<th>Total recovery (LSM ± S.E.) (n)</th>
<th>Abnormality rate (LSM ± S.E.) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>77</td>
<td>67.5 bc (52)</td>
<td>12.2 ± 1.1 cd (52)</td>
<td>9.4 ± 1.2 c</td>
</tr>
<tr>
<td>eCG treatment</td>
<td>First</td>
<td>35</td>
<td>91.4 a (32)</td>
<td>19.2 ± 1.4 b (31)</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>32</td>
<td>93.8 a (30)</td>
<td>11.0 ± 1.5 d (29)</td>
</tr>
<tr>
<td></td>
<td>Interchanged</td>
<td>17</td>
<td>94.1 ab (16)</td>
<td>16.6 ± 2.0 bc (16)</td>
</tr>
<tr>
<td>FSH treatment</td>
<td>First</td>
<td>30</td>
<td>53.3 c (16)</td>
<td>25.2 ± 2.0 a (16)</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>25</td>
<td>96.0 a (24)</td>
<td>11.4 ± 1.6 d (24)</td>
</tr>
<tr>
<td></td>
<td>Interchanged</td>
<td>24</td>
<td>54.1 c (13)</td>
<td>19.6 ± 2.3 ab (13)</td>
</tr>
</tbody>
</table>

**Table 1**
Receptivity, ovulation, recovery and abnormality rates as affected by treatments of eCG and FSH and recovery cycles

<table>
<thead>
<tr>
<th>Embryo donor ratea (n)</th>
<th>Normal embryosb (LSM ± S.E.)</th>
<th>Blastocyst ratec (LSM ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>78.8 (41)</td>
<td>8.4 ± 1.3 b</td>
</tr>
<tr>
<td>eCG treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First</td>
<td>80.6 (25)</td>
<td>14.1 ± 1.6 b</td>
</tr>
<tr>
<td>Second</td>
<td>62.1 (18)</td>
<td>6.3 ± 1.9 b</td>
</tr>
<tr>
<td>Interchanged</td>
<td>75.0 (12)</td>
<td>7.0 ± 2.3 b</td>
</tr>
<tr>
<td>FSH treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First</td>
<td>87.5 (14)</td>
<td>17.7 ± 2.2 a</td>
</tr>
<tr>
<td>Second</td>
<td>62.5 (15)</td>
<td>7.4 ± 2.1 b</td>
</tr>
<tr>
<td>Interchanged</td>
<td>84.6 (11)</td>
<td>12.2 ± 2.5 ab</td>
</tr>
</tbody>
</table>

**Table 2**
Embryo donor rate and normal recovered embryos by eCG and FSH treatment and recovery cycle

**LSM ± S.E.:** least square mean ± standard error; (n): number of does. Values with different letters (a, b, c) in the same column are statistically different (\(P < 0.05\)).

a Embryo donor rate: as percentage of donor does with at least one normal embryo respect to ovulating does.
b Normal embryos: normal embryos recovered in does with at least one normal embryo.
c Blastocyst rate as a percentage of embryos in blastocyst stage per donor with least one normal embryo.
The second treatments of FSH and eCG did not increase significantly the ovulation rate in comparison to the control group, or interchanged eCG or FSH groups, $P < 0.05$, Table 1). Significant differences were observed in the total recovery rate among the groups, first-eCG and first-FSH groups showed the highest total recovery ($15.4 \pm 1.6$ and $20.3 \pm 2.2$, respectively, Table 1), however, after including the ovulation rate as covariate in this analysis, these differences were not significant (ovulation rate coefficient: $-0.87 \pm 0.031$, $P < 0.001$, data not shown in tables). Abnormality rate was similar in the seven groups, the overall abnormality rate was $34.1 \pm 4.6\%$ (Table 1). Oocytes recovered from total recovery were similar among the groups ($25.4 \pm 4.6\%$, data not shown in tables), showing high fertilising failures in this rabbit line. The number of haemorrhagic follicles was higher in ovaries from first-FSH and interchanged-FSH groups ($5.7 \pm 1.0$ and $6.4 \pm 1.2$, respectively, data not shown in tables) than in the rest of groups ($0.9 \pm 0.5$, $1.9 \pm 0.7$, $1.9 \pm 0.8$, $0.8 \pm 1.0$ and $2.9 \pm 0.8$, control, first-eCG, second-eCG, interchanged-eCG and second-FSH, respectively, $P < 0.05$, data not shown in tables).

Overall embryo donor rate was $75.3\%$ (136/181), not significantly different among groups, however, embryo donor rate was slightly low in the second treatments of eCG and FSH groups ($62.1$ and $62.5\%$, respectively, Table 2). The number of normal embryos recovered per doe with at least one normal embryo was significantly higher in the first-FSH group than in eCG groups and the control group ($17.7 \pm 2.3$ versus $14.1 \pm 1.6$, $6.3 \pm 1.9$, $7.0 \pm 3.3$ and $8.4 \pm 1.3$, respectively, $P < 0.05$, Table 2), but similar to the interchanged-FSH group ($12.2 \pm 2.5$, Table 2). After covariance analysis including ovulation rate, these differences were not significant (covariate coefficient: $-0.47 \pm 0.044$, $P < 0.001$, data not shown in tables).

The percentage of normal embryos recovered in blastocyst stage was statistically similar among the groups. However, we observed that $19.4 \pm 3.4\%$ of normal embryos recovered from control group were in blastocyst stage versus $1.4 \pm 6.9$ to $5.6 \pm 4.4\%$ in the first-eCG, second-eCG, interchanged-eCG, first-FSH and interchanged-FSH groups ($P < 0.1$, data shown without angular transformation, Table 2), but similar to the second-FSH group ($14.3 \pm 5.7\%$).

In total, 598 recovered embryos were vitrified and the percentage of intact embryos was $79.4\%$. No significant differences in the percentage of intact embryos were observed between superovulation treatments ($81.1$, $78.2$ and $78.9\%$ in control, eCG and FSH groups, respectively, data not shown in tables).

The in vitro viability of non-vitrified and vitrified embryos recovered from does treated with eCG, FSH or controls is shown in Table 3. In general, the development of de-vitrified embryos to hatched blastocysts was significantly lower than non-vitrified embryos ($48.0$ versus $88.1$ development rate for vitrified versus non-vitrified embryos, $P < 0.05$, Table 3). The in vitro development rate of non-vitrified embryos recovered from superovulated does with eCG or FSH was lower than control, although this difference was not significant. On the other hand, vitrification of embryos recovered from superovulated or control does significantly decreased its in vitro viability in comparison with non-vitrified embryos ($67.9\%$ versus $92.1\%$, $34.3\%$ versus $84.4\%$ and $46.7\%$ versus $87.9\%$ in vitro development rate for vitrified versus non-vitrified embryos recovered from control, eCG and FSH groups, respectively, $P < 0.05$, Table 3).
The in vivo viability of non-vitrified and de-vitrified embryos recovered from does treated with eCG, FSH or controls is presented in Table 4. In general, 77.1% of recipient does were pregnant, and from 549 embryos transferred, 34.4% total born and 27.7% live born were scored. The vitrification of embryos significantly decreased the pregnancy rate after transfer (62.5% versus 91.7% for vitrified embryos versus non-vitrified embryos, respectively, \( P < 0.05 \), Table 4). The pregnancy rate in vitrified embryos tended to decrease more than in non-vitrified embryos when comparing within each superovulation treatment (87.5% versus 100% in control; 37.5% versus 87.5% in eCG group; 62.5% versus 87.5% in FSH group), although this difference was significant only in eCG group (Table 4). It was also observed that vitrification significantly decreased the total born rate (23.1% versus

### Table 3

in vitro development of rabbit embryos as influenced by superovulation treatments and vitrification

<table>
<thead>
<tr>
<th></th>
<th>Number of cultured embryos</th>
<th>Number of hatched blastocyst</th>
<th>In vitro development rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-vitrified embryos</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>63</td>
<td>58</td>
<td>92.1 a</td>
</tr>
<tr>
<td>eCG group</td>
<td>64</td>
<td>54</td>
<td>84.4 ab</td>
</tr>
<tr>
<td>FSH group</td>
<td>58</td>
<td>51</td>
<td>87.9 a</td>
</tr>
<tr>
<td>Total</td>
<td>185</td>
<td>163</td>
<td>88.1 A</td>
</tr>
<tr>
<td><strong>De-vitrified embryos</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>53</td>
<td>36</td>
<td>67.9 b</td>
</tr>
<tr>
<td>eCG group</td>
<td>70</td>
<td>24</td>
<td>34.3 c</td>
</tr>
<tr>
<td>FSH group</td>
<td>75</td>
<td>35</td>
<td>46.7 c</td>
</tr>
<tr>
<td>Total</td>
<td>198</td>
<td>95</td>
<td>48.0 B</td>
</tr>
</tbody>
</table>

Values with different letters (a, b, c) in the same column are statistically different \( (P < 0.05) \); values with different letters (A, B) in the same column are statistically different \( (P < 0.05) \).

### Table 4

Results of in vivo viability after transfer of non-vitrified and de-vitrified embryos recovered from does treated with eCG, FSH or control

<table>
<thead>
<tr>
<th></th>
<th>Number of recipients</th>
<th>Number of embryos transferred</th>
<th>Pregnancy rate (%)</th>
<th>Total born rate (%)</th>
<th>Live-born rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-vitrified embryos</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>94</td>
<td>100.0 a</td>
<td>43.6 a</td>
<td>39.4 a</td>
</tr>
<tr>
<td>eCG group</td>
<td>8</td>
<td>89</td>
<td>87.5 a</td>
<td>44.9 a</td>
<td>37.1 a</td>
</tr>
<tr>
<td>FSH group</td>
<td>8</td>
<td>89</td>
<td>87.5 a</td>
<td>49.4 a</td>
<td>42.7 a</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>272</td>
<td>91.7 A</td>
<td>46.0 A</td>
<td>39.7 A</td>
</tr>
<tr>
<td><strong>De-vitrified embryos</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>94</td>
<td>87.5 a</td>
<td>39.4 a</td>
<td>33.0 a</td>
</tr>
<tr>
<td>eCG group</td>
<td>8</td>
<td>89</td>
<td>37.5 b</td>
<td>16.8 b</td>
<td>5.6 b</td>
</tr>
<tr>
<td>FSH group</td>
<td>8</td>
<td>94</td>
<td>62.5 ab</td>
<td>12.8 b</td>
<td>8.5 b</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>277</td>
<td>62.5 B</td>
<td>23.1 B</td>
<td>15.9 B</td>
</tr>
</tbody>
</table>

Values with different letters (a, b) in the same column are statistically different \( (P < 0.05) \). Values with different letters (A, B) in the same column are statistically different \( (P < 0.05) \). Pregnancy rate percent: the number of recipient does with positive palpation/number of recipients \( \times 100 \). Total and live-born percent: the number of total or live born/the number of embryos transferred \( \times 100 \).
46.0% in vitrified versus non-vitrified embryos, Table 4) and the live-born rate (15.9% versus 39.7% in vitrified versus non-vitrified embryos, Table 4). The total and live-born rates were significantly lower in vitrified embryos than in non-vitrified embryos from superovulated does with eCG (16.8% versus 44.9% total born rate and 5.6% versus 37.1% live-born rate in vitrified versus non-vitrified embryos, respectively) or with FSH (12.8% versus 49.4% total born rate and 8.5% versus 42.7% live-born rate in vitrified versus non-vitrified embryos, respectively) (P < 0.05, Table 4). However, no significant differences in the total and live-born rates were observed between vitrified and non-vitrified embryos recovered from non-superovulated does (Table 4).

4. Discussion

In previous works, we found that induced superovulation in rabbit does selected for growth rate by subcutaneous administration of 50 IU of eCG did not much improve the recovery rates and the number of normal embryos recovered per donor doe [18,28] when comparing with results of embryo recovery in non-superovulated does [42–45]. In the present study, we tested other superovulation protocols by intramuscular administration of about 100 IU eCG/doe [27] or by three consecutive doses of 0.6 mg of FSH at 24 h intervals [30]. Our results were similar or lower when compared to those obtained when eCG was used with similar dose [27,30,31,46]. Similar findings were obtained by Schmidt et al. [30] and Kauffman et al. [2] when superovulation was induced by FSH. When higher doses of eCG or FSH were used, higher ovulation rate and total embryos were recorded [26]. However, we observed that 25% of donors failed in recovery, the number of haemorrhagic follicles increased and the embryonic development stage at recovery decreased when superovulation was induced by FSH (Table 2). These disadvantages of superovulation were previously reported for either eCG or FSH treatments [1,32,34,35].

In the present study, we found that repeated treatments of superovulation with eCG or FSH did not have the incremental effect on ovulation rate and the embryo recovery, as did the first treatments (Table 1). Renard et al. [1] observed a reduction in the number of recovered embryos and the embryo donor rate after repeating eCG and FSH treatments. The formation of anti-gonadotrophins antibodies in the blood of domestic animals repeatedly treated for superovulation [47–50] may explain the low response of donor does to repeated eCG and FSH treatments in our study. On the other hand, repeating the treatment of ovulation induction with hCG may provoke deleterious effects on the efficacy and the quality of recovered embryos [51,52] that could affect its in vitro or in vivo viability before and after vitrification. The change of the consecutive superovulation protocol for the same female may improve the ovulation rate and increase the number of normal embryos recovered per donor doe.

In the present study, 79.4% of 598 vitrified embryos were scored as intact embryos after de-vitrification. The same problem was reported for the R line by Vicente et al. [9], who observed 82% intact embryos of 382 vitrified embryos. Although we discarded all embryos with damaged zona pellucida to ensure the maximum in vivo development rate [53], we obtained a successful results of viability only in the control group (without superovulation treatment), 67.9% in vitro development to hatched blastocyst stage, 87.5% pregnancy rate,
39.4% total born and 33% live born after vitrification. Vicente and García-Ximénez [14], using the same protocol of vitrification in V line rabbits, obtained similar results in the in vitro development (64.6%) but higher survival rate at birth (57.6%). Vicente et al. [9] using the same vitrification protocol in R line, obtained similar results of in vivo viability after transfer (71% pregnancy rate, 42% total born and 39% live-born rates).

When superovulation with eCG or FSH was applied, the in vitro and in vivo viability of embryos were significantly decreased (Tables 3 and 4). In our work, the in vitro development rate of vitrified embryos was decreased to 34.3% in eCG group and 46.7% in FSH group in comparison with non-vitrified embryos (84.4% in eCG group and 87.9% in FSH group) (Table 3). After transfer of vitrified embryos from eCG or FSH groups, the total born and live-born rates were also decreased (16.8% versus 44.9% in eCG group; 12.8% versus 49.4% in FSH group for total born rate in vitrified versus non-vitrified embryos, respectively, Table 4). These results confirm and extend on previous reports concerning the superovulation and cryopreservation effects on viability of embryos [1,2,37,54]. For example, Renard et al. [1] reported the decrease of live-born rate from 38.5% in non-frozen superovulated embryos to 27.7% in frozen superovulated embryos, while Kauffman et al. [2] obtained 31% live born of non-superovulated vitrified embryos versus 20% live born of vitrified embryos from FSH group. Moreover, Renard et al. [1] observed a negative effect for superovulatory treatment on the live-born rate (56.3% versus 38.5% in non-frozen embryos).

Our embryo recovery data confirm the results of other studies showing that eCG [27,30] and FSH [2,30] increased the ovarian response and the number of recovered embryos. However, all of these reports with our results indicate that embryos recovered from superovulated donors, although they have normal morphological appearance, may exhibit lower developmental potential than embryos recovered from non-superovulated donors [2]. The higher sensitivity of embryos recovered from superovulated does to low temperatures leads to a decrease in their subsequent potential capacity for development after vitrification [21]. Yaakub et al. [55] observed in heifers that a higher superovulatory response could produce an inferior embryo quality. Another hypothesis supported by Vajta [56] may explain the lower viability rate after vitrification based on the number of trophoblastic cells and inner cell mass per embryo. The surviving blastomeres after vitrification procedures may be insufficient in number to permit the re-expansion of the blastocoelic cavity and continue the physiological development.

In conclusion, the first treatments with eCG or FSH increased the number of normal embryos recovered per donor doe, but these embryos are more sensitive to vitrification protocols and have low potential capacity to produce live pups. Repeating the same treatments of eCG or FSH had almost no effect on embryo recovery, but changing the consequent superovulation protocol for the same female may be used as a second primary treatment and so increase the number of normal embryos recovered per donor doe.

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References


