CRYOSURVIVAL OF RABBIT EMBRYOS OBTAINED AFTER
SUPEROVULATION WITH CORIFOLLITROPIN ALFA WITH or WITHOUT LH

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ABSTRACT

The efficiency of an embryo bank depends on provision of optimal conditions for recovery, cryopreservation and transfer to a breed or strain. In this sense, increasing the number of embryos available using superovulation should improve the cryobank efficiency. However, vagueness of response to conventional protocols to control or increase ovarian response and the quality of oocytes and embryos and their cryotolerance remain a challenge. The aim of our study was to evaluate the effect of corifollitropin alpha (CTP) and a recombinant human FSH (rhFSH), alone or supplemented with rhLH, on embryo cryosurvival by in vitro development and OCT4 and NANOG mRNA abundance at blastocyst stage and offspring rate. In vitro development of vitrified embryos was not significantly affected by superstimulation with or without rhLH supplementation, resulting in similar development rates to those of the control groups (fresh and vitrified embryos from non-superstimulated donor does). Blastocysts developed from vitrified embryos showed higher levels of OCT4 transcript abundance than fresh control, while NANOG transcript abundance was only higher in the blastocysts developed from vitrified embryos after superstimulation treatment in comparison with control groups. The implantation and offspring rates at birth were negatively affected by supplementation with rhLH. Both rhFSH or CTP vitrified embryo groups showed an implantation rate similar to those of the control groups, but an offspring rate lower than control. In conclusion, embryos produced using corifollitropin alpha did not compromise the cryosurvival of vitrified embryos in the rabbit. In addition, this study points out the negative effect of rhLH supplementation in terms of offspring rate on embryo vitrification.

Keywords: Superovulation, corifollitropin, rhLH, embryo, cryosurvival, rabbit.
INTRODUCTION

Embryo cryopreservation provides an important tool for animal breeding, enabling the establishment of genome cryobanking and saving embryos for an unlimited time. The efficiency of an embryo bank depends on the provision of optimal conditions for recovery, cryopreservation and transfer for each breed or strain. To this end, different laboratories have successfully developed embryo banks for a reduced number of breeds or strains (main laboratory and livestock species). In this sense, increasing the number of embryos using superovulation treatment will clearly reduce the number of donors, improve the efficiency of animal production (Takeo and Nagata, 2015) and minimise the number of animals in line with the 3Rs principle concept (Russell, 2005). Nevertheless, controlling reproductive events in unusual species or breeds remains a challenge, given the vagueness of response to conventional protocols to control or increase ovarian response and the quality of obtained oocytes and embryos and their cryotolerance in breeds or strains.

In rabbits, to ensure the maximum number of normal embryos recovered per donor, both eCG and pituitary derived FSH have commonly been used to induce superovulation (Kanayama et al. 1994, Kauffman et al. 1998, Hashimoto et al. 2004, Mehausen et al. 2005, 2006, Salvetti et al. 2007). However, superovulation treatments may generate a higher number of abnormal and immature follicles through either increasing the cytogenetic defects or abnormal steroidogenesis pattern, affecting the oocyte competence and consequently the future embryo development (Kennelly and Foote 1965, Fechheimer and Beatty 1974, Paufler et al. 1975, Taneja et al. 1990, Schmidt et al. 1992, Cheng et al. 1999, Hashimoto et al. 2004; Cortell et al. 2015).

Specifically, both oocytes and embryos obtained after FSH treatments seem to have advantages in terms of number and quality versus eCG, but due to the relatively short elimination half-life of FSH and rapid metabolic clearance, two daily injections are required to maintain the threshold level during ovarian superstimulation. Recently, recombinant human FSH (rhFSH) and a long-acting recombinant FSH named corifollitropin alpha (CTP) were successfully assayed in rabbit (Viudes-de-Castro et al. 2015 and 2017). CTP has a plasma half-life of approximately 65 hours and an almost four-fold extended time to peak serum levels in a single injection (Duijkers et al. 2002, Devroey et al. 2004, 2009) and provides both good superovulatory response and similar
in vivo survival rate to non superovulated embryos, especially when CTP is supplemented with LH (Viudes-de-Castro et al., 2017).

The use of LH in superovulation treatments is controversial and unclear; the quality of oocytes or embryos recovered after superovulation treatments varies, and one of the reasons for this may be the variable LH:FSH ratio. Low LH levels might intensify FSH sensitivity in granulosa cells by increasing androgen synthesis during the early stage of folliculogenesis, and this activity is required for normal follicle and oocyte development (Ruvolo et al. 2007, Durnerin et al. 2008). Moreover, high LH levels seem to be detrimental for follicular growth. In rabbits, the effect of LH on superovulation has been studied using purified porcine FSH, obtaining highly variable results (Hashimoto et al. 2004, Salvetti et al. 2007). An LH concentration window has been suggested in women below which estradiol production is not adequate and above which LH may be detrimental to follicular development (Borini and Dal Prato 2005, Balasch and Fábregues 2006). Our studies with recombinant human gonadotropins suggested that in the rabbit, the window of LH is FSH dose dependent, and the higher the concentration of FSH used, the more the LH window shifts to higher concentrations. When the FSH concentration used is low, 10% of LH supplement may exceed the LH requirements and the fertilisation and embryonic development is negatively affected. In contrast, when the FSH concentration used is high, 10% of LH supplementation is essential to increase the number of follicles recruited during the selection phase (Viudes-de-Castro et al. 2015).

Another concerning aspect is the negative interaction between superovulation and cryopreservation, making the embryos more sensitive to cryopreservation (Leoni et al. 2001, Belinger et al. 2004, Mehaisen et al. 2006, Forcada et al. 2011) and decreasing their ability to produce offspring (Maurer et al. 1968, Carney and Foote 1990, Kauffman et al. 1998, Mehaisen et al. 2006). Superovulated embryos seem to have a higher sensitivity to low temperatures and this leads to a decrease in their subsequent potential capacity for development after vitrification, based on a reduced number of trophoblastic cells and inner cell mass per embryo (Vajta 2000, and Leoni et al. 2001). 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.

The aim of the current study was to evaluate the effect of corifollitropin alpha (CTP) and a recombinant human FSH (rhFSH), alone or supplemented with recombinant
human LH, on embryo cryosurvival by in vitro development and mRNA abundance of
OCT4 and NANOG at blastocyst stage and live offspring rate at birth.

MATERIALS AND METHODS

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

Animals and ethical statement

The research was carried out at the experimental farm of the Institute of Science and
Animal Technology (ICTA), Polytechnic University of Valencia. All the experimental
procedures used in this study were performed in accordance with Directive 2010/63/EU
EEC for animal experiments and reviewed and approved by the Ethical Committee for
Experimentation with Animals of the Polytechnic University of Valencia, Spain
(research code: 2015/VSC/PEA/00061).

One hundred and twenty nulliparous does 18-20 weeks old were used. Does belonged to
a New Zealand White line selected for litter size at weaning (Line A, Estany et al.
1989). Animals were housed in flat-deck cages, fed with a standard pellet diet ad
libitum and had free access to water. An alternating cycle of 16 h lights and 8 h of dark
was used.

Hormonal treatment

Ovarian stimulation was induced using corifollitropin alpha (CTP, Elonva, Merck Sharp
& Dohme S.A.; Spain) and recombinant human FSH (rhFSH, Gonal-F 75; Serono
Europe Ltd., London, United Kingdom) either alone or in combination with
recombinant human LH (rhLH, Luveris 75; Serono Europe Ltd., London, United
Rabbit donors, weighing 3.9 to 4.2 kg, were assigned randomly to five experimental groups:

- **Group CTP:** 12 rabbit does were subcutaneously treated once with 3 µg of corifollitropin alpha.

- **Group CTP+rhLH:** 12 rabbit does were subcutaneously treated once with 3 µg of corifollitropin alpha and intramuscularly treated with a 10% of recombinant human LH, distributed in five equal doses at 12-hour intervals.

- **Group rhFSH:** 12 rabbit does were intramuscularly treated with 3 µg of recombinant human FSH, distributed in five equal doses at 12-hour intervals.

- **Group rhFSH+rhLH:** 12 rabbit does were intramuscularly treated with 3 µg of recombinant human FSH in combination with a 10% of recombinant human LH, distributed in five equal doses at 12-hour intervals.

- **Group non-superovulated donor does:** 36 receptive rabbit does without stimulation treatment were used to obtain fresh and vitrified embryos. Fresh embryos from non-superovulated does were used as control.

Superstimulated donor does were inseminated with 1 mL of sperm pool from fertile males of the same line 60 h after the first gonadotropin injection, and ovulation was induced with 1 µg buserelin acetate (Suprefact; Hoechst Marion Roussel, S.A., Madrid, Spain) given intramuscularly. Control group females were inseminated with the same sperm pool and ovulation was induced with 1 µg buserelin acetate. Sperm pool was constituted by 5-6 ejaculates from different males exhibiting a white colour and with a motility rate higher than 70% and morphological abnormality rate lower than 25%. The seminal dose used per donor doe was around 20-30 million sperm.
Embryo recovery

Females were euthanised 72 h after artificial insemination with an intravenous injection of 0.6 g pentobarbital sodium (Dolethal; Vetoquinol, Madrid, Spain) and the reproductive tract was immediately removed. Embryos were recovered by perfusion of each oviduct and uterine horn with 10 mL Dulbecco’s phosphate buffered saline (HyClone™ DPBS liquid without Calcium, Magnesium, Phenol Red; HyClone Laboratories, Logan, Utah, USA) containing 0.2% bovine serum albumin (AMRESCO® Albumin Bovine, (BSA); Solon, USA), 0.133 g/L CaCl₂, 0.100 g/L MgCl₂ and antibiotics (100 IU/mL Penicillin and 0.01 mg/mL Streptomycin, Sigma-Aldrich Quimica S.A., Spain). The recovered fluid was collected into sterile Petri dishes for examination under a stereomicroscope. Embryos were scored by morphologic criteria according to International Embryo Transfer Society classification (IETS). Briefly, only embryos in morula or early blastocyst stages with homogenous cellular mass and spherical mucin coat and zona pellucida were catalogued as normal embryos. In superstimulated groups, only embryos from donor does with more than 30 normal embryos were vitrified to avoid using non-superovulated embryos. Normal embryos from control group were split to immediately culture or transfer as fresh embryos or to vitrify.

A total of 1061 normal embryos were used, 171 non-superovulated fresh embryos and 890 as vitrified from different experimental groups.

Embryo vitrification procedure

Vitrification was carried out in 12 batches. A total of 890 embryos were vitrified and de-vitrified using the methodology described by Vicente et al. (1999). 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 10% (v/v) dimethyl-sulphoxide (1.75M DMSO, Sigma) and 10% (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% EG in DPBS supplemented with 0.2% (w/v) of BSA. Then, embryos suspended in the 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 directly plunged into liquid nitrogen.

De-vitrification procedure was performed by placing the French ministraws 10 cm from nitrogen vapour until the onset of ice formation in the vitrified fraction (milky appearance) and thawing by submerging the straws into a water bath at 20 °C for 10 sec. 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 0.33M sucrose for 5 min, and in the second step the embryos were washed in a DPBS solution for another 5 min.

Devitrified embryos were scored and only undamaged embryos were catalogued as transferable.

**In vitro culture until blastocyst stage**

In vitro culture was performed in 6 batches. A total of 536 embryos were cultured for 48 h in Tissue Culture Medium 199 (TCM199) + 10% Foetal Bovine Serum (FBS, Sigma-Aldrich Quimica S.A., Spain) supplemented with antibiotics (100 IU/mL Penicillin and 0.01 mg/mL Streptomycin, Sigma-Aldrich Quimica S.A., Spain) at 38.5 °C, 5% CO2 and saturated humidity. The in vitro development ability of embryos to hatched state (more than 50% of mass cell extruded to zona pellucida, Figure 1) was recorded.
mRNA expression of OCT4 and NANOG genes. Extraction and quantitative PCR analysis

PolyA RNA was extracted from pools consisting of 13 to 15 in vitro cultured embryos using the Dynabeads kit (Life Technologies, Carlsbad, CA, USA), following the manufacturer’s instructions. Four independent embryo pools were used for each experimental group. Then, reverse transcription was carried out using qScriptTMcDNA Synthesis kit (Quantabio, Beverly, MA, USA), according to the manufacturer’s instructions. Real-time polymerase chain reaction (PCR) reactions were conducted in an Applied Biosystems 7500 system (Applied Biosystems). Every PCR was performed from 5-mL diluted 1:10 complementary DNA (cDNA) template, 250-nM of forward and reverse specific primers (Table 1), and 15 mL of Power SYBR Green PCR Master Mix (Fermentas Gmbh, Madrid, Spain) in a final volume of 20 mL. The PCR protocol included an initial step of 50 °C (2 minutes), followed by 95 °C (10 minutes), and 42 cycles of 95 °C (15 seconds) and 60 °C (30 seconds). After quantitative PCR, a melting curve analysis was performed by slowly increasing the temperature from 65 °C to 95 °C, with continuous recording of changes in fluorescent emission intensity. The specificity was confirmed by melting curve analysis.

Relative expression levels were calculated by \(2^{-\Delta\Delta Ct}\) method adjusted for PCR efficiency (Livak and Schmittgen, 2001), applying the geometric average of H2AFZ (H2A histone family member Z (Mamo et al. 2008) and GAPDH glyceraldehyde-3-phosphate dehydrogenase (Llobat et al., 2012) as a housekeeping normalisation factor. Relative expression of cDNA pool from all samples was used as the calibrator to normalise all samples within one PCR run or between several runs. List of primers used for quantitative real-time polymerase chain reaction is shown in table 1.
Embryo transfer

A total of 491 embryos were transferred into 36 recipient females induced to ovulate 72 hours before transfer with 1 µg of buserelin acetate injected intramuscularly (Hoechst, Marion Roussel, Madrid, Spain). Synchronous females were anaesthetised by intramuscular injection of 16 mg of xylazine (Rompún, Bayer AG, Leverkusen, Germany) following intravenous injection of 16-20 mg ketamine hydrochloride (Imalgène, Merial SA, Lyon, France). Oviductal embryo transfer was performed using the laparoscopic technique described by Besenfelder and Brem (1993). The number of embryos transferred per recipient doe was from 12 to 15. At the end of the transfer, rabbit does were intramuscularly injected with 0.5 mL/doe of enrofloxacin (Baytril 5%, Bayer, Barcelona, Spain), brought back to the flat deck cages and fed a standard pellet diet ad libitum, having free access to water.

Embryo survival rate

Eleven days after ovulation induction, laparoscopy was performed on the recipient does and the number of implanted embryos per female was recorded. Animals were anaesthetised as described above. In vivo implantation rate was calculated as the successful implantation of the total transferred embryos per each recipient. At birth, litter size and individual pup weight were recorded. Offspring rate at birth was defined as the number of pups at birth related to the number of transferred embryos.

Statistical analysis

For development to hatched blastocyst, implantation and offspring rates at birth, a probit link with binomial error distribution was used, including the treatment group as fixed effect. Data on relative mRNA abundance were analysed by ANOVA using a GLM including the treatment group as fixed effect. NANOG data were normalised by an
Arctangent transformation for its subsequent analysis. All statistical analyses were performed with SPSS software (SPSS 16.0 software package; SPSS Inc., 2002, Chicago, IL, USA). Results were reported as least-square means (LSM) with standard error of the mean. LSM were separated using Fisher’s protected least significant difference test, with treatment effect declared significant at P < 0.05.

RESULTS

Of the 890 vitrified embryos, 856 (96.1%) had no damage to their covers and were cultured or transferred.

Effects of superstimulation treatment on in vitro development, implantation and offspring rates

As shown in Table 2, In vitro development of vitrified embryos was not significantly affected by superstimulation with or without rhLH supplementation, resulting in similar development rates to those of the control groups (fresh and vitrified).

The implantation and offspring rates at birth were negatively affected by superstimulation treatment supplemented with rhLH. Both rhFSH or CTP vitrified embryo groups showed an implantation rate similar to those of the vitrified and fresh control groups, but a survival rate at birth lower than the fresh control group (Table 2).

Effects of superstimulation treatment on OCT4 and NANOG relative expression

Blastocysts developed from vitrified embryos showed higher levels of OCT4 transcript abundance than fresh control, while NANOG transcript abundance was only higher in the blastocysts developed from vitrified embryos after superstimulation treatment, in comparison with the fresh and vitrified control groups (Table 2).
DISCUSSION

This study showed that rabbit embryos derived from a long acting rhFSH (CTP) provide a cryosurvival similar to that of fresh embryos derived from unstimulated donors in terms of implantation rate and similar survival rate at birth to those of vitrified embryos derived from unstimulated donors. In addition, the results indicate that superovulation treatment with CTP provides similar cryosurvival to that found when daily rhFSH is administered. Therefore, our results showed that superovulation treatment using CTP is a successful strategy for the establishment of GRBs in rabbit, reducing the level of distress for the animal and making its application more practical and efficient (Viuedes-de-Castro et al., 2017).

When CTP or rhFSH was supplemented with rhLH, implantation and offspring rate were lower. Nevertheless, no negative effects were detected either in the in vitro development or in the transcript abundance analysis of two candidate genes related to embryo development and cell pluripotentiality. The usual in vitro parameters to evaluate oocyte or embryo quality are based on morphological appearance at the recovery moment, development rate, metabolites and some gene expression levels such as factor octamer-binding 4 (OCT4), NANOG homeobox (NANOG) and SOX2, but they may not be enough. Changes in gene expression might trigger failures in development and implantation of the embryos and, consequently, in pregnancy loss. In this study, 72h superovulated vitrified embryos cultured for 48h had OCT4 and NANOG up-regulation versus non-superovulated fresh embryos, showing signs of alterations induced by vitrification process in these embryos, although no changes of in vitro developing rates were observed. These altered expressions were not observed when these genes were analysed in superovulated and non-superovulated fresh embryos (Viuedes-de-Castro et al., 2017). However, these results agree with the epigenetic changes in developing
vitrified mouse embryos at promoter region of three pluripotency genes, including OCT-4, observed by Wang et al. (2010) and Zhao et al. (2012). Only vitrified embryos from non-superovulated donors did not have NANOG over-expressed. In any case, the in vitro results were indicative of OCT4 and NANOG disturbances and this could explain the minor viability at birth of the vitrified embryos versus fresh control embryos, but did not support the lower viability of vitrified embryos obtained from superstimulation treatment supplemented with rhLH. It has been demonstrated that in vitro culture systems do not mimic the uterine environment and in vitro developed embryos differ from their in vivo counterparts, even after vitrification (Corcoran et al., 2006; Saenz-de-Juano et al., 2010, Asku et al., 2012, Marco-Jiménez et al., Vicente et al., 2013).

Vitrification and transfer procedures provide a sub-optimal condition and stressful environments to which the embryos must adapt (Saenz-de-Juano et al., 2016). This could contribute to the embryo-foetal programming and have consequences even in the neonatal period or adulthood (Pluess and Belsky, 2011). Previous studies in rabbit revealed that vitrification causes developmental differences in gastrulation, expression gene and proteomic disturbances in placental tissues with consequences on foetal mortality and differences in postnatal growth patterns (Mocé et al, 2010, Vicente et al, 2013, Saenz-de-Juano et al., 2014, 2015). The present study shows the difficulties of rabbit vitrified embryos in surviving until the end of gestation, but especially if embryos come from superstimulation treatment supplemented with rhLH. Some authors have shown beneficial effects of LH on ovarian response, oocyte maturation and in vivo survival (Sirard et al. 2006, Ruvolo et al. 2007, Viudes-de-Castro et al. 2015 and 2017 in rabbit). Viudes-de-Castro et al. (2017) showed that fresh embryos obtained from donors superstimulated with CTP and supplemented with rhLH reached similar implantation and birth survival rates to those of control embryos, suggesting a greater
embryo competence to implant than the other superstimulated groups with rhFSH with or without LH supplementation. However, in the present study, when CTP+rhLH was associated with the cryopreservation process, the in vivo cryosurvival or offspring rate of vitrified embryos was lower than for CTP alone and the vitrified control group. In the same way, rhFSH+rhLH group showed these detrimental effects on in vivo cryosurvival.

Taking into account the modifications in the expression of the genes and the highest percentage of implanted embryos observed after the transfer of vitrified embryos from CTP and rhFSH groups (66 and 73%), it will be necessary to define adequate asynchrony or the recipient doe strain in order to improve survival through the reduction of foetal losses. Marco-Jiménez et al (2013) observed a higher rate of embryos developed to term when 12h asynchrony between vitrified embryo and recipients was used. In this study, we decided to test the cryosurvival of superovulated rabbit embryos in a synchronic approach.

In conclusion, our findings indicate that embryos produced using corifollitropin alpha superovulation treatment do not compromise the cryosurvival of vitrified embryos in the rabbit. In addition, the present study clearly highlights the negative effect of rhLH supplementation in rhFSH superstimulation treatment in terms of offspring at birth, whereas there was no effect on in vitro embryo developmental rates. Therefore, corifollitropin alpha is a successful strategy for the establishment of genetic resource banks in the rabbit, reducing the level of distress for the animal and making its application more practical and efficient.
ACKNOWLEDGEMENTS

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Figure 1. Non-developed morula (A), hatching (B) and hatched (C) rabbit blastocyst embryos after 48h of in vitro culture.
Table 1. List of primers used for quantitative real-time polymerase chain reaction.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT4</td>
<td>CGAGTGAGAGGCAACTTGG</td>
<td>CGGTTACAGAACCACACACG</td>
<td>125</td>
</tr>
<tr>
<td>NANOG</td>
<td>CCAGGTGCTCTTACAGACA</td>
<td>TCACTACTCTGGGACTGGGA</td>
<td>104</td>
</tr>
<tr>
<td>H2AFZ</td>
<td>AGAGCCGGCTGCCAGTCC</td>
<td>CAGTCGCGCCCACACGTCC</td>
<td>85</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCCGCTTCTTCTCGTCAG</td>
<td>ATGGATCATTGATGGCGACAACAT</td>
<td>144</td>
</tr>
</tbody>
</table>

H2AFZ: H2A histone family member Z; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase;

OCT4: transcription factor octamer-binding 4; NANOG: NANOG homeobox.
Table 2. Effect of ovarian stimulation treatments on in vitro and in vivo development (least square mean ± standard error).

<table>
<thead>
<tr>
<th>Embryo type</th>
<th>Experimental groups</th>
<th>N</th>
<th>CE</th>
<th>Hatched OCT4</th>
<th>NANOG TE</th>
<th>Implantation rate</th>
<th>Offspring rate at birth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitrified</td>
<td>FSH-CTP</td>
<td>165</td>
<td>82</td>
<td>0.55 ± 0.06</td>
<td>0.87 ± 0.083&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.55 ± 0.084&lt;sup&gt;a&lt;/sup&gt;</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>FSH-CTP+LH</td>
<td>176</td>
<td>95</td>
<td>0.48 ± 0.05</td>
<td>0.85 ± 0.083&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.54 ± 0.084&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>FSH</td>
<td>175</td>
<td>98</td>
<td>0.60 ± 0.05</td>
<td>0.79 ± 0.083&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.62 ± 0.084&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77</td>
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<tr>
<td></td>
<td>FSH+LH</td>
<td>176</td>
<td>87</td>
<td>0.46 ± 0.05</td>
<td>0.76 ± 0.083&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.54 ± 0.084&lt;sup&gt;a&lt;/sup&gt;</td>
<td>89</td>
</tr>
<tr>
<td>Fresh</td>
<td>Non-superovulated</td>
<td>164</td>
<td>86</td>
<td>0.42 ± 0.05</td>
<td>0.73 ± 0.083&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.23 ± 0.084&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>Fresh Non-superovulated</td>
<td>171</td>
<td>88</td>
<td>0.57 ± 0.05</td>
<td>0.48 ± 0.083&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.28 ± 0.084&lt;sup&gt;b&lt;/sup&gt;</td>
<td>83</td>
</tr>
</tbody>
</table>

CTP: Corifollitropin α single-injection; CTP+rhLH: A single-injection of corifollitropin α and five equal doses at 12-hours interval of recombinant human LH; rhFSH: Five equal doses at 12-hour intervals of recombinant human FSH. rhFSH+rhLH: Five equal doses at 12-hours interval of recombinant human FSH plus recombinant human LH; Non-superovulated group: Five doses at 12-hours interval of saline solution; N: Number of embryos; CE: Number of cultured embryos; TE: Number of transferred embryos. <sup>a,b</sup>Values in the same column with different superscripts are statistically different (P<0.05).