**ADRIANA MOREIRA ZOLINI**

# L-CARNITINE AND *TRANS-10, CIS-12* CONJUGATED LINOLEIC ACID ON *IN VITRO* BOVINE EMBRYO PRODUCTION AND CRYOPRESERVATION

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Zootecnia, para obtenção do título de *Magister Scientiae*

VIÇOSA
MINAS GERAIS – BRASIL
2015

**Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa**

T
Z86l
2015

Zolini, Adriana Moreira, 1988-
L-carnitine and trans-10, cis-12 conjugated linoleic acid on in vitro bovine embryo production and cryopreservation / Adriana Moreira Zolini. – Viçosa, MG, 2015.
ix, 45f. : il. ; 29 cm.

Orientador: Ciro Alexandre Alves Torres.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Bovino - Reprodução. 2. Embrião - Criopreservação. 3. Fertilização *in vitro*. 4. L-carnitina. I. Universidade Federal de Viçosa. Departamento de Zootecnia. Programa de Pós-graduação em Zootecnia. II. Título.

CDD 22. ed. 636.20824

ADRIANA MOREIRA ZOLINI

**L-CARNITINE AND *TRANS-10, CIS-12* CONJUGATED LINOLEIC ACID ADDITION DURING BOVINE EMBRYO CULTURE ON EMBRYO DEVELOPMENT AND CRYOSURVIVAL**

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Zootecnia, para obtenção do título de *Magister Scientiae*

APROVADA: 26 de Junho de 2015.

José Domingos Guimarães

Carlos Antônio de Carvalho Fernandes

Ciro Alexandre Alves Torres
(Orientador)

***Tânia Moreira Zolini***, *meu pai* ***Adriano Espeschit Zolini***, *meus irmãos* ***Thiago Moreira Zolini*** *e* ***Rafael Moreira Zolini***, *ao meu grande amigo e companheiro* ***Victor Chiari Alves.***

*MUITO OBRIGADA!*

## AGRADECIMENTOS

Aos meus pais Adriano Espeschit Zolini e Tânia Moreira Zolini, pela exaustiva dedicação, privações e empenho em minha educação, pelos exemplos que me deram, e pelo amor com que sempre contei.

Aos meus irmãos Thiago Moreira Zolini e Rafael Moreira Zolini pelos momentos compartilhados que me deram força para seguir em frente;

Ao meu amigo e companheiro Victor Chiari Alves que ao longo de toda a minha trajetória acadêmica esteve sempre ao meu lado me apoiando e incentivando principalmente nos momentos de maiores incertezas. Muito obrigada pelas correções de todos os textinhos em inglês!

Ao professor Ciro Alexandre Alves Torres por toda liberdade, confiança e disposição em sempre me apoiar e auxiliar nos projetos e principalmente no intercâmbio para a Universidade da Flórida. Muito obrigado pelo grande exemplo de professor, pesquisador e pessoa.

Ao Dr. Jeremy Block e Dr. P J. Hansen pela abertura das portas de seus laboratórios para a realização do meu projeto de pesquisa no exterior. Por toda confiança e todos os ensinamentos em nossas reuniões semanais que possibilitaram um enorme crescimento científico, cultural e pessoal.

Aos amigos de Laboratório Carolina Rodriguez Jimenez, Jurandy Mauro Penitente filho, Jovana Azevedo e principalmente Erly Luisana Carrascal Triana que esteve ao meu lado me orientando (as vezes desorientando rsrs) em todas as etapas do mestrado; e aos amigos do laboratório no exterior Antonio Ruiz, Natália Frigone, Beatriz Leão, Sofia Ortega, Paula Tríbulo, Veronica Negrão, Jasmim e Luiz.

A todos os funcionários da Pós-graduação do Departamento de Zootecnia da UFV por toda sua receptividade, prontidão e eficiência.

Ao Departamento de Zootecnia da Universidade Federal de Viçosa, em especial ao Departamento de Reprodução Animal.

A Capes pelo apoio financeiro que possibilitou a elaboração desta dissertação.

**BIOGRAFIA**

Adriana Moreira Zolini, filha de Adriano Espeschit Zolini e Tânia Moreira Zolini, nasceu em Belo Horizonte – MG em 19 de setembro de 1988 e residiu em Contagem – MG até seus 16 anos, mudando-se posteriormente para sua cidade natal Belo Horizonte - MG.

Em 2008 ingressou no curso de Medicina Veterinária na Universidade Federal de Minas Gerais, Belo Horizonte - MG, graduando-se em fevereiro de 2013.

Em agosto de 2013 ingressou no programa de pós-graduação em Zootecnia na Universidade Federal de Viçosa, Viçosa-MG, na área de Reprodução Animal, tendo apresentado sua dissertação de Mestrado em junho de 2015.

**SUMÁRIO**

RESUMO ..... vi

ABSTRACT ..... viii

Introdução Geral: ..... 1

Referências ..... 2

EFFECTS OF L-CARNITINE ON DEVELOPMENT AND CRYOTOLERANCE OF *IN VITRO* PRODUCED EMBRYOS ..... 4

1. Introduction ..... 4
2. General description of L-carnitine ..... 5
3. L-carnitine role on lipid metabolism and on antioxidant mechanism ..... 6
5. Effect of L-carnitine supplementation during maturation and culture on IVP embryo cryosurvival ..... 10
6. Conclusion ..... 12
7. References ..... 12
1. Introduction ..... 18
2. General description of CLA ..... 19
3. CLA role on lipid metabolism ..... 19
4. Effects of CLA in the oocyte fatty acid profile ..... 21
5. Effects of CLA supplementation during IVC on embryo development and cryosurvival. ..... 22
6. Conclusion ..... 23
7. References ..... 23

L-CARNITINE AND *TRANS-10, CIS-12* CONJUGATED LINOLEIC ACID ON *IN VITRO* BOVINE EMBRYO PRODUCTION AND CRYOPRESERVATION ..... 29

1. Introduction ..... 30
2. Material and Methods ..... 31
3. Statistical Analysis ..... 33
4. Results ..... 34
5. Discussion ..... 38
6. Conclusion ..... 41
7. References ..... 41

Conclusões Gerais: ..... 45

**RESUMO**

ZOLINI, Adriana Moreira, M.Sc., Universidade Federal de Viçosa, junho de 2015. **L-carnitina e ácido linoléico conjugado *trans-10, cis12* na produção e criopreservação de embriões bovinos produzidos *in vitro*.** Orientador: Ciro Alexandre Alves Torres.

O objetivo deste trabalho foi avaliar os efeitos da adição de L-carnitina, um acelerador do metabolismo lipídico, e ácido linoléico conjugado (trans-10, cis-12) em diferentes fases da produção *in vitro* de embriões sobre o desenvolvimento e criotolerância embrionária. Em todos os experimentos, embriões foram produzidos *in vitro* utilizando-se complexos *cumulos*-oócitos (CCO) provenientes de ovários coletados em abatedouro. Foram calculadas as taxas de clivagem (dia 3 do cultivo), taxa de formação de blastocistos expandidos e blastocistos em estágio avançado de desenvolvimento (dia 7 do cultivo) em função do total de CCO inseminados. Embriões em estágio de blastocisto expandido foram coletados de cada tratamento no dia 7 do cultivo *in vitro* e submetidos ao congelamento lento. Avaliou-se a taxa de reexpansão e eclosão embrionária 24, 48 e 72 h após o descongelamento em função do total de embriões descongelados. No experimento 1, oócitos fertilizados foram incubados em meio de cultivo contendo diferentes concentrações de L-carnitina (0,00; 0,75; 1,50 ou 3,03 mM) suplementado ou não com 5 % de soro fetal bovino (SFB). A adição de L-carnitina ao meio de cultivo na concentração de 1,5 mM melhorou a taxa de formação de blastocistos em estágio avançado de desenvolvimento quando comparado ao grupo controle (15,2±2,0 vs. 11,2±1,5; P<0,05). A L-carnitina também apresentou efeito positivo sobre a reexpansão embrionária 24 e 48 h pós-descongelamento quando adicionada na concentração de 0,75 e 3,03 mM (77,4±4,5; 80,1±4,0 e 75,0±5,5; 78,0±4,2 vs. 64,0±4,7; 67,4±4,7) ao meio de cultivo (P<0,05). Não houve interação entre os efeitos da adição de L-carnitina e SFB ao meio de cultivo embrionário. Apesar da suplementação do meio de cultivo com SFB ter melhorado o desenvolvimento embrionário (27,2±1,1 vs. 19,4±0,9; P<0,01), houve uma redução das taxas de reexpansão 24, 48 e 72 h pós-descongelamento (65,8±2,9; 67,8±2,8; 66,0±3,0 vs. 78,1±3,8; 81,4±3,0; 79,1±3,5; P<0,01). No experimento 2, os embriões foram cultivados em meio contendo L-carnitina (0,75 mM) ou ácido linoleíco conjugado (CLA – 100 mM) durante as primeiras 96 h, últimas 72 h ou durante todo o cultivo *in vitro*. A suplementação do meio de cultivo com L-carnitina ou CLA durante diferentes

fases do cultivo *in vitro* não afetou o desenvolvimento e a criotolerância embrionária. No experimento 3, avaliou-se o desenvolvimento e a crioresistência embrionária quando oócitos foram maturados em meio suplementado com L-carnitina (3.03 mM) e/ou CLA (100 mM). L-carnitina e CLA não afetaram o desenvolvimento embrionário quando adicionados ao meio de maturação. Apesar da L-carnitina não ter afetado a a taxa de reexpansão embrionária pós-descongelamento, o CLA apresentou efeito negativo sobre a eclosão embrionária 72h pós-descongelamento (53,3±3,6 vs. 65,1±4,3; $P<0,05$) quando adicionado ao meio de maturação oócitaria. Como conclusão, a L-carnitina melhora a criotolerância de embriões produzidos *in vitro* quando adicionada à concentração de 0,75 mM ao meio de cultivo embrionário. Já o CLA apresenta efeito negativo sobre a sobrevivência embrionária após o descongelamento quando adicionado ao meio de maturação oócitaria.

**ABSTRACT**

ZOLINI, Adriana Moreira, M.Sc., Universidade Federal de Viçosa, June, 2015**. L-carnitine and trans-10, cis-12 conjugated linoleic acid on *in vitro* bovine embryo production and cryopreservation.** Advisor: Ciro Alexandre Alves Torres.

High lipid content in embryo is associated with low freezing tolerance. This study assessed the effects of exogenous L-carnitine and *trans-10, cis-12* (t10, c12) conjugated linoleic acid (CLA) on *in-vitro* development and cryotolerance of bovine embryos when added during different stages of *in vitro* production of embryos. For all experiments, embryos were produced *in vitro* using slaughterhouse cows oocytes. Cleavage rates on Day 3, blastocyst and advanced blastocyst (hatching/hatched blastocyst) formation rates on Day 7 were calculated from the total number of oocytes subjected to *in vitro* fertilization (IVF). Expanded blastocysts-stage embryos from each treatment were harvested on Day 7 and subjected to slow freezing. Embryo viability was assessed 24, 48 and 72 h after thawing. In experiment 1, fertilized oocytes were incubated with different L-carnitine concentrations (0.0, 0.75, 1.50 or 3.03 mM) in the presence or absence of fetal bovine serum (FBS). There was an improvement ($P<0.05$) on embryo development when 1.5 mM of L-carnitine was added to *in vitro* culture (IVC) medium. L-carnitine had also a positive effect ($P<0.05$) on post thaw embryo competence when supplemented at 0.75 and 3.03 mM during IVC. There was no interaction ($P>0.05$) between the effects of L-carnitine and FBS supplementation on IVC on embryo development and cryosurvival. Although FBS supplementation had increased blastocyst development ($P<0.05$), it reduced the reexpansion rates at 24, 48 and 72 h post thawing. In experiment 2, L-carnitine (0.75 mM) or CLA (100 mM) were supplemented during the first 96 h, last 72 h or throughout the entire IVC period. There was no effect of L-carnitine or CLA supplementation during different periods of IVC on embryo development and cryotolerance. In experiment 3, embryo development and cryosurvival were evaluated when oocytes were maturated in medium supplemented with L-carnitine (3.03 mM) or/and CLA (100 mM). No effect of L-carnitine and CLA supplementation during *in vitro* maturation (IVM) on IVP embryo development was detected. Although there was no effect ($P>0.05$) of L-carnitine supplementation on embryo cryotolerance, CLA showed a negative effect ($P<0.05$) on embryo cryosurvival when added during IVM. In conclusion, L-carnitine improved embryo cryosurvival

when added at 0.75 mM during IVC and CLA supplementation during IVM has a negative effect on post thaw embryo survival.

**Introdução Geral:**

A produção de embriões *in vitro* é uma das áreas mais desafiadoras dentre as biotecnologias da reprodução animal. Baixas taxas de concepção e falta de confiabilidade nos resultados de criopreservação embrionária são consequências da alta sensibilidade dos embriões produzidos *in vitro*. Esta susceptibilidade à criopreservação está relacionada às condições sub-ótimas dos meios de cultivo (Lonergan et al., 2003) que induz o acúmulo lipídico no citoplasma das células embrionárias principalmente quando os embriões são cultivaods em meio contendo soro fetal bovino (SFB). A retirada do soro do meio de cultivo reduz o acúmulo lipídico citoplasmático, melhorando a criotolerância de embriões bovinos. Entretanto, a ausência de soro no meio de cultivo compromete o desenvolvimento embrionário (Van Langendonckt et al., 1996; Yoshioka et al., 1997). Uma alternativa para contornar este problema é a suplementação dos meios de maturação e cultivo *in vitro* com substâncias que aceleram o metabolismo lipídico ou inibem a formação de acidos graxos no embrião.

O uso de acido linoléico conjugado (CLA) t10, c12 tem sido associado à redução da formação de gotas lipídicas em embriões bovinos (Pereira et al., 2007; 2008) ao inibir a absorção de ácidos graxos pelo embrião (Pariza et al., 2001). Além disso, a incorporação de CLA ao meio de cultivo embrionário parece aumentar a fluidez da membrana plasmática, conferindo, assim, uma maior resistência embrionária aos danos causados pelo congelamento (Pereira et al., 2007; 2008).

A L-carnitina, um componente do metabolismo lipídico, também está associada à redução do conteúdo lipídico em embriões bovinos, suínos e de ratos (Yamada et al., 2006; Dunning et al., 2010; Somfai et al., 2011). A L-carnitina atua no transporte dos ácidos graxos presentes no citosol para dentro da matriz mitocondrial onde ocorre a β-oxidação para geração de energia. Além dessa função metabólica, a L-carnitina atua também com um potente antioxidante (Gülçin, 2006), reduzindo o acúmulo de radicais livres e, consequentemente, reduzindo a apoptose (Pillich et al., 2005; Ye et al., 2010). O efeito antioxidante da L-carnitina foi observado em embriões bovinos, suínos e de camundongos (Takahashi et al., 2013; Wu et al., 2011).

A suplementação dos meios de maturação e cultivo embrionário com CLA e L-carnitina parece promissora. Ambas substâncias podem regular o metabolismo lipídico e, consequentemente, reduzir o acúmulo lipídico citoplasmático embrionário. Entretanto,

os efeitos da adição de CLA e L-carnitina, aos diferentes estágios da produção embrionária *in vitro*, no desenvolvimento e criotolerância embrionária são ainda desconhecidos.

**Referências**


Dunning K.R., Cashman K., Russell D.L., Thompson J.G., Norman R.J., Robker R.L. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. Biol. Reprod. 83, 909–918 (2010).

Gülçin I. Antioxidant and antiradical activities of L-carnitine. Life Sci. 78, 803-811 (2006).

Lonergan P., Rizos D., Kanka J., Nemcova L., Mbaye A.M., Kingston, M. Wade M., Duffy, P., and Boland M.P.Temporal sensitivity of bovine embryos to culture environment after fertilization and the implications for blastocyst quality. Reproduction 126(3), 337-46 (2003).

Pariza M.H., Park Y. and Cook M.E. The biologically active isomers of conjugated linoleic acid. Prog Lipid Res 40, 283–298 (2001).

Pereira R. M., Baptista M.C., Vasques M.I., et al. Cryosurvival of bovine blastocysts is enhanced by culture with *trans*-10 *cis*-12 conjugated linoleic acid (10t,12c CLA). Anim Reprod Sci, 98 (3–4), pp. 293–301 (2007).

Pereira R.M.; Carvalhais I.; Pimenta J.; Baptista M.C.; Vasques M. I.; Horta A. E. M.; Santos I. C.; Marques M. R.; Reis A.; Silva Pereira M.; Marques C. C. Biopsied and vitrified bovine embryos viability is improved by *trans*-10,*cis*-12 conjugated linoleic acid supplementation during *in vitro* embryo culture. Anim. Reprod. Sci.106: 322–32; (2008).

Pillich R.T., Scarsella G., Risuleo G. Reduction of apoptosis through the mitochondria pathway by the administration of acetyl-L- carnitine to mouse fibroblasts in culture. Exp. Cell Res. 306, 1-8 (200%).

Somfai T., Kaneda M., Akagi S., Watanabe S., Haraguchi S., Mizutani E., Dang-Nguyen T.Q., Geshi M., Kikuchi K., Nagai T. Enhancement of lipid metabolism with l-carnitine during *in vitro* maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes. Reprod Fertil Dev; 23: 912 920 (2011).

Takahashi T., Inaba Y., Somfai T., Kaneda M., Geshi M., Nagai T. and Manabe N. Supplementation of culture medium with L-carnitine improves development and

cryotolerance of bovine embryos produced *in vitro.* CSIRO PUBLISHING Reproduction, Fertility and Development, 25, 589–599 (2013).

Van Langendonckt A. Auquier P., Donnay I., Massip A., and Dessy F. Acceleration of in vitro bovine embryos development in the presence of fetal calf serum. Theriogenology 45, 194. [Abstract] (1996).

Wu G.Q., Jia B.Y., Li J.J., Fu X.W., Zhou G.B., Hou Y.P., Zhu S.E. L-carnitine enhances oocyte maturation and development of parthenogenetic embryos in pig. Theriogenology 76, 785–793 (2011).

Yamada T., Imai H., and Yamada M. Beneficial effects of acetyl-Lcarnitine treatment during IVM on post-fertilization development of bovine oocytes *in vitro*. Reprod. Fertil. Dev. 18, 280–281. [Abstract] (2006).

Ye J., Li J., Wei Q., Deng W., and Yu, L. L-carnitine attenuates oxidant injury in HK-2 cells via ROS- mitochondria pathway. Regul. Pept. 161, 58-66 (2010).

Yoshioka K., Othman A.M., Taniguchi T.,Yamanaka H., and Sekikawa K. (1997). Differential patterns of blastulation in bovine morulae cultured in synthetic oviduct fluid medium containing FCS or BSA. Theriogenology 48, 997–1006 (1997).

# EFFECTS OF L-CARNITINE ON DEVELOPMENT AND CRYOTOLERANCE OF *IN VITRO* PRODUCED EMBRYOS

**Abstract**

Lipids within oocytes and embryos are a potential energy source. Some species like bovine and swine accumulate large amounts of intracellular fatty acids in oocytes and embryos, which is related to the long time between ovulation and implantation in these species. Despite this relation between lipid content and the pre-implantation period, large amounts of intracellular lipid can compromise the success of cryopreservation and the removal of such lipids has been subject of considerable effort. L-carnitine has an effect as an activator of lipid metabolism and, consequently, ATP synthesis. Fatty acid metabolism occurs within the mitochondria via β-oxidation. The transport of active fatty acids into mitochondria is a limiting step catalyzed by L-carnitine. Once inside the mitochondria, fatty acids are oxidized to produce ATP. The potential to improve oocyte and embryo quality and cryotolerance by modulating fatty acid metabolism and β-oxidation with L-carnitine in culture media formulations has received little attention. This review investigated the developmental importance of L-carnitine on oocyte and embryo development and cryotolerance. Overall, there is some evidence that inclusion of L-carnitine to *in vitro* embryo production media improves embryo outcomes, most likely by supplying the oocyte and embryo with an essential co-factor required to utilize fatty acids. However, the effects of L-carnitine on oocyte and embryo cryosurvival is variable and studies evaluating the effects of *in vitro* maturation (IVM) and *in vitro* culture (IVC) media supplementation with L-carnitine on pregnancy rates are needed.

## 1. Introduction

L-carnitine is a naturally produced amine compound which plays an important role in fat metabolism. It is required for the transport of activated fatty acids into the mitochondria where ATP is generated via β-oxidation (Vanella et al., 2000). L-Carnitine is primarily derived from dietary sources with the highest levels found in meat and dairy products. If sufficient quantities are not obtained through the diet it can be synthesized from the amino acids methionine and lysine (Rebouche, 1992). L-carnitine also protects cell membrane and DNA against damage induced by reactive oxygen species (ROS) and has an important role in mitochondrial oxidation of fatty acids which

is a source of energy supply to the cell (Zhou et al., 2007). This unique dual effect of L-carnitine in terms of reducing cellular lipid content and providing anti-oxidative protection makes it a choice reagent for the non-invasive improvement of cryotolerance and developmental competence of embryos of livestock animals. Freezing tolerance is highly dependent on the amount of lipid in porcine and bovines embryos (Nagashima et al., 1995; Abe et al., 2002). Supplementation of IVM medium with L-carnitine reduced the amount of lipid droplets and changed their position from peripheral towards the center in porcine oocytes (Somfai, 2011). Similarly, supplementation of embryo culture medium with L-carnitine reduced lipid content of bovine embryos and increased cryotolerance (Takahashi et al., 2013). In this paper, the role of L-carnitine on lipid metabolism was first overviewed. Then, research history of the effects of L-canitine on oocyte maturation, *in vitro* embryo development and cryotolerance were summarized with special references to recent progress.

## 2. General description of L-carnitine

L-carnitine (β-hydroxy-γ-trimethyl-amino-butyric acid) is a small water-soluble molecule required for the entry of long-chain fatty acids (as acylcarnitine esters) into the mitochondria. Its name is derived from the fact that it was first isolated from meat (*carnus*) in 1905, and the L-isomer was only found to be bioactive.

Carnitine is primarily derived from dietary sources with the highest levels found in red meat (containing 500 to 1200 mg/kg), and milk (containing 16 to 64 mg/kg); Dietary carnitine is absorbed by gut epithelial cells and released into the blood-stream. If sufficient quantities are not obtained through the diet, carnitine can be synthesized from the essential amino acids methionine and lysine or obtained by renal re-absorption (reviewed by Bremer, 1983). The endogenous formation of carnitine occurs primarily in the liver, as well as in the kidneys and brain and it requires several co-factors including vitamin C, iron, B vitamins and niacin in the form of nicotinamide adenine dinucleotide (NAD). One of the earliest symptoms of vitamin C deficiency is fatigue which seems to be related to decreased synthesis of l-carnitine (Lohninger et al., 1987). After synthesis, l-carnitine in circulation is actively transported into tissues which generally contain at least 10-fold higher concentrations than blood plasma (Bremer, 1983); muscle, for instance, contains~1000-fold higher amounts. The sodium dependent plasma membrane transporter OCTN2 maintains high concentrations of carnitine within tissues and establishes a threshold for excretion of carnitine via urine

thus, maintaining appropriate stores of carnitine within the body (Wang et al., 1999, 2000; Tein, 2003). L-carnitine is concentrated in high energy demanding tissues such as skeletal and cardiac muscles and in a specialized reproductive tract organ, the epididymis. In 1973, Casillas showed that spermatozoa accumulate carnitine in mammalian epididymis, indicating that it is closely related with the development of spermatozoa fertilizing capacity. In the epididymis, free L-carnitine is taken up from the blood plasma and is transported via epididymal fluid. It is then passively diffused into the spermatozoa, where it accumulates both free and acetylated L-carnitine. L-carnitine in seminal plasma plays an essential role in male fertility (Ahmed et al., 2011). The initiation of sperm motility occurs in parallel with the increase of free L-carnitine concentration in the epididymal lumen (Chiu Ming et al., 2004). The high levels of L-carnitine present in seminal plasma, probably act as an antioxidant to facilitate preservation of sperm membranes and reduce sperm re-absorption, thereby increasing sperm concentration.

Carnitine biosynthesis and transport mechanisms have not been directly studied in ovarian cells. However, the amount of both free carnitine and acetyl-carnitine in rat ovaries increases dramatically following eCG/PMSG treatment (Costa and Stevenson, 1984), suggesting possible hormonal regulation of these processes. Carnitine could not be detected in follicular fluid or granulosa cells from pig ovaries (Lee at al., 1983), and evidence suggests that, after antral formation, granulosa cell development may not be dependent on fatty acid oxidation. On the other hand, during the luteal phase, ovarian development is characterized by active steroidogenesis which requires high oxygen consumption (Flint and Denton, 1970).

Changes in concentrations of total CoA and acetylcarnitine together with [free CoA] : [acetyl CoA] rates of more than 1:2, indicate that the major source of the increased oxygen consumption results from fatty acid oxidation. The increase in carnitine palmitoyltransferase activity in ovaries 5 days after gonadotropin stimulation also supports this assumption (Costa and Stevenson, 1984).

### 3. L-carnitine role on lipid metabolism and on antioxidant mechanism

L-carnitine has two major functions.

First, its role in the transport of long-chain fatty acids into mitochondria for its oxidation and ATP generation is well known. Long-chain fatty acid in cytoplasm, as coenzyme A esters are trans-esterified to L-carnitine in a reaction catalyzed by carnitine

palmitoyltransferase I, from the mitochondrial outer membrane. Long-chain acil-carnitine esters enter the mitochondria via specific carrier, carnitine-acylcarnitine translocase (Fritz and Yue, 1963). On the matrix side of the inner mitochondrial membrane the long-chain fatty acid is trans-esterified to intra-mitochondrial coenzyme A, catalyzed by carnitine-palmitiltransferase II, and releases L-carnitine which returns to cytoplasm. Long-chain fatty acid cannot enter mitochondria independent of its translocation as an ester of carnitine. Therefore, L-carnitine is essential in this case.

Besides ATP, the long-chain fatty acids metabolism also generate a large amount of ROS that inflicts oxidative damage on cell membrane and DNA.

The second L-carnitine function is related with its powerful antioxidant action (Gulçin, 2006) that reduces ROS accumulation and reduces apoptosis in animal cells (Pillich et al., 2005; Ye et al., 2010). L-carnitine has antioxidant activity that combines both ROS scavenging and metal-chelating properties. L-carnitine was found to be effective in reversing age-related trends and improving mitochondrial function during the aging process in rat skeletal muscle mitochondria (Panneerselvam and Kumaran, 2006). Mitochondria membrane stabilization leads to an increase in the energy supply to the organelle and protect the cell from apoptosis

4. **L-carnitine supplementation during *in vitro* maturation (IVM) and IVC on oocyte lipid metabolism and embryo development**

Oocytes store a large amount of lipids that have an important role in its maturation and developmental competence (Sturmey et al., 2009). Among the lipids, triglyceride is the main component of intracellular lipid in the oocyte (Homa et al., 1986) and thus provides a large potential energy reserve. Oocytes intracellular lipid levels differ dramatically among species (Genicot et al., 2005) and are more considerable in cow and pig oocytes (63 and 161 ng respectively). The amount of lipid present in the oocyte decreases during maturation *in vitro* what is followed by a significant rise in lipase activity in oocytes, indicating that fatty acid oxidation is occurring (Cetica et al., 2002). Studies using a variety of β-oxidation inhibitors have shown that lipid metabolism is essential for oocyte maturation (Dunning and Robker, 2012). The β-oxidation inhibition, by preventing entry of activated fatty acids into the mitochondria, has negative effect in meiotic resumption and on post-fertilization development (Dunning and Robker, 2012). Improvements on oocyte maturation and embryo development by supplementation of L-carnitine may result from the utilization of lipid via β-oxidation

to generate ATP, which is necessary for the resumption of meiosis and cytoplasmic maturation (Ferguson and Leese, 2006). Somfai et al. (2011) and Sutton-McDowall et al. (2012) observed a decrease in lipid droplets density in pig oocytes treated with L-carnitine during IVM and cow embryos treated during *in vitro* culture (IVC). However, other studies have not observed similar decrease in lipid content during oocyte IVM with or without L-carnitine treatment (Reader, 2014; Cran, 1985; Chankitisakul and Aardema, 2008). This may be due to the large variation in the amount of lipid among oocytes, overabundance of lipid which mask small changes in concentration, or the fact that most methods are destructive and measurements cannot be made in the same oocyte before and after IVM (Sturmey, 2009).

Considering the importance of lipid metabolism in oocyte developmental competence and the ability of L-carnitine to increase β-oxidation, studies have been investigating whether L-carnitine can improve embryo development when added to follicles growing *in vitro*, to *COCs* undergoing in vitro maturation or to *in vitro* produced embryos. The addition of L-carnitine (1 mM) to cultured mouse follicles for 12 days, from the preantral to large antral stage, increased β-oxidation (Dunning et al., 2011). In addition, oocytes isolated from the follicles treated with L-carnitine had greater rates of maturity (metaphase II/MII), higher fertilization rates and improved blastocyst development (Dunning et al., 2011).

In mice, supplementation of the IVM medium with L-carnitine improves spindle microtubule assembly and chromosome alignment in MII oocytes and increases subsequent embryonic development through apoptosis reduction (Mansour et al., 2009; Dunning et al., 2011). L-carnitine supplementation during IVM of bovine oocytes improved their nuclear maturation and subsequent embryo development after IVF (Phongnimitr et al., 2013). This findings coincides with the results of Somfai et al. (2011) reporting that maturation rate of porcine oocytes was increased in IVM medium supplemented with 0.6 – 5.0 mg/mL L-carnitine. In contrast, Wu et al. (2011) showed that oocyte maturation rates did not differ significantly between L-carnitine (0.25–1 mg/mL) and the control group. However, oocytes treated with 0.5 mg/mL L-carnitine during IVM, had a significant greater rate of blastocyst formation than that of control. The blastocyst rate was doubled in pre-pubertal sheep oocytes treated with acetyl-L-carnitine when compared to untreated oocytes (Reader, 2015). On the other hand, Chanktisakul et al., (2012) found no effect of L-carnitine supplementation during IVM on germinal vesicle breakdown, degenerated oocytes and maturation to MII rates in

bovine oocytes. Probably, the positive effects of L-carnitine was evident only after embryo culture when blastocyst rate and quality were accessed.

Besides its role in metabolism, L-carnitine acts as an antioxidant, reducing the levels of ROS in oocytes during IVM (Somfai et al., 2011; Wu et al., 2011). Incubation of oocytes and embryos with L-carnitine was reported to reduce cytoskeleton damage and decrease the level of apoptosis via its antioxidant action (Mansour et al., 2009). Oocytes matured in 1.25 mg/mL L-carnitine had significantly more active mitochondria, fewer lipid droplets and decreased ROS levels compared to controls (Somfai et al., 2011). Furthermore, the 0.5 mg/mL L-carnitine treatment also reduced oocyte ROS levels and increased production of the anti-oxidant glutathione during IVM (Wu et al., 2011).

In pigs, L-carnitine improves nuclear maturation in oocytes and increases embryonic cleavage rates, which are associated with an enhancement in mitochondrial activity and a decrease in intracellular lipid contents and $H_2O_2$ levels (Somfai, 2011). You et al. (2012) demonstrated that L-carnitine treatment during oocyte maturation improves somatic cell nuclear transfer (SCNT) embryonic development. These findings were associated with increase of the intracellular glutathione level of oocytes, which leads to ROS activity inhibition and stimulates expression of *POU5F1* and transcription factor genes during nuclear re-programming in SCNT pig embryos.

As in oocytes, β-oxidation also plays an important role in the early stages of the pre-implantation embryo development. Some mammals embryo cytoplasm, like cattle and pig (McEvoy, 2000), are rich in lipids, while others, such as human and mouse (Loewenstein, 1964), are not. This variation of intracellular lipid density of embryos among species is believed to be related to the interval between ovulation and implantation ("Time to attachment" theory, reviewed by Sturmey, 2009). Ruminant oocytes and embryos had higher lipid density compared to those of human and mouse (Sturmey, 2009). This is associated with an interval of 20 to 30 days between ovulation and implantation in cattle (Greenstein, 1968), compared to 4 to 6 days in mouse (Cross, 1994). Despite this relation between lipid content and the pre-implantation period, an inverse association between embryo quality and lipid content has been suggested, and factors that contribute to compromised embryo development and quality, such as *in vitro* embryo production (vs. *in vivo*) or serum-based culture conditions resulted in higher intracitoplasmatic lipid content (Thompson, 1995; Romek, 2010). Many factors that promote embryo development can be related to lipid metabolism, such as an increase in ATP production and conversion of lipids into steroids and hormones.

Furthermore, the absence of co-factors to facilitate lipid metabolism in media may be one of the most important contributing factor of compromised embryo development during *in vitro* culture when compared to *in vivo* development.

L-carnitine, as a β-oxidation stimulant, can be one of these co-factors L-carnitine supplementation (5 mM) increased bovine embryos development from zygote to morula stage in absence of carbohydrates, an effect that did not occur in the presence of the β-oxidation inhibitor etomoxin (Sutton-McDowall et al., 2012). Besides that, L-carnitine supplementation was also associated with a significant decrease in the intracellular lipid content in bovine embryos (Sutton-McDowall et al., 2012, Takahashi et al., 2013). Takahashi et al. (2013) reported that L-carnitine significantly increased rates of zygote development to blastocyst stage and blastocyst cell numbers when 1.5 and 3.03 mM L-carnitine were added to culture medium. L-carnitine supplementation also increased ATP levels and expression of metabolism-related genes (Takahashi et al., 2013) which suggests that lipid content in embryos may decreased by enhanced lipid metabolism by L-carnitine. According to Biggers et al. (1967), mouse zygotes cultured in absence of carbohydrates did not go through cleavage, but supplementation with L-carnitine dose-dependently increased the number of zygotes cleaving to the 2-cell stages (Dunning et al., 2010). These results suggest that embryotrophic effects of L-carnitine are mediated by stimulating the utilization of intracellular lipid storage.

L-carnitine may also act as a protector against oxidative stress in embryos. L-carnitine treatment of paternogenic porcine embryos significantly decreased ROS and apoptosis in blastocysts (Wu et al., 2011). In mouse, L-carnitine significantly improved blastocyst development rate and reduced apoptosis level when 1.5 mM was added to culture medium (Abdelrazik et al., 2009). L-carnitine supplementation was also able to neutralize embryotoxic effects on exogenous induction of oxidative stress by 500 µmol/L $H_2O_2$ , to reverse the anti-proliferative effect of TNF-α and to reduce DNA damage (Abdelrazik et al., 2009).

## 5. Effect of L-carnitine supplementation during maturation and culture on IVP embryo cryosurvival

Slow freezing and vitrification are the main methods for gamete cryopreservation. Both techniques have been used for oocyte and embryo cryopreservation in several species with relatively satisfactory results (Saragusty end Arav, 2011). The blastocyst development percentage from vitrified IVM oocytes was significantly lower than those

non-vitrified IVM oocytes (Dinnyés et al., 2000) and similarly IVP embryos are less resistant to cryopreservation procedures (Rizos et al., 2002). The large oocyte volume makes them very sensitive to chilling and extremely susceptive to intracellular ice formation (Arav et al., 1996, Zeron et al., 1999). Besides that, they are surrounded by *zona pellucida* which makes it difficult to water and cryoprotectants movement in and out of oocytes. *Zona pellucida* fractures are often observed in oocytes after freezing and thawing which is associated with the pre-mature release of cortical granules causing zona hardening, reducing sperm penetration and subsequent embryo development (Pereira and Marques, 2008; Vincent et al., 1990). Cryopreservation induces meiotic spindle abnormalities causing chromosomal aneuploidy in resultants embryos (Chen, 2003; Schatten, 1998). Oocytes also have higher cytoplasmatic lipid content that increases its sensitivity to chilling injuries during cryopreservation (Ogawa et al., 2010). In livestock oocytes, most of the lipid droplets are in close proximity to plasma membrane – the primary site of cryo-injuries – and other organelles such as mitochondria and endoplasmatic reticulum (Kikuchi et al., 2012; Kruip, 1983). This damage of mitochondria and endoplasmatic reticulum by cryopreservation has been reported to reduce ATP content (Manipalviratn et al., 2011; aZhao, 2011; bZhao 2011) which also affects fertilization (Van Blerkom, 1995; Van Blerkom and Davis, 2007). Similarly to oocytes, embryos with high intracellular lipid content are more prone to cryoinjury. In order to solve this problem, several methods to decrease these cytoplasmatic lipid content have been attempted, among them the use of L-carnitine. It is possible that L-carnitine can improve oocyte quality (Mansour et al., 2009) and/or reduce the intracellular lipid content in oocytes (Somfai et al., 2011) which would suggest that their improved tolerance to cryo-damage results in high oocyte survival rate and their subsequent development after vitrification. Supplementation of IVM medium with L-carnitine reduced the amount of lipid droplets and changed their position from the periphery towards the center in porcine oocytes (Somfai et al., 2011). Similarly, embryo culture medium supplementation with L-carnitine reduced lipid content in bovine embryos and increased freezing tolerance (Takahashi, 2013). Moawad et al. (2013) demonstrated that supplementation of L-carnitine during both vitrification and IVM improves pre-implantation development from vitrified germinal-vesicle-oocytes in *COCs*. On the other hand, results from our laboratory showed that supplementation of maturation medium with L-carnitine did not improve embryo re-expansion and hatching rates after thawing (unpublished data).

## 6. Conclusion

A variety of studies using different experimental approaches has showed evidence that L-carnitine is able to increase fatty acid metabolism and reduce oxidative stress in oocytes and early embryos. However, effects of L-carnitine on embryo development and cryotolerance show great variability among research and also, until now, no experiment evaluated the effects of IVM and IVC media supplementation with L-carnitine on pregnancy rates. Facing that, further research is needed.

## 7. References


Abdelrazik, Sharma R., Mahfouz R., Agarwal A. L-carnitine decreases DNA damage and improve the *in vitro* blastocyst development rate in mouse embryos. Fertility and Sterility Vol. 91, N°. 2, (2009).

Ardema H., Knijn H. M., Oei C. H. Y., Vos P.L.A.M.,Gadella B. M. Lipid droplet dynamics during in vitro maturation of bovine oocytes. Reprod Domest Anim, 43 p.76, (2008).

Ahmed S.D., Karia K. A., et al.; Role of L-carnitine in male infertility; J Pak Med Assoc. 61(8):732-6, (2011).

Arav A., Zeron Y., Leslie S.B., Behboodi E., Anderson G.B. Crowe JH. Phase transition temperature and chilling sensitivity of bovine oocytes. Cryobiology 33 589–599, (1996).

Bremer J.,a. Carnitine—metabolism and functions. Physiol. Rev. 63, 1420–1480 (1998).

Casillas E.R. Accumulation of carnitine by bovine spermatozoa during maturation in the epididymis. J Biol Chem; 248 : 8227-32 (1973).

Cetica P., Pintos L., Dalvit G., Beconi M. Activity of key enzymes involved in glucose and triglyceride catabolism during bovine oocyte maturation in vitro. Reproduction 124, 675–681 (2002).

Chankitisakul V.,Somfai T.,Inaba Y.,Techakumphu M.,Takashi N. Supplementation of maturation medium with L-carnitine improves cryo-tolerance of bovine *in vitro* matured oocytes. Theriogenology. Volume 79, Pag. 590–598 (2013).

Chen S.U., Lien Y.R., Chao K.H., Ho H.N.,Yang Y.S., Lee T.Y. Effects of cryopreservation on meiotic spindles of oocytes and its dynamics after thawing: clinical implications in oocyte freezing—a review article. Mol Cell Endocrinol, 202, pag. 101–107(2003).

Chiu Ming N.G., Blackman M.R., Wang C., Swerdloff S. The role of carnitine in the male reproductive system. Ann N Y Acad Sci; 1033: 177-88 (2004).

Costa N.D. Stevenson P.M. Changes in coenzyme A and carnitine concentrations in superovulated rats. Biochim. Biophys. Acta 792, 130–134 (1984).

Cran D. G. Qualitative and quantitative structural changes during pig oocyte maturation. J Reprod Fertil, 74, pp. 237–245 (1985).

Cross J.C., Werb Z., Fisher S.J. Implantation and the placenta: key pieces of the development puzzle. Science; 266:1508 –18 (1985).

Dinnyés A., Dai Y., Jiang S.,Yang X. High developmental rates of vitrified bovine oocytes following parthenogenetic activation, in vitro fertilization, and somatic cell nuclear transfer. Biol Reprod, 63, pp. 513–518 (2000).

Dunning K.R., Cashman K., Russell D.L., Thompson J.G., Norman R.J., Robker R.L. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. Biol. Reprod. 83, 909–918 (2000).

Dunning K.R., Akison L.K., Russell D.L., Norman R.J., Robker R.L. Increased beta-oxidation and improved oocyte developmental competence in response to l-carnitine during ovarian in vitro follicle development in mice. Biol. Reprod. 85, 548–555 (2011).

Dunning K. R. and Robker R. L. Promoting lipid utilization with l-carnitine to improve oocyte quality. Animal Reproduction Science Volume 134, Issues 1–2, Pages 69–75 (2012).

Ferguson E.M. and Leese H.J. A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. Mol. Reprod. Dev. 73, 1195–1201 (2006).

Fritz and Yue. Long chain carnitine acyltransferase and the role of acylcarnitine derivatives in the catalytic increase of fatty acid oxidation induced by carnitine. J. Lipid Res., 4, pp. 288–297 (1963).

Flint A. P. F., Denton Biochem R. M.. J., 117, pp. 73–83 (1970).

Genicot G., Leroy J.L., Van Soom A., Donnay I. The use of a fluorescent dye, Nile red, to evaluate the lipid content of single mammalian oocytes. Theriogenology 63, 1181–1194 (2005).

Greenstein J.S. Current bibliographic index of mammalian reproduction. V. Int J Fertil;13:157– 88 (1968).

Gülçin I. Antioxidant and antiradical activities of L-carnitine. Life Sci. 78, 803-811. doi:10.1016/ J. LFS. 2005.05.103 (2006).

Homa S.T., Racowsky C., McGaughey R.W.Lipid analysis of immature pig oocytes. J Reprod Fertil 77, 425–434 (1986).

Kikuchi K., Ekwall H.,Tienthai P., Kawai Y., Noguchi J., Kaneko H., *et al.* Morphological features of lipid droplet transition during porcine oocyte fertilisation and early embryonic development to blastocyst *in vivo* and *in vitro*. Zygote, 10, pp. 355–366 (2002).

Kruip T.A.M., Cran D.G.,Van Beneden T.H., Dieleman S.J.Structural changes in bovine oocytes during final maturation *in vivo*. Gamete Res, 8, pp. 29–47 (1983).

Lee G., Pearce P.H., Ghisalberti A.V., Costa N.D., Stevenson P.M. Biochim. Biophys. Acta, 763, pp. 237–244 (1983).

Loewenstein J.E., Cohen A.I., Mass D. Dry mass, lipid content and protein content of the intact and zona-free mouse ovum. J Embryol Exp Morphol;12:113–21 (1964).

Lohninger A., Kaiser E., Legenstein E., Staniek H. Carnitine, metabolism and function. In: Kaiser, E., Lohninger, A. (Eds.), Carnitine—Its Role in Lung and Heart Disorders. Karger Press, Basel, pp. 1–25 (1987).

Manipalviratn S., Tong Z.-B., Stegmann B.,Widra E., Carter J., DeCherney A. Effect of vitrification and thawing on human oocyte ATP concentration. Fertil Steril, 95, pp. 1839–1841 (2011).

Mansour G., Abdelrazik H., Sharma R.K., Radwan E., Falcon T., l-carnitine supplementation reduces oocyte cytoskeleton damage and embryo apoptosis induced by incubation in peritoneal fluid from patients with endometriosis. Fertil Steril; 91: 2079 2086 (2009).

McEvoy T.G., Coull G.D., Broadbent P.J., Hutchinson J.S., Speake B.K. Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. J Reprod Fertil; 118:163–70 (2000).

Moawad A. R., Tan S. L., Xu B., Chen H. Y., and Taketo T. l-Carnitine Supplementation During Vitrification of Mouse Oocytes at the Germinal Vesicle Stage Improves Preimplantation Development Following Maturation and Fertilization In Vitro. *Biology of Reproduction* April 1, vol. 88 no. 4 104 (2013).

Ogawa B.,Ueno S., Nakayama N., Matsunari H., Nakano K., Fujiwara T., *et al.* Developmental ability of porcine in vitro matured oocytes at the meiosis II stage after vitrification. J Reprod Dev, 56, pp. 356–361 (2010).

Panneerselvam K.S., Kumaram S. L-carnitine and alpha-lipoic acid improves mitochondrial function during ageing process. Clin Nutr (2006).

Pereira R., Marques C. Animal oocyte and embryo cryopreservation. Cell Tissue Bank, 9, pp. 267–277 (2008).

Phongnimitr T., Liang Y., Srirattana K., Panyawai K., Sripunya N., Treetampinich C., Rarnpai R. Effect of L-carnitine on maturation, cryo-tolerance and embryo developmental competence of bovine oocytes. Animal Science Journal. 84, 719-725 (2013).

Pillich, R.T., Scarsella, G., Risuleo, G. Reduction of apoptosis through the mitochondria pathway by the administration of acetyl-L- carnitine to mouse fibroblasts in culture. Exp. Cell Res. 306, 1-8 (2005).

Reader K. L., Cox N. R., Stanton J.A., Juengel J.L. Mitochondria and vesicles differ between adult and prepubertal sheep oocytes during IVM Reprod Fertil Dev (2014).

Reader K.L. , Cox N. R., Stanton J. L, Juengel J. L. Effects of acetyl-L-carnitine on lamb oocyte blastocyst rate, ultrastructure, and mitochondrial DNA copy number. Theriogenology (2015).

Rizos D.; Fair T.; Papadopoulos S.; Boland M.P.; Lonergan P. Developmental, qualitative, and ultrastructural differences between ovine and bovine embryos produced in vivo or in vitro. Mol. Reprod. Dev., v. 62, p. 320-7, (2002).

Romek M., Gajda B., Krzysztofowicz E., Smorag Z. Changes of lipid composition in non-cultured and cultured porcine embryos. Theriogenology;74:265–76 (2010).

Saragusty J. end Arav A. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. Reproduction, 141, pp. 1–19 (2011).

Schatten G.,Simerly C., Schatten H. Microtubule configurations during fertilization, mitosis, and early development in the mouse and the requirement for egg microtubule-mediated motility during mammalian fertilization. Proc Natl Acad Sci U S A, 82, pp. 4152–4156 (1985).

Somfai T., Kaneda M., Akagi S., Watanabe S., Haraguchi S., Mizutani E., Dang-Nguyen T.Q., Geshi M., Kikuchi K., Nagai T. Enhancement of lipid metabolism with l-carnitine during *in vitro* maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes. Reprod Fertil Dev; 23: 912 920 (2011).

Stanley C.A. Carnitine disorders. Adv. Pediatr. 42, 209–242 (1995).

Sturmey R.G., Reis A., Leese H.J., McEvoy T.G. Role of fatty acids in energy provision during oocyte maturation and early embryo development. Reprod Domest Anim; 44:Suppl 3:50 – 8 (2009).

Sutton-McDowall M. L., Feil D., Robker R.L.,Thompson J.G., Dunning K.R.Utilization of endogenous fatty acid stores for energy production in bovine preimplantation embryos. Theriogenology, 77, pp. 1632–1641 (2012).

Takahashi T., Inaba Y., Somfai T., Kaneda M., Geshi M., Nagai T. and Manabe N. Supplementation of culture medium with L-carnitine improves development and cryotolerance of bovine embryos produced *in vitro.* CSIRO PUBLISHING Reproduction, Fertility and Development, 25, 589–599 (2013).

Tein I. Carnitine transport: pathophysiology and metabolism of known molecular defects. J. Inherit. Metab. Dis. 26, 147–169 (2003).

Thompson J.G., Gardner D.K., Pugh P.A., McMillan W.H., Tervit H.R. Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. Biol Reprod;53:1385–91 (1995).

Van Blerkom J. and Davis P. Mitochondrial signaling and fertilization. Mol Hum Reprod, 13, pp. 759–770 (2007).

Van Blerkom J., Davis P.W., Lee J. Fertilization and early embryology: ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. Hum Reprod, 10, pp. 415–424 (1995).

Vanella A., Russo A., Acquaviva R., Campisi A., Di Giacomo C., Sorrenti V. et al. L-Propionyl-carnitine as superoxide scavenbger, antioxidant, and DNA cleavage protector. Cell Biol. Toxicol; 16:99-104 (2000).

Vincent C., Pickering S.J., Johnson M.H. The hardening effect of dimethylsulphoxide on the mouse zona pellucida requires the presence of an oocyte and is associated with a reduction in the number of cortical granules present. J Reprod Fertil, 89, pp. 253–259 (1990).

Wang Y., Taroni F., Garavaglia B., Longo N. Functional analysis of mutations in the OCTN2 transporter causing primary carnitine deficiency: lack of genotype–phenotype correlation. Hum. Mutat. 16, 401–407 (2000).

WangY.,Ye J., Ganapathy V., Longo N. Mutations in the organic cation/carnitine transporter OCTN2 in primary carnitine deficiency. Proc. Natl. Acad. Sci. U.S.A. 96, 2356–2360 (1999).

Wu G.Q., Jia B.Y., Li J.J., Fu X.W., Zhou G.B., Hou Y.P., Zhu S.E. L-carnitine enhances oocyte maturation and development of parthenogenetic embryos in pig. Theriogenology 76, 785–793 (2011).

Ye J., Li J., Wei Q., Deng W., and Yu, L. L-carnitine attenuates oxidant injury in HK-2 cells via ROS- mitochondria pathway. Regul. Pept. 161, 58-66 (2010).

You J., Lee J., Hyun S.H., Lee E. l-carnitine treatment during oocyte maturation improves *in vitro* development of cloned pig embryos by influencing intracellular glutathione synthesis and embryonic gene expression. Theriogenology; 78: 235 243 (2012).

Zeron Y., Pearl M., Borochov A., Arav A. Kinetic and temporal factors influence chilling injury to germinal vesicle and mature bovine oocytes. Cryobiology 38 35–42 (1999).

Zhao X. M., Du W. H., Wang D., Hao H.S., Liu Y., Qin T., *et al.* Effect of cyclosporine pretreatment on mitochondrial function in vitrified bovine mature oocytes. Fertil Steril, 95, pp. 2786–2788 (2011a).

Zhao X. M., Du W. H., Wang D., Hao H.S., Liu Y., Qin T., et al. Recovery of mitochondrial function and endogenous antioxidant systems in vitrified bovine oocytes during extended *in vitro* culture. Mol Reprod Dev, 78, pp. 942–950 (2011b).

Zhou X., Liu F., Zhai S. Effect of L-carnitine in nutrition treatment for male infertility: a systemic review. Asia Pac J Clin Nutr; 16( Suppl 1):383-90 (2007)

## EFFECTS OF CONJUGATED LINOLEIC ACID (CLA) ON DEVELOPMENT AND CRYOTOLERANCE OF IN VITRO PRODUCED EMBRYOS

**Abstract**

*In vitro*-produced (IVP) embryos is one of the most challenging areas in animal reproduction biotechnologies. Although countless pregnancies have been obtained, IVP embryos have lower quality and viability when compared with *in vivo*-produced embryos. Besides that, studies have demonstrated that IVP embryos are more susceptible to cryopreservation damages, which are associated to morphological and physiological patterns. Many quality differences can be related to culture conditions, especially to serum addition, which has been reported to be responsible for the increase in cytoplamatic lipid accumulation in IVP embryos. In order to solve this problem, some studies proposed the supplementation of *in vitro* maturation (IVM) medium and/or *in vitro* culture (IVC) medium with conjugated linoleic acid (CLA) to improve oocyte and embryo quality and cryotolerance, by modulating fatty acid metabolism. CLA isomers can affect the lipid profile and signaling of cells and thereby alter their function *in vitro*. Regarding changes in lipid metabolism and composition, CLA isomers have become relevant for the improvement of IVP embryos competence and cryotolerance. This review investigated the importance of CLA on oocyte and embryo development and cryotolerance. Overall, there is some evidence that inclusion of CLA to *in vitro* embryo growth media improves embryo outcomes. However, the effect of CLA on oocyte and embryo cryosurvival is variable and studies evaluating the effects of IVM and IVC media supplementation with CLA on pregnancy rates are needed.

### 1. Introduction

Conjugated linoleic acid isomers comprise a group of polyunsaturated fatty acids (PUFA) derived from linoleic acid (C18:2n-6; Parodi, 1994) or from vaccenic acid (trans-11 18:1; Bougnoux et al., 2010). These CLA isomers have diverse effects on lipid metabolism, depending on the isomer type, dose, duration of treatment, metabolic status and species of subject. One of these effects is the reduction in cytoplasmatic lipid droplet formation (Pereira et al., 2007; 2008) and the change in membrane lipid profile (Pereira et al., 2008; Al Darwich et al., 2010; Stinshoff et al., 2014). Regarding this dual effect, CLA isomers have become relevant for the improvement of assisted reproduction technologies like IVP embryos and cryopreservation of IVP embryos.

Excessive levels of fatty acids in embryos compromise mitochondrial activity and metabolism (Van Hoeck et al., 2013) resulting in reduced cryotolerance (Pereira and Marques, 2008). In this paper, the role of CLA on oocyte maturation, *in vitro* embryo development and cryotolerance was summarized with special references to recent progress.

## 2. General description of CLA

CLA isomers is a non-specific term that comprises a group of PUFA derived from linoleic acid (C18:2n-6; Parodi, 1994) or from $\Delta^9$-desaturation of vaccenic acid (trans-11 18:1; Bougnoux et al., 2010). CLA is naturally present in many types of meat and dairy products. Ruminant tissues contain more CLA than non-ruminant tissues, with average values of 4.5 and less than 1 mg CLA/g of fat respectively (Ha et al., 1989). Dairy products are also a rich natural source of CLA. However, the content varies widely depending on roughage quality ingested by the animal (Parodi, 1994). Conjugated linoleic acid may also be present in some vegetable at low concentrations (Fogerty et al., 1988). Non-ruminants incorporate CLA into their tissues through diet and through bacterial isomeration, that produces small amounts of linoleic acid isomers. Ruminants, however, incorporate CLA primarily during biohydrogenation of linoleic acid by ruminal flora. The c9, t11 form of CLA is produced as a first intermediate in the ruminal biohydrogenation of dietary linoleic acid by *Butyrivibrio fibrisolvens* (Parodi, 1994). Thus, c9, t11 is the predominant isomer in milk. Besides that, CLA can also be manufactured through several methods using different substrates (O'Quinn, 2000) and it is used in supplement feed and experimental conditions.

## 3. CLA role on lipid metabolism

CLA has diverse effects on lipid metabolism, depending on the isomer type, dose, duration of treatment, metabolic status and species of subject. Park et al. (1997) demonstrated that cultures of mature murine 3T3-L1 adipocytes treated with 20–200 μM of crude mixture of CLA isomers for 2 days had 8 % less lipid, 66 % lower lipoprotein lipase (LPL) activity, and 22 % more glycerol release (e.g., lipolysis) compared to control cultures. Subsequently, Park et al. (1999) reported that the cultures of mature 3T3-L1 adipocytes treated with 44 μM trans-10, cis-12 isomer of CLA or 100 μM of a crude mixture of CLA isomers had 66% less LPL activity, 55% less t*riglyceride* (TG) content, and 1.8-fold more lipolysis than control cultures. In contrast,

treating cultures with cis-9, trans-11 or trans-9, trans-11 isomers had no impact on cellular lipid status. Similar findings have been reported by Lin et al. (1999). CLA isomer also decreased the expression of hepatic stearoyl-CoA desaturase (key enzyme in fatty acid metabolism) mRNA in mice, whereas enzymatically synthesized cis-9, trans-11 CLA did not show the same effect (Lee et al., 1998).

The inhibitory effect of CLA on the lipogenesis has been observed in several species. Supplementation of Sprague-Dawley rats diet with 0.25–0.5 % (w/w) of a crude mixture of CLA isomers for 5 weeks reduced retroperitoneal and parametrial fat pad weights without affecting growth rate or food intake (Azain et al., 2000). Pigs fed with 0.05–1.0 % (w/w) mixed CLA isomers also increased feed efficiency and reduced back fat without affecting total body weight (Cook et al., 1999). Similarly, feeding growing pigs for 8 weeks with 0.07–0.5 % (w/w) mixed CLA isomers increased feed efficiency and lean body mass while reducing fat deposition compared to control pigs (Ostrowska et al., 1999). In cows, feeding 10 g/day of the t10, c12 CLA isomer decreased lipogenesis and increased plasma nonesterified fatty acid levels (Baumgard et al., 2000; Baumgard et al., 2002). A similar study has shown that supplementation with 100 g/day of CLA for 1 day decreased fatty acid synthesis and desaturation in cows (Loor and Herbein, 1998). Other studies using cultures of human preadipocytes (Evans, 2001; Evans, 2002) and stromal vascular cells from human adipose tissue (Brown, et al., 2001) agree with these findings.

Data from these studies suggest that CLA acts in different cell mechanisms in order to decrease TG content. It probably has an inhibitory effect on fatty acid synthesis, uptake and esterification into TG by adipocytes. Increasing levels of trans-10, cis-12, but not cis-9, trans-11, CLA in cultures of human preadipocytes decreased *de novo* lipogenesis (Brown et al., 2001a) and TG esterification (Brown et al., 2001b). Supporting these data, supplementation with 1 % CLA markedly down regulated GLUT-4 mRNA levels, and consequently glucose uptake in white and brown adipose tissues, but upregulated GLUT-4 mRNA levels in skeletal muscle in mice (Tsuboyama-Kasaoka, 2000; Gavino, 2000). These reductions of GLUT-4 mRNA level in adipose tissue by CLA indicate an inhibition of CLA on the conversion of glucose into fat.

CLA may also inhibit lipogenesis and TG esterification through a disruption in the fatty acid desaturation process. I*n vivo* studies have determined that CLA treatment changed the proportion of saturated vs. unsaturated fatty acids. Pigs fed 1 to 5 % mixed CLA isomers showed increased levels of saturated fatty acids and decreased levels of

unsaturated fatty acids intramuscularly (Joo, et al. 2002). Sows fed 2 % mixed CLA isomers for 35 days showed more 16:0 (saturated) (g/100g lipid) and less 18:1 (unsaturated) in their backfat as well as increased 16:0 and decreased 16:1 (unsaturated) in their milk. (Bee and Dietary, 2000). Similarly, cows fed with 50–100 g/day of mixed CLA isomers for 5 days produced milk with a similar increase in 16:0/16:1 and 18:0/18:1 (Chouinard et al., 1999).

Studies *in vitro* have showed similar increase in the saturated:unsaturated fatty acids concentrations. Lee et al. (Lee et al., 1998) demonstrated that H2.35 liver cells treated with 150 μM mixed CLA isomers for 20 h increased 16:0 and 18:0, while decreasing 18:1 levels. Agreeing with these findings, Brown et al. (2002) found that differentiating cultures of human preadipocytes treated with trans-10, cis-12 CLA for 12 days had significantly higher saturated fatty acids concentrations (combined C16 and C18 fatty acids) in their phospholipid and neutral lipid fractions, respectively, compared to control cultures. These data shows evidence that CLA incorporates into cellular lipids and alters its fatty acid composition.

**4. Effects of CLA in the oocyte fatty acid profile**

Besides the role of CLA on fatty acid metabolism described above, the *trans-10, cis-12* CLA isomer (t10, c12 CLA) has been shown to regulate cyclo-oxygenase (COX), lipo-oxygenase pathways (Pariza et al., 2001; Kim et al., 2005; Bauman et al., 2008) and enhance plasma concentrations of IGF-1 (Castanheda-Gutierrez et al., 2007), with a promising shortening of time to conception and increased pregnancy rates in lactating dairy cows fed with supplemented diets. (Bern al-Santos et al., 2003; Castaneda-Gutierrez et al., 2005; de Veth et al., 2009). This positive effect on reproduction is in part related to the presence of t10, c12 in the fatty acid profile of oocytes matured in t10, c12 CLA supplemented medium.

Maturation environment has great influence on oocyte development into blastocysts and studies suggest a regulator and metabolic role for lipids during their maturation. In bovine, supplementation of oocyte culture medium with 10, c12 CLA increased the rate of very good quality embryos on day 8 post-insemination. However, it had no effect on embryo development. According to Absalón-Medina (2014) supplementation with either t10, c12 or c9, t11 during IVM had no effect on maturation, cleavage, or blastocyst rates. Results from our laboratory agree with these findings (unpublished data).

Although supplementation with *trans*-10, *cis*-12 CLA isomer in culture medium reduced the lipid content of *in vitro* produced bovine embryos by reducing the gene expression of 1-acylglycerol 3-phosphate 0-acyltransferase (AGPAT) enzyme (which is involved in triglycerides synthesis; Batista et al., 2014), total fatty acid in bovine oocytes matured in medium supplemented with t10, c12 CLA did not differed from control (Lapa et al., 2011). In pigs, t10, c12 CLA was able to modify the distribution and morphology of cytoplasmic lipid droplets during oocyte maturation, thus reducing its lipid content when added during 44 to 48 h of maturation (Prates et al., 2012). According to Prates et al. (2013) porcine cumulus-oocyte complexes (COC) can uptake and accumulate t10, c12 CLA from the culture media, being *cumulus* cells more susceptible to fatty acid and dimethylacetal modifications induced by this CLA isomer than oocytes.

**5. Effects of CLA supplementation during IVC on embryo development and cryosurvival.**

Regarding changes in lipid composition and lipid metabolism, CLA isomers become relevant for improvement of IVP embryo development and cryotolerance (Pereira et al., 2007; 2008; Pereira and Marques, 2008). Excessive levels of fatty acids in embryos impair mitochondrial activity and metabolism (Van Hoeck et al., 2013) resulting in reduced cryotolerance (Pereira and Marques, 2008). Although serum-supplemented media may generate higher blastocyst rates, it can increase lipid accumulation and alter mitochondrial metabolism which reduces bovine embryo developmental competence (Abe et al., 2002; Reis et al., 2003). Alternatively, Pereira et al. (2007; 2008) showed that culture medium supplemented with CLA t10, c12 reduced lipid droplets formation in bovine embryos. Besides that, embryos produced in this medium had greater resistance to freezing damage, which was related to the ability of CLA to change lipid composition and probably to increase the membrane fluidity (Pereira et al., 2007; 2008). These results differ from those described by Al Darwich et al. (2010) that found lower fatty acid desaturase 2 (FADS2) transcript levels in embryos cultivated in IVC medium supplemented with 100 μM t10, c12. $FDS_2$ is a critical enzyme in the biosynthesis pathways of polyunsaturated fatty acids that contribute to increase membrane fluidity and so improve embryo resistance and cryotolerance (Hoci et al., 1999; Marques et al., 2007). Besides that, those authors also described a decrease in blastocyst development rate and on embryo survival rate when 100 μM 12t, 10c was supplemented during

embryo culture. Similarly, Stinshoff et al. (2014) also found a negative effect of 12t, 10c IVC medium supplementation on embryo development. We also found no effect of 12t, 10c supplementation during different periods on IVC on embryo development and cryotolerance (unpublished data). These contrasting findings may be due to different incubation and media conditions among the experiments.

In contrast to previous effects of CLA isomers on lipid metabolism, Absalón-Medina V.A. et al. (2014) found a higher lipid content in embryos treated with 15 and 25 μM of t10, c12 during IVC. According to these authors, CLA isomers, especially t10, cis 12, may direct glucose towards triglyceride synthesis pathway. Considering that higher lipid content has negative effect on embryo cryotolerance, 15 μM t10, c12 CLA treatment group had lower post-thawing blastocyst survival rates. Besides that, the transcript abundance of 2 antiapoptotic markers, whose expression is related to embryo survival (BIRC5 and HSPA1A), was reduced by t10, c12.

## 6. Conclusion

A variety of studies, each using different experimental approaches, has provided evidence that CLA has diverse effects on lipid metabolism, depending on the isomer type, dose, duration of treatment, metabolic status and species of subject. However, effects of CLA on embryo metabolism, development and cryotolerance showed great variability among research and also, until now, no experiment evaluated effects of IVM and IVC media supplementation with CLA on pregnancy rates. Facing that, further research is needed.

## 7. References

Abe H.,Yamashita S., Itoh T., Satoh T., Hoshi H. Accumulation of cytoplasmatic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free medium or in serum-containing medium. Mol. Reprod. Dev., 61, pp. 57–66 (2002).

Absalón-Medina V.A. Bedford-Guaus S.J., Gilbert R.O., Siqueira L.C. Esposito G., Schneider A., Cheong S.H., Butler W.R. The effects of conjugated linoleic acid isomers *cis*-9,*trans*-11 and *trans*-10, *cis*-12 on in vitro bovine embryo production and cryopreservation. J. Dairy Sci. 97:6164-6176. American Dairy Science Association, (2014).

Absalón-Medina V.A., Bedford-Guaus S.J., Gilbert R.O., Siqueira L.C., Esposito G., Schneider A., Cheong S.H. and Butler W.R. The effects of conjugated linoleic acid isomers *cis*-9, *trans*-11 and *trans*-10,*cis*-12 on *in vitro* bovine embryo production and cryopreservation. J. Dairy Sci. 97:6164-6176, (2014).

Al Darwich A. Perreau C., Petit M. H., Papillier P.,Dupont J., Guillaume D., Mermillod P., Florence Guignot F. Effect of PUFA on embryo cryoresistance, gene expression and AMPKα phosphorylation in IVF-derived bovine embryos. Prostaglandins & Other Lipid Mediators Volume 93, Issues 1–2, Pages 30–36 (2010).

Azain M., Hausman D., Sisk M., Flatt W., Jewell D. Dietary conjugated linoleic acid reduces rat adipose tissue cell size rather than cell number. J Nutr, 130, pp. 1548–1554 (2000).

Batista R. I. T. P., N. R. B, Campos-Junior P. H. A., Pereira M. M, Camargo L. S. A., Carvalho B. C., Gama M. A. S., and Viana J. H. M*Trans*-10, *cis*-12 conjugated linoleic acid reduces neutral lipid content and may affect cryotolerance of *in vitro*-produced crossbred bovine embryos. J Anim Sci Biotechnol. 5(1): 33 (2014).

Bauman D.E., Perfield J.W., Harvatine K.J., Baumgard L.H. Regulation of fat synthesis by conjugated linoleic acid: lactation and the ruminant model. J Nutr 138, 403–409 (2008).

Baumgard L.H., Corl B.A., Dwyer D.A., Saebo A., Bauman D.E. Identification of the conjugated linoleic acid isomer that inhibits milk fat synthesis. Am J Physiol Regul Integr Comp Physiol; 278: R179–R184 (2000).

Baumgard L.H., Matitashvili E., Corl B.A., Dwyer D.A., Bauman D.E. *Trans*-10, *cis*-12 conjugated linoleic acid decreases lipogenic rates and expression of genes involved in milk lipid synthesis in dairy cows. J Dairy Sci; 85: 2155–2163 (2002).

Bee G. and Dietary G. Conjugated linoleic acids alter adipose tissue and milk lipids of pregnant and lactating sows. J Nutr, 130, pp. 2292–2298 (2000).

Bernal-Santos G., Perfield J.W., Barbano D.M., Bauman D.E., Overton T.R. Production responses of dairy cows to dietary supplementation with conjugated linoleic acid (CLA) during the transition period and early lactation. J Dairy Sci 86, 3218–3228 (2003).

Bougnoux, P., Hajjaji N., Maheo K.,Couet C., and Chevalier S. Fatty acids and breast cancer. Sensitization to treatments and prevention of metastatic re-growth. Prog. Lipid Res. 49:76 86 (2010).

Brown J., Halverson Y. D., Lea-Currie R., Geigerman C., McIntosh M. *Trans*-10, *cis*-12, but not cis-9, trans-11, conjugated linoleic acid attentuates lipogenesis in primary cultures of stromal vascular cells from human adipose tissue. J Nutr, 131, pp. 2316–2321 (2001a).

Brown J , Lea-Currie Y., Sen A., McIntosh M. *Trans*-10, *cis*-12 conjugated linoleic acid suppress fatty acid incorporation into lipid in cultures of human preadipocytes. Obesity Res, 9, p. 142S (2001b).

Brown J., Fabiyi O., Lea-Currie R., Storkson J., Pariza M., McIntosh M. *Trans*-10, *Cis*-12 Conjugated linoleic acid decreases triglyceride content by altering lipid metabolism, but not pparγ or c/ebpα expression, in primary cultures of human (pre)adipocytes. FASEB J, 16, p. 1015 (2002).

Castaneda-Gutierrez E., Overton T.R., Butler W.R., Bauman D.E. Dietary supplements of two doses of calcium salts of conjugated linoleic acid during the transition period and early lactation. J Dairy Sci 88, 1078–1089 (2005).

Chouinard P., Courneau L., Barbano D., Metzger L., Bauman D. Conjugated linoleic acids alter milk fatty acid composition and inhibit milk fat secretion in dairy cows. J Nutr, 129, pp. 1579–1584 (1999).

Cook M., Jerome D., Crenshaw T., Buege P., Pariza M., Albright K., Schmidt S., Scimeca J., Lotgren P., Hentges E. Feeding conjugated linoleic acid improves feed efficiency and reduces carcass fat in pigs, Adipocyte Biology and Hormone Signaling Symposium. University of Wisconsin-Madison. Dept. Biochemistry, p. P67 (1999).

de Veth M.J., Bauman D.E., Koch W., Mann G.E., Pfeiffer A.M., Butler W.R. Efficacy of conjugated linoleic acid for improving reproduction: a multi-study analysis in early-lactation dairy cows. J Dairy Sci 92, 2662–2669 (2009).

Evans M., Park Y., Pariza M., Curtis L., Kuebler B., McIntosh M. *Trans*-10, *cis*-12 conjugated linoleic acid reduces triglyceride content while differentially affecting peroxisome proliferator activated receptor gamma2 and aP2 expression in 3T3-L1 preadipocytes. Lipids; 36: 1223–1232 (2001).

Evans M., Brown J., McIntosh M. Isomer-specific effects of conjugated linoleic acid (CLA) on adiposity and lipid metabolism. J Nutr Biochem,13: 508–516 (2002).

Fogerty A.C., Ford G.L., Svoronos D. Octadeca-9,11-dienoic acid in foodstuffs and in the lipids of human blood and breast milk. Nutr. Rep. Int. 38:937–44 (1988).

Gavino V., Gavino G., LeBlanc M., Tuchweber B. An isomeric mixture of conjugated linoleic acids but not pure *cis*-9, *trans*-11-octadecodienoic acid affects body weight gain and plasma lipids in hamsters. J Nutr, 130, pp. 27–29 (2000).

Hochi S., Kimura K., Hanada A. Effect of linoleic acid-albumin in the culture medium on freezing sensitivity of in vitro-produced bovine morulae. Theriogenology, 52 (3), pp. 497–504 (1999).

Joo S., Lee J., Ha Y., Park G. Effects of dietary conjugated linoleic acid on fatty acid composition, lipid oxidation, color, and water-holding capacity of pork loin. J Anim Sci, 80, pp. 108–112 (2002).

Lapa M., Marques C.C., Alves S.P., Vasques M.I., Baptista M.C., Carvalhais I., Horta A.E.M., Bessa R.J.B., Pereira R.M. *Trans*-10 *cis*-12 conjugated linoleic acid (CLA) reduces fatty acid (FA) content of bovine matured oocytes. Reprod Dom Anim 44, 87 (2009).

Lapa M.; Marques C. C.; Alves S. P.; Vasques M. I.; Baptista M. C.; Carvalhais I.; Silva Pereira M.; Horta A. E. M.; Bessa R. J. B.; Pereira R. M. Effect of *trans*-10, *cis*-12 conjugated linoleic acid on bovine oocyte competence and fatty acid composition. Reprod.Domest. Anim. 46: 904–10; (2011).

Lee K. N., Pariza M. N., Ntambi J. M. Conjugated linoleic acid decreases hepatic stearoyl-CoA desaturase mRNA expression. Biochem Biophys Res Commun 248:817-821(1998).

Lin Y.G., Draijer R., Kreeft A., de Deckere E. A. M., Rudrum M., Tijburg L. Defferential effects of conjugated linoleic acid isomers on lipids metabolism *in vitro*. FASEB J13:LB207 (1999).

Loor J. and Herbein J. Exogenous conjugated linoleic acid isomers reduce milk fat concentration and yield by inhibiting *de novo* fatty acid synthesis. J Nutr, 128, pp. 2411–2419 (1998).

Marques C., Baptista M.,Vasques M., Horta A., Pereira R.. Effect of polyunsaturated fatty acids (PUFA) on bovine oocyte *in vitro* maturation and subsequent embryo development and freezability. Reprod Dom Anim, 42, pp. 108–109 (2007).

O'Quinn P.R., Nelssen J.L., Goodband R.D, Tokach M. D. Conjugated linoleic acid. Animal Health Research Reviews 1(1); 35–46 (2000).

Ostrowska E., Muralitharan M., Cross R., Bauman D., Dunshea F. Dietary conjugated linoleic acids increase lean tissue and decrease fat deposition in growing pigs. J Nutr, 129, pp. 2037–2042 (1999).

Pariza M.H., Park Y and Cook M.E.The biologically active isomers of conjugated linoleic acid. Prog Lipid Res 40, 283–298 (2001).

Park Y., Albright K., Liu W., Storkson J., Cook M., Pariza M. Effect of conjugated linoleic acid on body composition in mice Lipids, 32, pp. 853–85 (1997).

Park Y., Albright K., Storkson J., Liu W., Cook M., Pariza M. Changes in body composition in mice during feeding and withdrawl of conjugated linoleic acid Lipids, 34, pp. 243–248 (1999).

Parodi P.W. Conjugated linoleic acid: an anticarcinogenic fatty acid present in milk fat. Australian Journal of Dairy Technology 49: 93–97 (1994).

Pereira R. M., Baptista M.C., Vasques M.I., et al. Cryosurvival of bovine blastocysts is enhanced by culture with *trans*-10 *cis*-12 conjugated linoleic acid (10t,12c CLA). Anim Reprod Sci, 98 (3–4), pp. 293–301 (2007).

Pereira R. M. and Marques C. C. Animal oocyte and embryo cryopreservation. Cell Tissue Bank.9: 267–77; (2008).

Pereira R.M.; Carvalhais I.; Pimenta J.; Baptista M.C.; Vasques M. I.; Horta A. E. M.; Santos I. C.; Marques M. R.; Reis A.; Silva Pereira M.; Marques C. C. Biopsied and vitrified bovine embryos viability is improved by *trans*-10,*cis*-12 conjugated linoleic acid supplementation during *in vitro* embryo culture. Anim. Reprod. Sci.106: 322–32; (2008).

Prates E. G.; Marques C. C.; Baptista M. C.; Vasques M. I.; Carolino N.; Horta A. E. M.; Charneca R.; Nunes J. T.; Pereira R. M. Fat area and lipid droplets morphology of porcine oocytes during in vitro maturation with *trans*-10, *cis*-12 conjugated linoleic acid (t10,c12 CLA) and forskolin. Animal 7: 602–9; (2012).

Prates E.G., Alves S.P., Marques C.C., Baptista M.C., Horta A.E.M., Bessa R.J.B., Pereira R.M. Fatty acid composition of porcine cumulus oocyte complexes (COC) during maturation: effect of the lipid modulators *trans*-10,*cis*-12 conjugated linoleic acid (t10,c12 CLA) and forskolin. In Vitro Cell.Dev.Biol. Animal. 49:335–345(2013)

Reis A., Rooke J.A., McCallum G.J., Staines M.E., Ewen M., Lomax M.A., McEvoy T.G. Consequences of exposure to serum, with or without Vitamin E supplementation, in terms of the fatty acid content and viability of bovine blastocysts produced in vitro. Reprod. Fertil. Dev., 15, pp. 275–284 (2003).

Stinshoff H., wilkening S., Hanstedt A., Bollwein H., and Wrenzycki C. Dimethylsulfoxide and conjugated linoleic acids affect bovine embryo development *in vitro*. Reprod. Fertil. Dev. 26:502-510 (2014).

Tsuboyama-Kasaoka N., Takahashi M., Tanemura K., Kim H., Tange T., Okuyama H., Kasai M., Ikemoto S., Ezaki O. Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. Diabetes, 49, pp. 1534–1542 (2000).

Van Hoeck V., Leroy J. L. M. R. , Arias Alvares M., Rizos D., Gutierrez-Adan A., Schnorbusch K., Bols P.E.J.; Leese H. J., and Sturmey R.G. Oocytes development failure in response to elevated nonesterified fatty acid concentrations: Mechanistic insights. Reproduction 145:33-44 (2013).

**L-CARNITINE AND *TRANS-10, CIS-12* CONJUGATED LINOLEIC ACID ON *IN VITRO* BOVINE EMBRYO PRODUCTION AND CRYOPRESERVATION**

**Abstract**

High lipid content in embryo is associated with low freezing tolerance. This study assessed the effects of exogenous L-carnitine and *trans-10, cis-12* (t10, c12) conjugated linoleic acid (CLA) on *in-vitro* development and cryotolerance of bovine embryos when added during different stages of *in vitro* production of embryos. For all experiments, embryos were produced *in vitro* using slaughterhouse cows oocytes. Cleavage rates on Day 3, blastocyst and advanced blastocyst (hatching/hatched blastocyst) formation rates on Day 7 were calculated from the total number of oocytes subjected to *in vitro* fertilization (IVF). Expanded blastocysts-stage embryos from each treatment were harvested on Day 7 and subjected to slow freezing. Embryo viability was assessed 24, 48 and 72 h after thawing. In experiment 1, fertilized oocytes were incubated with different L-carnitine concentrations (0.0, 0.75, 1.50 or 3.03 mM) in the presence or absence of fetal bovine serum (FBS). There was an improvement ($P<0.05$) on embryo development when 1.5 mM of L-carnitine was added to *in vitro* culture (IVC) medium. L-carnitine had also a positive effect ($P<0.05$) on post thaw embryo competence when supplemented at 0.75 and 3.03 mM during IVC. There was no interaction ($P>0.05$) between the effects of L-carnitine and FBS supplementation on IVC on embryo development and cryosurvival. Although FBS supplementation had increased blastocyst development ($P<0.05$), it reduced the reexpansion rates at 24, 48 and 72 h post thawing. In experiment 2, L-carnitine (0.75 mM) or CLA (100 mM) were supplemented during the first 96 h, last 72 h or throughout the entire IVC period. There was no effect of L-carnitine or CLA supplementation during different periods of IVC on embryo development and cryotolerance. In experiment 3, embryo development and cryosurvival were evaluated when oocytes were maturated in medium supplemented with L-carnitine (3.03 mM) or/and CLA (100 mM). No effect of L-carnitine and CLA supplementation during *in vitro* maturation (IVM) on IVP embryo development was detected. Although there was no effect ($P>0.05$) of L-carnitine supplementation on embryo cryotolerance, CLA showed a negative effect ($P<0.05$) on embryo cryosurvival when added during IVM. In conclusion, L-carnitine improved embryo cryosurvival when added at 0.75 mM during IVC and CLA supplementation during IVM has a negative effect on post thaw embryo survival.

## 1. Introduction

*In vitro*-production (IVP) of embryos is one of the most challenging areas on animal reproduction biotechnologies. Low pregnancy rates and lack of reliability over the results after IVP embryos cryopreservation are pointed as a direct reflex of higher sensibility of these embryos. This susceptibility to cryopreservation is related to sub-optimal cultivation conditions (Lonergan et al., 2003) that leads to cytoplasm lipid accumulation in embryos when they are cultured in media containing fetal bovine serum (FBS). The serum withdrawal from culture medium reduces the accumulation of lipids, improving cryotolerance of bovine embryos. However, it compromises blastocyst development (Van Langendonckt et al., 1996; Yoshioka et al., 1997). An alternative to solve this problem is the addition of substances to maturation and culture media in order to accelerate lipid metabolism or inhibit its formation.

The use of *t10, c12* conjugated linoleic acid (CLA) has been reported to reduce lipid droplet formation in bovine embryos (Pereira et al., 2007; 2008) through fatty acids uptaking reduction (Pariza et al., 2001). Besides that, CLA incorporation in culture media seems to increase membrane fluidity, thus conferring embryos great resistance to cryopreservation-induced damage (Pereira et al., 2007; 2008).

L-carnitine, a component of lipid metabolism, also reduced lipid contend in bovine, swine and mouse oocytes (Yamada et al., 2006; Dunning et al., 2010; Somfai et al., 2011). It has a role in the transport of fatty acids from the cytosol to the mitochondria where they will be used as a source of energy for the β-oxidation. Besides its metabolic function, L-carnitine is a powerful antioxidant (Gülçin, 2006), reducing the accumulation of reactive oxygen species (ROS) and lowering the frequency of apoptosis in animal cells (Pillich et al., 2005; Ye et al., 2010). The antioxidant effect of L-carnitine has been observed in bovine, swine and mouse embryos (Takahashi et al., 2013; Wu et al., 2011).

Maturation and culture media supplementation with CLA and L-carnitine seems promising since both substances can regulate lipid metabolism and, consequently, reduce cytoplasmatic lipid acumulation. However, the effects of CLA and L-carnitine supplementation during different stages of bovine *in vitro* production on subsequent embryo development and cryotolerance rates remain unknown. Facing that, the aim of the present study was to investigate the effects of L-carnitine supplementation in the presence or absence of FBS and the effects of CLA and L-carnitine supplementation

during maturation and on different stages of embryo culture on *in vitro* development and cryotolerance of bovine embryos.

## 2. Material and Methods

### 2.1 Embryo Production

*In vitro* production (IVP) of bovine embryos was performed as previously described by Fields et al. (2011) unless otherwise specified. Briefly, *cumulus* oocyte complexes were obtained by cutting the surface of slaughterhouse cows ovaries with a scalpel and vigorously rinsing the ovary through a bath of oocyte washing medium (Mofa®). Groups of 10 *cumulus* oocyte complexes were matured in 50 µL droplets of oocyte maturation medium - tissue culture medium-199 with Earle salts (Invitrogen), 10 % bovine steer serum, 2 µg/mL estradiol-17b, 20 µg/mL bovine follicle-stimulating hormone (Bioniche Life Sciences), 22 µg/mL sodium pyruvate, 50 µg/mL gentamicin sulfate and 1 mM glutamine - covered with mineral oil for 20 h at 38.5 $^{o}$C and placed in a humidified atmosphere of 5 % $CO_2$. Oocytes were fertilized with isolate-purified sperm (1 x $10^6$ /mL) for 18 h at 38.5 °C in synthetic oviduct fluid-fertilization medium (Sakatani et al., 2012). *Cumulus* cells were denuded after fertilization by vortexing in 600 µL HEPES-TALP containing 10 000 U/mL hyaluronidase. Groups of 15 putative zygotes were then cultured in 25 µL microdrops of synthetic oviduct fluid-bovine embryo 1 (SOF-BE1 – Fields et al., 2011) covered with mineral oil at 38.5 °C in a humidified atmosphere of 5 % $O_2$ and 5 % $CO_2$ with the balance being $N_2$. Blastocysts were harvested at Day 7 post-insemination.

### 2.2 Embryo freezing and survival evaluation

Expanded blastocysts graded excellent according to the guidelines of the International Embryo Transfer Society (Stringfellow and Seidel, 1998) were selected on Day 7 (Day 0 = day of IVF) and used for freezing. They were frozen with ethylene glycol plus sucrose (Vigro$^{TM}$). Embryos were washed 3 times in holding medium (Vigro$^{TM}$) , transferred directly into the freezing medium, and then 5-10 embryos were loaded into a 0.25 mL straw and allowed to equilibrate for 10 min. The straws were then put into a – 6 °C nitrogen freeze machine of a programmable freezer (Freeze Control®) and seeded 2 min after that. The straws were maintained at this temperature for 10 min, and then cooled to -32 °C at a rate of -0.3 °C/min. The straws were maintained at -32 °C for 10 min, and then plunged into liquid nitrogen. After being stored for at least 1 day,

the embryos were warmed by air thawing for 5 s and then immersed in a water bath at 29 °C for 20 s. After thawing, the embryos were washed 3 times in HEPES-TALP and cultured for 72 h in groups of 15 in 25 µL microdrops of SOF-BEI with 5 % FBS and 1 % dithiothreitol (Sigma) covered with mineral oil at 38,5 °C in a humidified atmosphere of 5 % (v/v) $O_2$ and 5 % (v/v) $CO_2$ with the balance being $N_2$. The percentages of embryos that developed to the reexpanded, hatching and hatched blastocyst stages were determined 24, 48 and 72 h post thawing, respectively.

The slow freezing method was chosen over vitrification because frozen embryos through this technique can be thawed without the need of laboratory conditions and specialized technicians. Therefore, more likely to be used in farm conditions.

2.3 Experimental design

**Experiment 1: Effect of L-carnitine and fetal bovine serum (FBS) supplementation during *in vitro* culture (IVC) on development and cryotolerance of IVP bovine embryos.**

Fertilized oocytes (Day 1) were pooled and randomly subjected to IVC in medium supplemented with 0.0 mM, 0.75 mM, 1.5 mM or 3.03 mM of L-carnitine (Sigma, ref.C0283) based on the report by Abdelrazik et al. (2009) in the presence or absence of 5 % FBS. A total of 2,768 oocytes were used in this study. Cleavage rates on Day 3, blastocyst and advanced blastocyst (hatching and hatched blastocyst) formation rates on Day 7 were calculated from the total number of oocytes subjected IVF. The experiment was replicated ten times.

Expanded blastocyst stage embryos from each treatment were harvested on Day 7 and subjected to slow freezing. After thawing, embryo viability was assessed. The experiment was replicated nine times.

**Experiment 2: Effect of L-carnitine and *t10, c12* CLA supplementation during different periods of *in vitro* culture (IVC) on development and cryotolerance of IVP bovine embryos.**

Fertilized oocytes (2,804) were pooled and randomly cultured in IVC medium supplemented with 0.75 mM L-carnitine (based on results from first experiment) or 100 mM *t10, c12* CLA (Cayman, ref. 90145; Pereira et al., 2004) during the first 96 h, last 72 h or through the entire IVC period according with each treatment (Table 1). Cleavage rates on Day 3, blastocyst and advanced blastocyst (hatching and hatched blastocyst)

formation rates on Day 7 were calculated from the total numbers of oocytes subjected to IVF.

Expanded blastocyst stage embryos from each treatment were harvested on Day 7 and subjected to slow freezing. After thawing, embryo viability was assessed. The experiment was replicated fourteen times.

**Table 1**. L-carnitine and *t10, c12* CLA supplementation during different periods of IVC

| Treatment | First 4 days of culture | Last 3 days of culture |
|---|---|---|
| 1 | - | - |
| 2 | - | CLA |
| 3 | - | L- carnitine |
| 4 | CLA | - |
| 5 | CLA | CLA |
| 6 | L-carnitine | - |
| 7 | L-carnitine | L- carnitine |

**Experiment 3: Effect of L-carnitine and *t10, c12* CLA supplementation during *in vitro* maturation (IVM) on development and cryotolerance of IVP bovine embryos.**

A total of 1,796 oocytes were pooled and randomly maturated in 4 different treatments including control, L-carnitine (3.03 mM), *t10,c12* CLA (100 mM), L-carnitine (3.03 mM) + CLA (100 mM). L-carnitine concentration was based on the report by Takahashi et al. (2013). Cleavage rates on Day 3 and blastocyst and advanced blastocyst (hatching and hatched) formation rates on Day 7 were calculated from the total numbers of oocytes subjected to IVF. The experiment was replicated five times.

Expanded blastocyst stage embryos harvested from four treatments were subjected to slow freezing. After thawing, embryo viability was assessed. The experiment was replicated five times.

### 3. Statistical Analysis

Data were statistically analyzed using Statistical Analysis System (SAS) version 9.3 (SAS institute Inc., Cary, NC). Because data did not show a normal distribution, they were arcsine transformed before analysis. All parameters were subjected to ANOVA (GLM procedure). The limit of significance was set at $P<0.05$.

## 4. Results

**Experiment 1**: **Effect of L-carnitine and fetal bovine serum (FBS) supplementation during *in vitro* culture (IVC) on development and cryotolerance of IVP bovine embryos.**

Supplementation with L-carnitine during IVC did not affect cleavage rates on day 3. On day 7, significantly higher ($P<0.05$) percentages of embryos developed to the advanced blastocyst stage in the presence of 1.5 mM L-carnitine than in its absence. The advanced blastocyst rate in the 3.03 mM L-carnitine group was lower ($P<0.1$) than 1.5 mM mg/mL L-carnitine group. (Table 2).

**Table 2.** Effect of L-carnitine supplementation during *in vitro* culture (IVC) on development of IVP bovine embryos (10 replicates).

| L-carnitine (mM) | Inseminated oocytes (n) | Cleavage rate (%) | D7 Blastocyst rate (%) | D7 advanced rate (%) |
|---|---|---|---|---|
| 0 | 683 | 77.6±1.9 | 24.8±1.7 | 11.2±1.5[a] |
| 0.75 | 646 | 75.9±2.4 | 24.2±1.0 | 13.7±1.6[ab] |
| 1.5 | 705 | 77.4±2.4 | 24.6±1.3 | 15.2±2.0[b] |
| 3.03 | 734 | 78.9±2.2 | 19.8±1.7 | 8.3±1.2[a] |

[ab]Values with different superscripts within columns differ significantly; Data within same columns with different superscripts letters (a, b and c) are statistically different ($P< 0.05$); D7 blastocyst rate (%): Percentage of expanded blastocyst on Day 7 of culture; D7 advanced rate (%) : Percentage of Hatching and hatched blastocyst on Day 7 of culture.

**Commented [D1]:** Use the Word's Table option to do the tables. Tables should not be a figure. For all of them.

Blastocysts developed in the presence of 0.75 and 3.03 mM L-carnitine exhibited higher ($P < 0.01$) survival rates at 24 h and 48 h (table 3) post thawing than control blastocysts.

**Table 3.** Effect of L-carnitine supplementation during *in vitro* culture (IVC) on IVP bovine embryos cryotolerance (9 replicates)

| L-carnitine (mM) | N°. freeze embryos (n) | 24 hours (%) | | 48 hours (%) | | 72 hours (%) | |
|---|---|---|---|---|---|---|---|
| | | Reexpanded | Hatched | Reexpanded | Hatched | Reexpanded | hatched |
| 0 | 129 | 64.0±4.7[a] | 24.7±4.3 | 67.4±4.7[a] | 41.5±6.1 | 68.7±5.3 | 46.7±5.9 |
| 0.75 | 117 | 77.4±5.1[b] | 28.8±5.4 | 80.1±4.0[b] | 47.3±6.4 | 76.3±4.7 | 57.5±5.9 |
| 1.5 | 108 | 71.5±4.5[ab] | 27.2±5.8 | 73.1±4.1[ab] | 50.7±6.7 | 70.8±4.8 | 55.8±5.6 |
| 3.03 | 112 | 75.0±5.5[b] | 24.1±4.2 | 78.0±4.2[b] | 50.2±5.7 | 74.5±4.8 | 55.7±5.6 |

[ab]Values with different superscripts within columns differ significantly

Although blastocyst rates were higher (P<0.01) in the groups supplemented with FBS (Table 4), no interaction was observed between L-carnitine and FBS effects.

**Table 4.** Effect fetal bovine serum (FBS) supplementation during *in vitro* culture (IVC) on IVP bovine embryos development. (10 replicates).

| Fetal Bovine Serum (%) | Inseminated oocytes (n) | Cleavage rate (%) | D7 Blastocyst rate (%) | D7 advanced blastocyst rate (%) |
|---|---|---|---|---|
| 0 | 1473 | 77.2±1.5 | 19.4±0.9[a] | 10.1±1.1[a] |
| 5 | 1622 | 77.8±1.6 | 27.2±1.1[b] | 14.2±1.4[b] |

[ab]Values with different superscripts within columns differ significantly; D7 blastocyst rate (%): Percentage of expanded blastocyst on Day 7 of culture; D7 advanced blastocyst rate (%) : Percentage of Hatching and hatched blastocyst on Day 7 of culture.

Although serum supplementation had increased blastocyst development (Table 4), it reduced the reexpansion rates (P<0.01) at 24, 48 and 72 h post thawing (table 5).

**Table 5**. Effect fetal bovine serum (FBS) supplementation during *in vitro* culture (IVC) on IVP bovine embryos cryotolerance. (9 replicates)

| Fetal Bovine Serum (%) | N°. freeze embryos (n) | 24 hours (%) | | 48 hours (%) | | 72 hours (%) | |
|---|---|---|---|---|---|---|---|
| | | Reexpanded | Hatched | Reexpanded | Hatched | Reexpanded | Hatched |
| 0 | 199 | 78.1±3.8[a] | 27.0±3.6 | 81.4±3.0[a] | 53.8±4.4 | 79.1±3.5[a] | 61.8±4.1[a] |
| 5 | 267 | 65.8±2.9[b] | 25.4±3.3 | 67.8±2.8[b] | 41.0±4.1 | 66.0±3.0[b] | 46.1±3.5[b] |

[ab]Values with different superscripts within columns differ significantly

**Experiment 2**: **Effect of L-carnitine and CLA supplementation during different periods of *in vitro* culture (IVC) on development and cryotolerance of IVP bovine embryos.**

There were no differences in cleavage, Day 7 blastocyst and Day 7 advanced blastocyst rates across groups (P>0.05 - Table 6). Also, L-carnitine and CLA did not affected reexpansion and hatching rates after thawing when added during different periods of IVC (Table 7).

**Table 6**. Effect of L-carnitine and CLA supplementation during different periods of *in vitro* culture (IVC) on IVP bovine embryos development (14 replicates).

| Treatment | Inseminated oocytes (n) | Cleavage rate (%) | D7 Blastocyst rate (%) | D7 advanced blastocyst rate (%) |
|---|---|---|---|---|
| 1 | 393 | 82.7±1.9 | 20.3±1.3 | 11.3±2.4 |
| 2 | 379 | 85.3±2.0 | 17.5±1.2 | 8.2±1.5 |
| 3 | 374 | 83.1±2.2 | 16.3±1.0 | 9.9±1.8 |
| 4 | 423 | 85.4±1.4 | 15.8±0.9 | 10.0±1.6 |
| 5 | 424 | 85.1±1.7 | 17.4±1.3 | 11.3±2.2 |
| 6 | 418 | 86.0±1.3 | 22.4±2.1 | 13.8±0.3 |
| 7 | 393 | 87.1±2.4 | 18.6±1.0 | 11.0±1.8 |

Treatments = 1: control; 2: control followed by L-carnitine treatment; 3: control followed by CLA treatment; 4: CLA treatment followed by control; 5: CLA treatment for the entire culture period; 6: L-carnitine treatment followed by control; 7: L-carnitine for the entire culture period. There were no significant difference between treatments (P>0.05). D7 blastocyst rate (%): Percentage of expanded blastocyst on Day 7 of culture; D7 advanced blastocyst rate (%) : Percentage of Hatching and hatched blastocyst on Day 7 of culture.

**Table 7.** Effect of L-carnitine and CLA supplementation during different periods of *in vitro* culture (IVC) on IVP bovine embryos cryotolerance (14 replicates)

| Treatment | N°. freeze embryos (n) | 24 hours (%) | | 48 hours (%) | | 72 hours (%) | |
|---|---|---|---|---|---|---|---|
| | | Reexpanded | Hatched | Reexpanded | Hatched | Reexpanded | Hatched |
| 1 | 90 | 80.7±4.6 | 39.5±7.8 | 75.7±5.0 | 54.5±8.1 | 68.4±7.9 | 62.8±8.3 |
| 2 | 70 | 88.1±5.2 | 31.4±5.6 | 77.7±5.7 | 58.6±8.0 | 75.9±5.4 | 63.8±8.6 |
| 3 | 67 | 92.1±4.2 | 33.9±8.4 | 87.4±5.1 | 69.4±7.4 | 85.0±6.2 | 73.4±7.7 |
| 4 | 68 | 88.6±5.1 | 31.6±7.4 | 77.9±8.9 | 52.2±8.8 | 76.1±9.8 | 57.8±10.1 |
| 5 | 78 | 88.3±3.9 | 31.3±6.6 | 72.0±7.6 | 55.0±8.4 | 67.8±8.5 | 59.0±7.9 |
| 6 | 92 | 83.1±3.9 | 29.9±7.6 | 82.3±4.4 | 54.2±8.0 | 79.6±4.4 | 63.0±6.8 |
| 7 | 72 | 85.6±7.6 | 33.3±8.2 | 77.0±7.9 | 50.3±7.2 | 78.6±8.6 | 58.1±9.7 |

Treatments = 1: control; 2: control followed by L-carnitine treatment; 3: control followed by CLA treatment; 4: CLA treatment followed by control; 5: CLA treatment for the entire culture period; 6: L-carnitine treatment followed by control; 7: L-carnitine for the entire culture period. There were no significant difference between treatments (P>0.05).

**Experiment 3: Effect of L-carnitine and *t10, c12* CLA supplementation during *in vitro* maturation (IVM) on development and cryotolerance of IVP bovine embryos.** Supplementation of maturation medium with L-carnitine or CLA had no effect on cleavage rates on day 3 and on embryo and advanced embryo formation rates on day 7 (Table 8 and 9). Also, there was no interaction between the effects of L-carnitine and CLA supplementation during maturation on cleavage rates on Day 3 and on embryo and advanced embryo formation rates on Day 7.

**Table 8.** Effect of L-carnitine supplementation during *in vitro* maturation (IVM) on IVP bovine embryos development (5 replicates).

| Treatment | Inseminated oocytes (n) | D3 Cleavage rate (%) | D7 Blastocyst rate (%) | D7 advanced blastocyst rate (%) |
|---|---|---|---|---|
| Control | 403 | 83.2±1.4 | 19.4±4.1 | 11.8±2.2 |
| L-carnitine | 443 | 84.4±1.4 | 17.1±3.0 | 9.7±2.3 |

There were no significant difference between treatments (P>0.05). D7 blastocyst rate (%): Percentage of expanded blastocyst on Day 7 of culture; D7 advanced blastocyst rate (%) : Percentage of Hatching and hatched blastocyst on Day 7 of culture.

**Table 9.** Effect of *t10, c12* CLA supplementation during *in vitro* maturation (IVM) on IVP bovine embryos development (5 replicates).

| Treatment | Inseminated oocytes (n) | D3 Cleavage rate (%) | D7 Blastocyst rate (%) | D7 advanced blastocyst rate (%) |
|---|---|---|---|---|
| Control | 403 | 83.2±1.4 | 19.4±4.1 | 11.8±2.2 |
| CLA | 449 | 80.9±2.4 | 15.6±3.2 | 10.4±2.6 |

CLA = Trans-10 cis-12 conjugated linoleic acid. There were no significant difference between treatments (P>0.05).=; ). D7 blastocyst rate (%): Percentage of expanded blastocyst on Day 7 of culture; D7 advanced blastocyst rate (%) : Percentage of Hatching and hatched blastocyst on Day 7 of culture.

Although L-carnitine had no effect on post thawing reexpansion and hatching rates when added during maturation (table 10), CLA supplementation during IVM significantly decreased hatching rates at 48 h and 72 h after thawing (P<0.05; table 11). There was no interaction between the effects of L-carnitine and CLA supplementation during maturation on post thawing reexpansion and hatching rates.

**Table 10**. Effect of L-carnitine supplementation during *in vitro* maturation (IVM) on IVP bovine embryos cryotolerance (5 replicates).

| Treatment | N°. freeze embryos (n) | 24 hours (%) | | 48 hours (%) | | 72 hours (%) | |
|---|---|---|---|---|---|---|---|
| | | Reexpanded | Hatched | Reexpanded | Hatched | Reexpanded | Hatched |
| Control | 61 | 79.8±7.4 | 42.4±5.3 | 79.8±5.1 | 65.1±4.3 | 75.0±6.8 | 65.1±4.3 |
| L-carnitine | 61 | 71.1±9.2 | 27.3±7.8 | 83.1±4.6 | 53.4±10.2 | 82.0±4.7 | 62.8±7.4 |

There were no significant difference between treatments (P>0.05).

**Table 11**. Effect of *t10, c12* CLA supplementation during *in vitro* maturation (IVM) on IVP bovine embryos cryotolerance (5 replicates).

| Treatment | N°. freeze embryos (n) | 24 hours (%) | | 48 hours (%) | | 72 hours (%) | |
|---|---|---|---|---|---|---|---|
| | | Reexpanded | Hatched | Reexpanded | Hatched | Reexpanded | Hatched |
| Control | 61 | 79.8±7.4 | 42.4±5.3 | 79.8±5.1 | 65.1±4.3$^{a}$ | 75.0±6.8 | 65.1±4.3$^{a}$ |
| CLA | 67 | 61.9±7.1 | 21.7±6.2 | 68.8±6.6 | 47.5±7.1$^{b}$ | 66.3±5.2 | 53.3±3.6$^{b}$ |

CLA = Trans-10 cis-12 conjugated linoleic acid; $^{ab}$Values with different superscripts within columns differ significantly (P<0.05)

## 5. Discussion

The main objective of the current study was to analyze the effects of L-carnitine and CLA supplementation during different stages of *in vitro* embryo production process on embryo development and competence development after cryopreservation. In the first experiment, supplementation with 1.5 mM of L-carnitine during IVC resulted in significant improvement of advanced blastocyst rates, which is similar to that previously reported by Abdelrazik et al., 2009 and Takahashi et al., 2013. This positive effect of L-carnitine on embryo development has been related to its role in the transport of long-chain fatty acids into mitochondria for its β-oxidation and, consequently, ATP generation (Dunning et al., 2010; Takahashi et al., 2013).

Besides ATP, long-chain fatty acids β-oxidation also generates reactive oxygen species (ROS) that can compromise oocyte and embryo quality. Although L-carnitine has antioxidant activity reducing intracellular concentrations of ROS in oocytes and embryos (Somfai T. et al. 2011; Wu G.Q. et al. 2011; Abdelrazik et al. 2009), the IVC medium supplementation with 3.03 mM L-carnitine tended to reduce embryo development probably because the high ROS prodution by the over-stimulated β-oxidation was not completely scavenged by L-carnitine.

This dual effect of L-carnitine in terms of reducing cellular lipid content and providing anti-oxidative protection makes it a choice reagent for the improvement of cryotolerance and developmental competence in livestock embryos. In the present experiment, developmental competence was improved 24 and 48 h post thawing in embryos treated during IVC with 0.75 and 3.03 mM of L-carnitine. There was no difference in re-expansion and cleavage rates between treatments 72 h post thawing probably because even the bad quality embryos were able to develop after this long cuture time. Similarly, embryo culture medium supplemented with L-carnitine reduced lipid content in bovine embryos and increased freezing tolerance (Takahashi et al., 2013). Freezing tolerance is highly dependent on the amount of lipid present in porcine and bovines embryos (Nagashima et al., 1995; Abe et al., 2002). Although serum supplementation may generate higher blastocyst rates, removal of serum from culture media reduced lipid content and improved cryotolerance of bovine embryos (Abe et al., 2002). These findings agree with the present study in which FBS supplementation reduced post thawing embryo cryotolerance and developmental competence. Serum supplementation increases cytoplasmic lipid uptake and lowers phosphatidyl choline and cholesterol esters resulting in reduced cryotolerance (Pereira et al., 2007; Al Darwich et al., 2010; Pereira and Marques, 2008). Considering that L-carnitine increases lipid metabolism reducing cellular fatty acid content we investigated the interaction between L-carnitine and FBS on embryo cryotolerance when added to IVC medium. Contrary to our expectations, L-carnitine supplementation did not avoid the deleterious effects of FBS on embryo cryotolerance. Serum supplementation may also induce excessive lipid accumulation through alterations of mitochondrial metabolism (Pereira and Marques et al., 2008) what may also compromise L-carnitine effects on lipid metabolism.

This study also evaluated the possible effects of L-carnitine or CLA supplementation for the first 96 h, last 72 h or through the entire IVC period. In experiment 2, L-carnitine supplementation either throughout the entire IVC period or during only the first 96 h or last 72 h of IVC did not affect embryonic development and cryotolerance. These results contrast with the result from experiment 1 in which L-carnitine supplementation during the IVC significantly improved post thawing embryo development and developmental competence. This conflicting report probably happened due to the substitution of the

old culture medium to a new one on day 4 of embryo culture in experiment 2. With this exchange, L-carnitine concentration was re-estabilished and it may have compromised the positive effects of L-carnitine on embryo development and cryotolerance shown in experiment 1. In this study CLA supplementation during the first, last or entire IVC period also had no effect on embryo development and cryosurvival. CLA has an important role in lipid metabolism. It seems to exerts specific effects on adipocytes by inhibiting the expression of several genes coding lipids synthesis enzymes (Peterson et al., 2003; Granlund et al., 2005), as well as protein lipase activity (Park et al., 1999). Consequently, CLA supplementation may inhibit fatty acids synthesis and uptake reducing intracytoplasmatic lipid content. Besides that, it has been reported that CLA can change lipid composition and probably increase the membrane fluidity when added to embryo culture medium (Pereira et al., 2007; 2008). Reduced intracellular lipid content and increased membrane fluidity could contribute to improve embryo resistance to cryopreservation. However, previous studies supplementing culture media with CLA have generated contradictory results in relation to embryo criotolerance. Criosurvival and viability improvement of post thawing bovine embryos when CLA was added to culture were demonstrated in reports by Pereira et al. (2007, 2008). On the other hand, recent reports found lower embryo survival after cryopreservation when CLA was added during IVC (Al Darwich et al., 2010). Furthermore, in a similar study in which the CLA was not conjugated with FBS, embryo development and cryosurvival were not improved when CLA was supplemented through the entire IVC period or during 36 h before vitrification (Absalón-Medina et al., 2014).

Considering the importance of lipid metabolism in oocyte developmental competence and the ability of L-carnitine to increase β-oxidation and CLA to reduce fatty acids synthesis and uptake, the effects of L-carnitine or/and CLA supplementation during the IVM on embryo development and cryosurvival were evaluated in this study. Although Somfai et al. (2011) had found that swine oocytes matured in medium supplemented with L-carnitine had significantly more active mitochondria, fewer lipid droplets and decreased ROS levels compared to controls, further development to the blastocyst stage was not improved by L-carnitine supplementation. Chankitisakul et al. (2013) also found no effect of L-carnitine supplementation during IVM on bovine embryo development. In agreement with these results we found no effect of L-carnitine maturation medium supplementation on bovine embryo cleavage, blastocyst and advanced blastocyst development. Also L-carnitine had no effect on post thawing

embryo survival. Considering these results, it is possible that enhancing lipid metabolism during IVM does not have a significant effect on embryo development and criosurvival.

In bovine, supplementation of oocyte culture medium with 10, c12 CLA increased the rate of very good quality embryos on day 8 post-insemination (Lapa et al., 2011). On the other hand, according to Absalón-Medina (2014) supplementation with either t10, c12 or c9, t11 during IVM had no effect on embryo development and decreased post thawing embryo survival. Similarly, supplementation of IVM medium with CLA did not improved embryo development and had a negative effect on post thawing embryo competence. Furthermore, it is possible that reducing lipid content in oocyte through IVM medium supplementation may compromise embryo quality reducing its cryotolerance.

**Commented [D2]:** It's just a review. Why CLA did not improve embryo development and cryosurvival? Probably, changes in lipids metabolism caused by CLA are detrimental for embryos.

## 6. Conclusion

Supplementation of IVC medium with L-carnitine improved blastocyst development and cryotolerance, but had no effect on blastocyst rates and post thawing embryo developmental competence when added to IVM medium or during different periods of IVC. Treatment with CLA did not improve embryo development and cryotolerance when added during different periods of IVC. Also CLA had no effect on blastocyst development and decreased post thawing embryo competence when added during IVM.

## 7. References


Abdelrazik H., Sharma R., Mahfouz R., Agarwal A. L-carnitine decreases DNA damage and improve the *in vitro* blastocyst development rate in mouse embryos. Fertility and Sterility Vol. 91, N°. 2 (2009).

Abe H., Yamashita S., Satoh T., and Hoshi H. Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. Mol. Reprod. Dev. 61, 57–66 (2002).

Absalón-Medina V.A. Bedford-Guaus S.J., Gilbert R.O., Siqueira L.C. Esposito G., Schneider A., Cheong S.H., Butler W.R. The effects of conjugated linoleic acid isomers *cis*-9,*trans*-11 and *trans*-10, *cis*-12 on in vitro bovine embryo production and cryopreservation. J. Dairy Sci. 97:6164-6176 (2014).

Al Darwich A. Perreau C., Petit M. H., Papillier P.,Dupont J., Guillaume D., Mermillod P., Florence Guignot F. Effect of PUFA on embryo cryoresistance, gene expression and AMPKα phosphorylation in IVF-derived bovine embryos. Prostaglandins & Other Lipid Mediators Volume 93, Issues 1–2, Pages 30–36 (2010).

Chankitisakul V., Somfai T., Inaba Y., Techakumphu M., Nagai T. Supplementation of maturation medium with L-carnitine improves cryo-tolerance of bovine in vitro matured oocytes. Theriogenology. Mar 1;79(4):590-8 (2013).

Dunning K.R., Cashman K., Russell D.L., Thompson J.G., Norman R.J., Robker R.L. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. Biol. Reprod. 83, 909–918 (2010).

Fields S.D., Hansen P.J., Ealy A.D.: Fibroblast growth factor requirements for *in vitro* development of bovine embryos. Theriogenology, 75:1466–1475 (2011)

Granlund L., Pedersen J. I., Nebb H. I. Impaired lipid accumulation by *trans-10, cis-12* during adipocyte differentiation is dependent on timing and length of treatment. Biochim. Biophys. Acta 1687, 11-22 (2005).

Gülçin I. Antioxidant and antiradical activities of L-carnitine. Life Sci. 78, 803-811 (2006).

Lapa M.; Marques C. C.; Alves S. P.; Vasques M. I.; Baptista M. C.; Carvalhais I.; Silva Pereira M.; Horta A. E. M.; Bessa R. J. B.; Pereira R. M. Effect of *trans*-10, *cis*-12 conjugated linoleic acid on bovine oocyte competence and fatty acid composition. Reprod.Domest. Anim. 46: 904–10 (2011).

Lonergan P., Rizos D., Kanka J., Nemcova L., Mbaye A.M., Kingston, M. Wade M., Duffy, P., and Boland M.P.Temporal sensitivity of bovine embryos to culture environment after fertilization and the implications for blastocyst quality. Reproduction 126(3), 337-46 (2003).

Nagashima H., Kashiwazaki N., Ashman R. J., Grupen C. G., and Nottle M. B. Cryopreservation of porcine embryos. Nature 374(6521), 416 (1995).

Pariza M.H., Park Y. and Cook M.E. The biologically active isomers of conjugated linoleic acid. Prog Lipid Res 40, 283–298 (2001).

Park Y., Storkson J.M., Albright K.J., Liu W., Pariza M.W. Evidence that the *trans-10,cis-12* isomers of conjugated linoleic acid induces body composition changes in mice. Lipids 34, 235-241 (1999).

Pereira R.M., Baptista M.C. Vasques M.I., Horta A.E.M., Portugal P.V. Bessa R.J.B., Marques C.C. Post-thawing resistance of bovine embryos is improved by *trans-10,cis-*

*12* conjugated linoleic acid (CLA). In:Procceedings of 15th International Congress on Animal Reproduction (ICAR), vol. 2, Porto Seguro, Brazil, August 8th-12th, p.538 (2004).

Pereira R. M., Baptista M.C., Vasques M.I., et al. Cryosurvival of bovine blastocysts is enhanced by culture with *trans*-10 *cis*-12 conjugated linoleic acid (10t,12c CLA). Anim Reprod Sci, 98 (3–4), pp. 293–301 (2007).

Pereira R.M.; Carvalhais I.; Pimenta J.; Baptista M.C.; Vasques M. I.; Horta A. E. M.; Santos I. C.; Marques M. R.; Reis A.; Silva Pereira M.; Marques C. C. Biopsied and vitrified bovine embryos viability is improved by *trans*-10,*cis*-12 conjugated linoleic acid supplementation during *in vitro* embryo culture. Anim. Reprod. Sci.106: 322–32; (2008).

Pereira R. M. and Marques C. C. Animal oocyte and embryo cryopreservation. Cell Tissue Bank.9: 267–77 (2008).

Peterson D. G., Matitashvili E.A., Bauman D. E. Diet-induced milk fat depression in dairy cows results in increased *trans-10,cis-12* CLA in milk fat and coordinate suppression of mRNA abundance for mammary enzymes involved in milk fat synthesis. J. Nutr.133, 3098-3102 (2003).

Pillich R.T., Scarsella G., Risuleo G. Reduction of apoptosis through the mitochondria pathway by the administration of acetyl-L- carnitine to mouse fibroblasts in culture. Exp. Cell Res. 306, 1-8 (200%).

Sakatani M, Alvarez NV, Takahashi M, Hansen PJ: Consequences of physiological heat shock beginning at the zygote stage on embryonic development and expression of stress response genes in cattle. J Dairy Sci, 95:3080–3191 (2012).

Somfai T., Kaneda M., Akagi S., Watanabe S., Haraguchi S., Mizutani E., Dang-Nguyen T.Q., Geshi M., Kikuchi K., Nagai T. Enhancement of lipid metabolism with l-carnitine during *in vitro* maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes. Reprod Fertil Dev; 23: 912 920 (2011).

Stringfellow D. A., and Seidel S. M. 'Manual of the International Embryo Transfer Society: A Procedural Guide and General Information for the Use of Embryo Transfer Technology Emphasizing Sanitary Procedures'. (International Embryo Transfer Society: Savoy, IL, USA (1998).

Takahashi T., Inaba Y., Somfai T., Kaneda M., Geshi M., Nagai T. and Manabe N. Supplementation of culture medium with L-carnitine improves development and

cryotolerance of bovine embryos produced *in vitro.* CSIRO PUBLISHING Reproduction, Fertility and Development, 25, 589–599 (2013).

Van Langendonckt A. Auquier P., Donnay I., Massip A., and Dessy F. Acceleration of in vitro bovine embryos development in the presence of fetal calf serum. Theriogenology 45, 194. [Abstract] (1996).

Wu G.Q., Jia B.Y., Li J.J., Fu X.W., Zhou G.B., Hou Y.P., Zhu S.E. L-carnitine enhances oocyte maturation and development of parthenogenetic embryos in pig. Theriogenology 76, 785–793 (2011).

Yamada T., Imai H., and Yamada M. Beneficial effects of acetyl-Lcarnitine treatment during IVM on post-fertilization development of bovine oocytes *in vitro*. Reprod. Fertil. Dev. 18, 280–281. [Abstract] (2006).

Ye J., Li J., Wei Q., Deng W., and Yu, L. L-carnitine attenuates oxidant injury in HK-2 cells via ROS- mitochondria pathway. Regul. Pept. 161, 58-66 (2010).

Yoshioka K., Othman A.M., Taniguchi T.,Yamanaka H., and Sekikawa K. (1997). Differential patterns of blastulation in bovine morulae cultured in synthetic oviduct fluid medium containing FCS or BSA. Theriogenology 48, 997–1006 (1997).

**Conclusões Gerais:**

Estudos utilizando diferentes delineamentos experimentais têm mostrado que a L-carnitina e o CLA são capazes de alterar o metabolismo lipídico e reduzir a quantidade de ácidos graxos em oócitos e em embriões. Entretanto, tanto os efeitos da L-carnitina quanto do CLA sobre o desenvolvimento e criotolerância embrionária apresentam grande variabilidade de resultados entre as pesquisas e, sob nosso conhecimento, até o presente momento nenhum experimento avaliou os resultados da suplementação dos meios de maturação e cultivo com L-carnitina e CLA na taxa de gestação. Dessa forma, mais estudos devem ser realizados para que o real beneficio dessas substâncias sobre a qualidade embrionária seja determinado.