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Biopsied and vitrified bovine embryos viability is improved by \textit{trans}10, \textit{cis}12 conjugated linoleic acid supplementation during in vitro embryo culture

R.M. Pereira\textsuperscript{1*}, I. Carvalhais\textsuperscript{1}, J. Pimenta\textsuperscript{1}, M.C. Baptista\textsuperscript{1}, M.I. Vasques\textsuperscript{1}, A.E.M. Horta\textsuperscript{1}, I.C. Santos\textsuperscript{1}, M.R. Marques\textsuperscript{1}, A. Reis\textsuperscript{2}, M. Silva Pereira\textsuperscript{1}, C.C. Marques\textsuperscript{1}

\textsuperscript{1}Estação Zootécnica Nacional – INIAP, 2005-048 Vale de Santarém, Portugal.

\textsuperscript{2} University of Newcastle, Institute for Cell and Molecular Biosciences, Framlington Place, Newcastle upon Tyne NE2 4HH, UK.

*e-mail: rosalnp@gmail.com

Fax: 351 243 767 307; Phone: 00351 243 767 316.

Abstract.

Bovine embryos cultured in serum-containing media abnormally accumulate lipids in the cytoplasm. This is well known to contribute to their higher susceptibility to cryopreservation and biopsied embryos are even further susceptible. We aimed to improve in vitro produced (IVP) embryos resistance to micromanipulation and cryopreservation by supplementing serum-containing media with \textit{trans}-10, \textit{cis}-12 conjugated linoleic acid (\textit{t}10\textit{c}12 CLA). The effect of \textit{t}10\textit{c}12 CLA on lipid deposition and embryonic development was also tested. After in vitro maturation and fertilization (\textit{IVF} day=D0), zygotes were cultured on granulosa cells+M199+10\% serum+100 \textmu M GSH supplemented with 100 \textmu M of \textit{t}10\textit{c}12 CLA (CLA group, \textit{n}=1394) or without supplementation (control group, \textit{n}=1431). Samples of D7/D8 embryos were observed under Nomarsky microscopy for lipid droplets evaluation while others were biopsied and vitrified (group B-Control, \textit{n}=24; group B-CLA, \textit{n}=23). Non-biopsied embryos were also frozen (group NB-Control, \textit{n}=49; group NB-CLA, \textit{n}=45). Biopsied cells were used for embryo sex determination. Post-warming embryo survival and viability were determined at 0 and 24h of culture, respectively.
1. Introduction

Improvements on embryo micromanipulation techniques led to the use of embryo bisection technology in commercial embryo transfer programs, and made possible the direct genetic analysis of preimplantation bovine embryos after biopsy. These analysis have been used mostly to determine the sex of embryos but can also detect genetic markers linked to economic trait loci for improving productivity or disease-encoding genes to prevent genetic diseases (Chrenek et al., 2001; Lopes et al., 2001; Kageyama et al., 2006).

Both in vivo and in vitro produced (IVP) embryos are currently used for commercial transfer in cattle industry. In-vivo produced bovine embryos have been transferred after sexing and cryopreservation in some embryo transfer units (Bredbacka et al., 1994, Thibier and Nibart 1995, Roschlau et al., 1997, Shea 1999). However, IVP embryos are rarely cryopreserved for sexing (Agca et al., 1998) because they are more fragile to micromanipulation and more sensitive to freezing than the in-vivo counterparts (Leibo and Loskutoff, 1993, Hasler et al., 1995).
The reduced cryotolerance of IVP embryos, specially those cultured in serum supplemented media, seems directly correlated to an excessive accumulation of lipids during in vitro embryo development. In fact, bovine embryo culture in serum free systems reducing the accumulation of cytoplasmic lipid droplets in blastocysts or the mechanical removal of excessive intracellular lipids from early embryos significantly improve their resistance to cryopreservation (Ushijima et al., 1999, Diez et al., 2001, Abe et al., 2002a). As well as influencing cryotolerance, excessive accumulation of cytoplasmic lipids in bovine embryos has been associated with abnormal or immature mitochondria, shorter and less numerous microvilli, poor developed junctional complexes, disruption of stress responsive genes impairing embryo quality (Abe et al., 2002a,b, Rizos et al., 2003).

The majority of intracellular lipids in oocytes and embryos are triacylglycerols (McEvoy et al., 2000, Ferguson and Leese 1999, Charpigny et al., 2003). However, while triacylglycerols accounted for 40-50% of the total lipid mass of bovine in vivo embryos in in vitro produced embryos it can reach 88% (Charpigny et al., 2003). Moreover, Ferguson and Leese (1999) reported that triacylglycerols concentrations in in vivo cattle embryos remain stable from the two cell to the blastocyst stage, whereas in vitro the triacylglycerols reserves can double by the blastocyst stage in embryos exposed to serum (10%, v/v) from the four-cell stage.

Recently new strategies based on emerging studies in the field of lipid research have been used to reduce intracellular lipid content in porcine and bovine embryos and therefore increase their tolerance to cryopreservation. Forskolin, a lipolytic agent capable of stimulating lipolysis of triacylglycerols, was used by Men et al. (2006). This agent promoted the cryosurvival of porcine IVP embryos after partial delipidation through chemical stimulation of intracellular lipolysis. Furthermore, the addition of a conjugated isomer of linoleic acid, the trans-10, cis-12 octadecadienoic (t10 c12 CLA) to serum supplemented media results in improving the survival rate of bovine blastocysts after slow-freezing and thawing (Pereira et al., 2006a). This CLA isomer reduced embryo lipid accumulation during in vitro culture. At our knowledge this strategy was
never tested in biopsied embryos. Our objectives were to improve IVP embryos resistance to micro-manipulation and cryopreservation by supplementing serum-containing media with \( t_{10},c_{12} \) CLA. \( t_{10},c_{12} \) CLA effect on lipid deposition and embryonic development was also tested.

2. Materials and Methods

2.1 Embryo production

Bovine ovaries collected immediately after slaughtering at a local abattoir, were stored at 37 °C in Dulbecco’s PBS (Gibco, ref. 14040-91) during transport to the laboratory within 2.5 hours. PBS was supplemented with 0.15% of bovine serum albumine (w/v, BSA, Fraction V, Sigma, ref. A-7888) and 0.05 mg mL\(^{-1}\) of kanamycin (Sigma, ref. K-4000). At the laboratory, cumulus oocytes complexes (COCs) were aspirated from follicles with 2-6 mm in diameter. Oocytes with at least three layers of compact cumulus cells and an evenly granulated cytoplasm were washed and selected for maturation.

In vitro maturation, fertilization and co-culture procedures which were used have been described previously (Pereira et al., 2006b). Briefly, selected COCs were matured in TCM199 with Earle’s salts, L-glutamine and 25 mM Hepes (GibCo, ref.22340-020). This medium was supplemented with 10% of superovulated oestrus cow serum (SOCS), 10 \( \mu \)g mL\(^{-1}\) FSH (Sigma, ref. -2293), 100 UI mL\(^{-1}\) penicillin and 100 mg mL\(^{-1}\) streptomycin (ref. P0781, Sigma). Maturation was accomplished in an incubator at 39 °C with humidified atmosphere in air and 5% CO2, for 22-24 hours. Fertilization was performed with frozen thawed semen submitted to swim-up. In vitro fertilization medium consisted of modified Tyrode’s medium supplemented with 5.94 UI mL\(^{-1}\) of heparin (Sigma, ref. H-3393), 10 mM of penicillamine (Sigma, ref. P-4875), 20 mM of hypotaurine (Sigma, ref. H-1384) and 0.25 mM of epinephrine (Sigma, ref. E-1635). Sperm concentration was adjusted to \( 10^6 \) spermatozoa mL\(^{-1}\). Sperm and COCs co-incubation lasted for 22 hours. Following co-incubation, presumptive zygotes (n=2931) were randomly placed in 100 µL droplets of a granulosa cell
monolayer cultured with TCM199 + 10% SOCS + 100 µM glutathione (GSH, Sigma, ref. G-6013) supplemented with 100 µM \textit{10t,12c} CLA (Matreya, ref. 001249; Pereira et., 2004) (CLA group) or without supplementation (control group), under paraffin oil, where embryo culture proceeded for 8 days. Atmospherical conditions for fertilization and embryo co-culture were 39°C and 5% CO2 in air with humidified atmosphere.

Cleavage was assessed 48 hours after fertilization (D0=insemination day). On day 7 and day 8, embryos were evaluated for development and morphological status (Grade 1 = excellent, grade 4 = bad). Embryo rates at D7 and D8 were calculated as number of morulae and blastocysts at those days per number of 2-4 cell embryos at cleavage.

2.2 Embryo biopsy and cryopreservation.

Excellent and good early to expanded blastocysts were placed into microdrops (Vigro, ref. EVM062) for embryo biopsy. The biopsy was carried out using an Olympus inverted microscope and a Narishige micromanipulator with a microblade (AB technology, ref. ESE020). The biopsied cells (8 to 10 blastomeres) were removed from the trophectoderm, emerged in liquid nitrogen and stored at -20°C until sex determination. Biopsied embryos were immediately cryopreserved using a vitrification procedure (Mermillod et al., 1997). Non-biopsied morulae and blastocysts classified as excellent, good and fair were also cryopreserved using the same procedure. Briefly, embryos were kept at room temperature for 5 min in PBS supplemented with 20% new-born calf serum (NBCS). They were then vitrified in three steps at room temperature as follows: 10% glycerol for 5 min, 10% glycerol and 20% ethylene glycol (EG) for 5 min, and finally 25% glycerol and 25% EG for 30 s in PBS–NBCS. During the last step, embryos were quickly aspirated into the center of a 0.25 mL plastic straw (IVM, L’Aigle, France) within 20–30 µL of vitrification solution. Embryos were separated by two air bubbles from two surrounding segments of PBS–NBCS containing 0.8 M galactose. The straws were sealed and immediately plunged directly into liquid nitrogen.
2.3 Assessment of embryo post-thawed viability

For assessment of in vitro survival, biopsied and non-biopsied blastocysts were warmed and transferred to granulosa cells monolayer cultured with TCM199, 10% SOCS and 100 µM GSH. Groups of embryos cultured with or without \( t_{10, c_{12}} \) CLA were compared in grade 1 and 2 biopsied embryos (B-CLA and B-control groups respectively, 5 replicates; experiment 1) and in grade 1, 2 and 3 non-biopsied embryos (NB-CLA and NB-control groups respectively, 6 replicates; experiment 2). In each session, straws were removed from the liquid nitrogen and held 5 s in air followed by 15 s in a 22ºC water bath. The straw content was expelled and embryos were incubated during 5 min in this content and then washed twice for 5 min in 3 mL PBS to eliminate cryoprotectants before transferred to the monolayer. After initial assessment, blastocysts were allowed in culture for additional 24 hours. Blastocysts that regained their original shape with a fully re-expanded blastocoel were regarded as viable blastocysts (Gardner et al., 2003).

2.4 Sex determination

DNA was extracted from the biopsied samples and amplified according to the method reported previously (Forrell, 2001, Carvalhais et al., 2006). Briefly, each biopsy sample was treated with 0.16 \( \mu \)g \( \mu \)L\(^{-1}\) of proteinase K at 37°C for 1 hour followed by 20 minutes at 95°C to inactivate the enzyme. Genomic DNA isolated from the blood of male and female cattle (4 ng \( \mu \)L\(^{-1}\)) was used as positive controls from embryo sexing. For sex determination, a pair of bovine Y chromosome-specific \( (Y_1Y_2 174 \text{ bp fragment}, \text{Macháty et al., 1993}) \) primers and co-amplification of bovine autosomal bovine DNA \( (C_1C_2 216 \text{ bp fragment, Peura et al. 1991}) \) primer pair were used in a multiplex PCR. Their sequences were \( Y_1Y_2 5´-\text{ CCC} \)
\begin{align*}
  &\text{TTC CAG CTG CAG TGT CA - 3´ and 5´- GAT CTG TAA CTG CAA ACC TGG C – 3´; } \\
  &\text{C_1C_2 5´- TGG AAG CAA AGA ACC CCG CT - 3´ and 5´-TCG TGA GAA ACC GCA CAC TG3´. The } \\
\end{align*}
PCR reactions were performed in 25 \( \mu \)L containing 1 X PCR buffer without MgCl\(_2\) (Fermentas, ref. B15), 1.5 mM MgCl\(_2\) (Fermentas, ref. EP0402), 50 mM KCl (Fermentas, ref. EP0402), 10 mM
dNTP (Fermentas, ref. RO192), 10 µg BSA (Fraction V, Sigma, ref. A-7888), 5 unit Taq-DNA Polymerase (Fermentas, ref. EP0402), 10 pM of C1C2 and 30 pM of Y1Y2 primers. The DNA was amplified under PCR conditions of an initial incubation at 94ºC for 5 min, and 40 cycles at 94ºC for 60 sec, 56ºC for 30 sec, and 72ºC for 60 sec, followed by a final elongation step at 72ºC for 30 min. The primer pair C1C2 was included in the tubes containing the reaction mixture only after the seventh cycle of the PCR. The products of PCR amplification were electrophoresed on 2.5% agarose gels containing ethidium bromide (0.5 µg mL⁻¹, GibcoBRL, ref.15585-011), and analyzed and photographed using a UV transilluminator.

2.5 Lipid Evaluation

Samples of excellent, good and fair quality blastocysts (control group n=10 and CLA group n=11) were used for Nomarski differential interference contrast microscope observation in fresh (Abe et al., 1999, 2002b). Embryos were photographed using an Olympus camera attached to the microscope and then analyzed using ImageJ software (version 1.19q, National Institutes of Health, USA). At least 100 lipid droplets randomly distributed at different focal planes were evaluated, and total area occupied by lipid droplets and total area of each embryo were measured. Following image analysis, 3 variables were considered: fat embryo index (a = area occupied by lipid droplets per total embryo area X 100), number of lipid droplets with an area < 4 µm² (b) and number of lipid droplets with an area ≥ 7 µm² (c). Embryos were classified as fat, intermediate or lean using the classification equations obtained from a discriminate analysis running with data variables (a, b, c) of bovine embryos produced in different culture conditions (Pereira et al, 2006a).

These equations were: Fat = -72.07 + 1.49 a + 0.50 b + 1.17 c; Intermediate = -38.09 + 0.72 a + 0.62 b + 0.92 c; Lean = -30.41 + 0.14 a + 0.74 b + 0.74 c.

2.6 Statistical analysis

Data representing 13 replicates for embryo production and 5 or 6 replicates for post-thawing
embryo survival were analyzed using one way ANOVA. ANOVA was also used to compare fat embryo index between groups.

Comparisons of lipid droplets areas frequency distribution and of embryo sex ratio between groups were carried out by the Chi-square test. Mann-Whitney U Test was used to analyze data from embryo lipid content classification into 3 categories (fat, intermediate and lean). Differences were considered significant when $P \leq 0.05$ (Statsoft Inc, 1995).

3. RESULTS

3.1 Embryo production and sex determination

A total of 2825 in vitro fertilized COCs were assessed for cleavage. Cleavage rates and D7/D8 embryo rate did not vary between treatments (table 1). Equally $10t,12c$ CLA supplementation did not interfered ($P>0.05$) on the quality or sex ratio of the produced embryos. Biopsied embryos after culture in the absence or presence of $10t,12c$ CLA were 52.2% males and 48.8% female and 45.5% males and 54.5% female, respectively. From the 47 biopsied embryos (B-CLA n=23; B-Control n=24) only 2, one from each group, remained without an identified sex.

3.2 Embryo lipid content evaluation

$10t,12c$ CLA supplementation to serum-containing media decreased ($P=0.008$) embryo lipid content (table 2, figure 1). Besides presenting a smaller fat embryo index ($a=19.3 \pm 2.9\%$, CLA group vs $a=36.1 \pm 2.3\%$, control group, $P<0.001$), these embryos presented also (figure 2) a higher number of small droplets (<2 $\mu m^2$ and 2-4 $\mu m^2$) than control ($P<0.001$ and $P=0.001$, respectively). In contrast, embryos from control group had predominantly more large lipid droplets (6-8 $\mu m^2$, $P<0.001$; 8-10 $\mu m^2$, $P=0.001$; 18-20 $\mu m^2$, $P=0.002$; 28-30 $\mu m^2$, $P=0.003$; > 38 $\mu m^2$, $P<0.001$).

3.3 Post-thawing embryo survival

After warming, survival rate (table 3) was higher ($P<0.001$) in biopsied embryos cultured with
1 $10t,12c$ CLA than without (95% vs 62.5%, respectively). $10t,12c$ CLA presence also improved the re-expanding rates after 24 h of culture of either biopsied (B-CLA= 64.6% vs B-control=27.5%, P=0.01) or non-biopsied (NB-CLA=86% vs NB-control=68.6%, P=0.05) embryos as compared to controls (table 3).

4. Discussion

Results show for the first time that $10t,12c$ CLA supplementation to serum-containing media more than doubles post-warming biopsied embryo viability. In a lesser extent non-biopsied bovine embryos cultured with this CLA isomer also re-expanded better after warming than control. Moreover, the addition of $10t,12c$ CLA to culture medium did not affect cleavage rate, embryo sex ratio, quality or development to the blastocyst stage but significantly reduced the accumulation of lipids as confirmed by Nomarski embryo observation.

Others have confirmed that mechanical delipidation through centrifugation and micromanipulation has been successfully applied to the cryopreservation of in vitro produced and cloned bovine embryos (Ushijima et al., 1999, Tominaga et al., 2000, Diez et al., 2001). However, besides being an invasive method extremely labor-intensive and time-consuming, mechanical delipidation alters the developmental potential of the delipidated blastocysts after transfer to recipient heifers (Diez et al., 2001). Equally, embryo culture in serum free systems reducing the accumulation of cytoplasmic lipid droplets in the blastocysts improved their resistance to cryopreservation (Abe et al. 1999, 2002b). Nevertheless, higher blastocyst developmental rates are observed in serum supplemented media by many authors (Pinyopummintr and Bavister, 1991, Eckert and Niemann, 1995; Cho et al., 2002, Pereira et al., 2006a). An alternative approach to reduce lipid content of bovine embryos is described in this study. Besides enhancing embryo cryosurvival $10t,12c$ CLA supplementation in serum containing media significantly reduces lipid droplet size and fat embryo index. Control embryos were fatter than the former embryos.

Although excessive accumulation of lipid during in vitro embryo production in the presence of
serum or their damaging influence on embryo quality and cryopreservation have been reported extensively for cattle (Shamsuddin and Rodriguez-Martinez, 1994; Ferguson and Leese, 1999, Abe et al., 1999, 2002a, Rizos et al., 2003, Reis et al., 2003), the information available on the significance of lipid droplets in the mammalian oocytes and embryos remains insufficient to date. Lipid droplets can be found in nearly all organisms as an integral facet of energy storage in the cell and contain primarily triacylglycerols and cholesteryl esters. Newly synthesized lipid is incorporated into existing cytoplasmic droplets, forms a discrete cytoplasmic droplet, or forms a small inclusion that fuses with an existing droplet. The lipid in these droplets undergoes turnover via hydrolysis and resynthesis being metabolically active (Murphy and Vance, 1999; Kellner-Weibel et al., 2001). Flynn and Hillman (1978, 1980) showed that preimplantation mouse embryos are able to synthesize lipid de novo from exogenous glucose or fatty acids present in the microenvironment. Neutral lipids such as triacylglycerol are the major lipid synthesized and stored. Reis et al. (2005) confirmed that the lipid droplets in ovine embryos are mostly composed of neutral lipids. Moreover, the extent of lipid droplets accumulation in in vitro blastocysts can be modified by using different culture conditions affecting embryo cryotolerance (Abe et al., 2002b, Reis et al., 2005, Pereira et al., 2006a). Nevertheless the minor size of lipid droplets and reduced fat embryo index from embryos cultured in serum containing medium and $10t,12c$ CLA are probably not the only mechanisms involved in the embryo cryoprotective action of this isomer. Previous results from our laboratory showed that post-thawing integrity and re-expansion rates of embryos cultured in serum containing medium and $10t,12c$ CLA were better than those obtained with a defined medium without serum although these last embryos had the minor lipid accumulation (Pereira et al., 2006a). There are several possible mechanisms by which $10t,12c$ CLA may interfere on embryo lipid content and cryotolerance. Conjugated linoleic acid isomers have anti-carcinogenic properties, anti-atherogenic and anti-obesity effects (Pariza et al., 2001). The $10t,12c$ CLA exerts specific effects on the adipocytes by inhibiting the expression of several genes coding lipids synthesis enzymes (Peterson et al., 2003; Granlund et al., 2005), as well as lipoprotein lipase activity (Park et al.,
Consequently, \(10\alpha,12\beta\) CLA supplementation inhibits synthesis and uptake of fatty acids, resulting in a net loss of triacylglycerols due to normal turnover. As referred bovine embryos cultured in serum containing medium tend to store large amounts of triacylglycerols (Ferguson and Leese 1999, Charpigny et al., 2003). Reduced triacylglycerols content induced by \(10\alpha,12\beta\) CLA during embryo culture could contribute to the increased embryo resistance to cryopreservation. Foremost bovine embryos generated in the presence of serum selectively accumulate an excess of a few familiar fatty acids, most of them saturated, until the blastocyst stage and this is suggested to contribute to their high susceptibility to cryopreservation (Reis et al., 2003, Lapa et al., 2005). Besides reducing the total amounts of fatty acids, \(10\alpha,12\beta\) CLA is also responsible for inducing modification in fatty acid profiles in tissues namely by interfering in the above triacylglycerols or in saturated and unsaturated fatty acids levels (Park et al., 1999, 2004; Alasnier et al., 2002). Moreover a direct incorporation of the polyunsaturated \(10\alpha,12\beta\) CLA into the embryo membranes during culture might contribute to an increased membrane fluidity (unsaturation level) and so improving embryo resistance to cryopreservation. Previous investigations showed that linoleic acid added to serum-containing embryo culture medium improved post-thawing survival of morulae (Hochi et al. 1999). Also an increase in the ratio of unsaturated to saturated phospholipids fatty acids in bovine oocytes during winter improved oocyte membrane integrity following chilling (Zeron et al., 2001). A close relationship among cold susceptibility, lipid phase transition and lipid profile was already identified in animal gametes (Arav et al., 2000, Zeron et al., 2001).

Another potential mechanism for the response to \(10\alpha,12\beta\) CLA is an altered formation of cyclooxygenase and lipoxygenase metabolites (Kim et al., 2005, Rodriguez-Sallaberry et al 2006, Wang et al., 2006), eventually attenuating cell damage and inflammatory response after embryo biopsy. The identified reduction of PGF\(2\alpha\) synthesis due to \(10\alpha,12\beta\) CLA (Rodriguez-Sallaberry et al., 2006) could also improve embryo quality as this prostaglandin exerts detrimental effects on embryo quality and development (Scenna et al., 2004; Pereira et al., 2005). Similar mechanisms could be applied to PGE2 (Pereira et al., 2005, Wang et al., 2006) although these \(10\alpha,12\beta\) CLA
effects on bovine embryos need further studies.

The mechanism of action of $10t,12c$ CLA is not fully clarified at present as in vitro and in vivo studies are not always in agreement, and possibly because CLA isomers act in different ways and with different consequences when administered to different species. Nevertheless, the present study not only confirms earlier reports of $10t,12c$ CLA cryoprotective action on bovine embryos but it also establishes an efficacious protocol for micromanipulated in vitro embryo production more than doubling post-thawing viability after vitrification.

In conclusion, supplying CLA to serum-containing media decreased embryo cytoplasmic lipid deposition during in vitro culture and significantly improved the resistance of IVP embryos to micromanipulation and cryopreservation.

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Figure 1. Nomarski differential interference contrast micrographs (400×) of lean (left) and fat (right) blastocysts cultured in serum containing media supplemented with C18:2 trans-10, cis-12 or without supplementation, respectively. The classification equations used for embryo lipid content classification were: Fat = -72.07 + 1.49a + 0.50b + 1.17c; Intermediate = -38.09 + 0.72a + 0.62b + 0.92c; Lean = -30.41 + 0.14a + 0.74b + 0.74c (a = area occupied by lipid droplets per total embryo area ×100; b = number of lipid droplets with an area < 4 µm²; c = number of lipid droplets with an area ≥ 7 µm²).
Figure 2. Lipid droplets area (µm$^2$) frequency distribution in blastocysts produced in serum containing media supplemented with (CLA group, 1100 droplets) or without (control group, 1000 droplets) C18:2, $trans$-10 $cis$-12.
Table 1. Embryo production yields expressed as percentages of in vitro fertilized COCs or of cleaved embryos, respectively for cleavage and Days 7 + 8 embryo rates (13 replicates).

<table>
<thead>
<tr>
<th></th>
<th>Fertilized COCs (n)</th>
<th>Cleavage</th>
<th>Days 7 +8 embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>% (mean±sem)</td>
<td>n</td>
</tr>
<tr>
<td>control</td>
<td>1431</td>
<td>954</td>
<td>68.0 ± 2.8</td>
</tr>
<tr>
<td>CLA</td>
<td>1394</td>
<td>977</td>
<td>70.5 ± 2.9</td>
</tr>
</tbody>
</table>

CLA = C18:2 trans-10, cis-12;
Table 2. Embryo distribution according to classification functions based on lipid content evaluation (Nomarski microscope).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Embryo classification (%)</th>
<th>U Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fat</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Control (n=10)</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>CLA (n=11)</td>
<td>9.1</td>
<td>72.7</td>
</tr>
</tbody>
</table>

U Test: Groups with different letters are statistically different (P≤0.05)

CLA= C18:2 trans-10, cis-12,

Fat=-72.07+1.49a +0.50b+1.17c; Intermediate=-38.09+0.72a +0.62b+0.92c; Lean = -30.41+0.14a +0.74b+0.74c

c (a= area occupied by lipid droplets per total embryo area ×100; b= number of lipid droplets with an area < 4 µm²; c= number of lipid droplets with an area ≥ 7 µm²)
Table 3. Survival and viability (re-expansion) of vitrified biopsied (grade 1 and 2; experiment 1 - 5 replicates) and non-biopsied embryos (grade 1, 2 and 3; experiment 2 - 6 replicates) after warming and 24 hours of co-culture.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Thawed embryos (n)</th>
<th>Post-warming survival*</th>
<th>Re-expansion after 24h*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>% (mean±sem)</td>
<td>n</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-control</td>
<td>24</td>
<td>62.5 ± 7.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7</td>
</tr>
<tr>
<td>B-CLA</td>
<td>23</td>
<td>95.0 ± 7.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NB-control</td>
<td>49</td>
<td>83.9 ± 9.1</td>
<td>36</td>
</tr>
<tr>
<td>NB-CLA</td>
<td>45</td>
<td>91.7 ± 2.9</td>
<td>39</td>
</tr>
</tbody>
</table>

Data within the same columns and experiment with different superscripts are statistically different (P<0.05)

CLA= C18:2 trans-10, cis-12

*calculated as percentage of thawed embryos