Accepted Manuscript

Supplementation of L-carnitine during *in vitro* maturation improves embryo development from less competent bovine oocytes

Drahomira Knitlova, Pavlina Hulinska, Michal Jeseta, Katerina Hanzalova, Bartosz Kempisty, Marie Machatkova

PII: S0093-691X(17)30300-X

DOI: 10.1016/j.theriogenology.2017.06.025

Reference: THE 14163

To appear in: *Theriogenology*

Received Date: 9 February 2017

Revised Date: 12 June 2017

Accepted Date: 28 June 2017

Please cite this article as: Knitlova D, Hulinska P, Jeseta M, Hanzalova K, Kempisty B, Machatkova M, Supplementation of L-carnitine during *in vitro* maturation improves embryo development from less competent bovine oocytes, *Theriogenology* (2017), doi: 10.1016/j.theriogenology.2017.06.025.

revised highlighted

~~Presence of L-carnitine during maturation of oocytes with different meiotic competence enhances *in vitro* development of bovine embryos and accelerates their differentiation~~

Supplementation of L-carnitine during *in vitro* maturation improves embryo development from less competent bovine oocytes

Drahomira Knitlova[a], Pavlina Hulinska[a], Michal Jeseta[a], Katerina Hanzalova[a], Bartosz Kempisty[b] and Marie Machatkova[a,*]

[a] *Department of Genetics and Reproduction, Veterinary Research Institute, Brno, Czech Republic;*

[b] *Department of Histology and Embryology, Department of Anatomy, Poznan University of Medical Science, Poznan, Poland*

[*]Corresponding author: Marie Machatkova, Dr. Ph.D., Department of Genetics and Reproduction, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic, Phone: +420 533 331 418, Fax: +420 541 211 229, E-mail address: machatkova@vri.cz

ABSTRACT

The present study was designed to ~~characterize~~ define the impact of L-carnitine, supplemented during maturation, on bovine oocytes with different meiotic competence in terms of their IVF outcomes. Meiotically more competent (MMC) and less competent (MLC) oocytes were obtained separately from differently sized follicles at selected phases of folliculogenesis. The oocytes were matured with or without L-carnitine, ~~were~~ fertilized and cultured to the blastocyst stage. The oocytes were examined for nuclear maturation, mitochondrial cluster formation, lipid consumption, fertilization and embryo development. ~~Even though no significant differences in proportions of oocytes at metaphase II were found among MMC and MLC oocytes matured with or without L-carnitine.~~ The proportion of oocytes at metaphase II was significantly higher in the L-carnitine-treated MMC than that in the L-carnitine-treated MLC oocytes. However in comparison with the untreated controls, the proportion of MII oocytes with mitochondrial clusters was significantly higher only in the L-carnitine-treated MLC oocytes, which also showed a significantly lower mean lipid content. The L-carnitine-treated MLC oocytes showed significantly higher fertilization and syngamy rates than the untreated MLC oocytes. On the other hand, in the L-carnitine-treated MMC oocytes, the fertilization rate was similar to that of the untreated controls and the syngamy rate was significantly delayed. Although no significant differences in cleavage on Day 2 were found among all oocyte categories, L-carnitine treatment resulted in a significantly higher blastocyst yield in the MLC oocytes on Day 7 and Day 8 and a significantly higher proportion of expanded blastocysts in relation to the total number of blastocysts in MMC oocytes on Day 8. It can be concluded that L-carnitine supplementation during maturation improves the development of bovine embryos from meiotically less competent oocytes and accelerates blastocyst formation from more competent oocytes.

Keywords: bovine oocytes, meiotic competence, L-carnitine, maturation, embryo development, blastocyst differentiation

1. Introduction

~~Both the nuclear and the cytoplasmic maturation of oocytes are important for effective fertilization and embryo production in mammals.~~For effective fertilization and embryo production in mammals, both the nuclear maturation as well as cytoplasmic maturation of oocytes are important. The activation of pathways involved in protein synthesis and phosphorylation is indispensable for oocyte cytoplasmic maturation and subsequent embryo development [1]. It is generally known that deficiencies in cytoplasmic maturation are responsible for reducing the development of embryos from prepubertal oocytes. Differences in the number and distribution of cytoplasmic organelles between prepubertal and adult oocytes matured under *in vitro* conditions have been reported. Prepubertal calf and lamb oocytes have mitochondria of lower volume density, fewer in number and smaller in size, compared with their adult counterparts [2,3].

In adult cyclic cows, the developmental potential of oocytes is changing in accordance with follicular waves emerging during the ovarian cycle. In each wave, the developmental competence of oocytes rises with an increasing size of follicles, stagnates due to dominant follicle (DF) selection and subsequenly decreases during DF growth. This influences the developmental competence of other oocytes from subordinate follicles [4–6]. Bovine oocytes undergo changes in the number and distribution of cytoplasmic organelles in a manner specific for each phase of follicular development. While in the growth phase, oocytes show little contact of mitochondria with lipid droplets, both sparsely distributed in the oocyte periphery, in the static phase they exhibit an increased number of mitochondria associated with lipid droplets, also in the periphery. In the regression phase, mitochondria in tight contact with lipid droplets are evenly distributed in the whole oocyte [7].

~~Among cytoplasmic organelles participating in the acquisition of oocyte developmental competence, mitochondria and lipids are crucial to energy production.~~ Mitochondria and lipids are crucial to energy production and supplementation of energy resources of oocyte. The ability of mitochondria to balance ATP supply is considered the most critical factor in relation to oocyte fertilization and embryo development [8,9].

In vitro matured bovine oocytes differ in mitochondrial patterns and ATP production [10]. More competent oocytes from medium follicles activate mitochondria twice during maturation, before meiosis resumption and before completion of maturation, while less competent oocytes do it only once, before completion of maturation [11].

Lipid metabolism regulators added to culture media can influence the expresion of lipid metabolism-regulating genes and thus can improve the developmental competence and quality of embryos [12,13]. L-carnitine is intrinsically involved in mitochondrial function and lipid metabolism via the transport of fatty acids to mitochondria and its involvement in fatty acid β-oxidation. It also participates in the regulation of vital cellular functions such as apoptosis. Although positive effects of L-carnitine on oocytes and embryos have been described in cattle [14,15], sheep [16], pigs [17,18] and mice [19–21], no information is available about L-carnitine impact on bovine oocytes with different meiotic and developmental competences. ~~The aim of this study was to investigate utilization of L-carnitine, supplemented during maturation, by bovine oocytes with different meiotic and developmental competence in relation to their IVF outcomes. We assumed that meiotically less competent oocytes would chiefly use L-carnitine and thus improve their developmental competence.~~ It is generally known that oocytes recovered from larger follicles have a greater

capability to complete nuclear and cytoplasmic maturation, undergo successful fertilization and develop to the blastocyst stage than have oocytes from smaller follicles.

Therefore, the aim of this study was to investigate the effect of L-carnitine, supplemented during maturation, on subpopulations of bovine oocytes differing in their meiotic and developmental competence. We hypothesized that a) a response of more competent oocytes to L-carnitine is different from that of less competent oocytes; b) less competent oocytes utilize L-carnitine more effectively than do more competent oocytes; c) L-carnitine supplementation during maturation improves embryo development chiefly from less competent oocytes.

2. Materials and methods

All the chemicals used in this study were purchased from Sigma-Aldrich Chemical Co. (Prague, Czech Republic) unless otherwise stated.

2.1. Oocyte collection

Slaughtered Holstein dairy cows (n = 181), aged 4 to 6 years, with a checked ovarian cycle stage, served as donors. Ovaries in the growth, stagnation and regression phases, after dominant phase elimination, defined by follicle population and corpus luteum morphology, were used for oocyte recovery. Meiotically more competent (MMC) oocytes were collected ~~by aspiration~~ from medium follicles (6 to 10 mm) by aspiration and meiotically less competent (MLC) oocytes were subsequently collected from small follicles (2 to 5 mm) by slicing of the ovarian cortex. Only healthy cumulus-oocyte complexes with the homogenous ooplasm, surrounded by compact multiple layers of cumulus cells, were selected from each oocyte category and used in experiments.

2.2. Oocyte maturation

In each category, one half of the oocytes was matured in 500 µL of TCM-199 medium (M4530; Earle's salts), with 20 mM sodium pyruvate, 50 IU/mL penicillin, 50 µg/mL streptomycin, 5% oestrus cow serum (ECS; Sevapharma, Prague, Czech Republic) and gonadotropins (P.G. 600 15 IU/mL; Intervet, Boxmeer, Holland) supplemented with 2.5 mM L-carnitine (C0283). The concentration of 2.5 mM L-carnitine was selected as being most effective for maturation of bovine oocytes on the basis of our unpublished results. The other half oocytes was matured in the same medium, but without L-carnitine, and served as a control. Maturation took place in four-well plates (Nunclon Intermed, Roskilde, Denmark) under a humidified atmosphere of 5% CO_2 in air at 38.8 °C for 24 h. Aliquot parts ~~Adequate numbers~~ of L-carnitine-treated and untreated matured oocytes, in which the first polar body had been extruded, were examined. Before their examination, the oocytes were denuded from cumulus cells by vortexing in TCM-199 medium containing 0.1% (w/v) hyaluronidase.

2.3. Maturation assessment

For ~~chromation~~ chromatin and mitochondria evaluation, the oocytes were first stained in PBS supplemented with 0.4% BSA and 200 nM MitoTracker Orange CMTMRos dye (Molecular Probes, Eugene, OR, USA) for 30 min at 38.8 °C. Subsequently, ~~they~~ the oocytes were washed in PBS, fixed in 3.7% paraformaldehyde for 60 min at room temperature and washed again. Avoiding compression, the oocytes were mounted on slides using Vectashield medium (Vector Lab, Burlingame, CA, USA) containing l µM of DNA dye (SYTOX Green;

Invitrogen; Carlsbad, CA, USA). They were examined with a laser scanning confocal microscope (Leica TCS SP2 AOBS; Leica, Heidelberg, Germany) equipped with Ar and DPSS lasers. The 40 × HCX PL APO CS objective, pinhole, offsets, gain and AOBS were adjusted. The 488 nm excitation band and a 490 to 515 nm detector and the 561 nm excitation band and a 565 to 600 nm detector were used for chromatin and mitochondria detection, respectively. The oocytes were scanned in equatorial optical sections and processed by NIS-ELEMENTS AR 3.0 software (Laboratory Imaging, Prague, Czech Republic).

The oocytes at metaphase II (MII) and those with mitochondrial clusters (Fig. 1) were considered to have completed nuclear and cytoplasmic maturation, respectively [11]. The proportion of oooytes with completed maturation was expressed as a proportion of MII oocytes with mitochondrial clusters in relation to the total number of MII oocytes.

2.4. Lipid assessment

The matured oocytes were fixed in 3.7% paraformaldehyde solution for 60 min at room temperature, washed in PBS and permeabilized with 1% Triton X-100 for 1 h. To stain lipid droplets, the oocytes were incubated in PBS supplemented with 0.4% BSA and 1 µM Nile red dye (Invitrogen, Molecular Probes, Oregon, USA) for 10 min at room temperature. They were washed three times in PBS and mounted on slides, avoiding oocyte compression, using Vectashield medium (Vector Laboratories., Burlingame, CA, USA) with 1 µM of DNA dye TO-PRO-3 (Invitrogen, Molecular Probes, Oregon, USA) to visualize chromatin configuration. They were examined with a confocal microscope adjusted to the same parameters as described above. The 488 nm excitation band and a 540 to 600 nm detector and the 638 nm excitation band and a 640 to 710 nm detector were used for lipid droplets and chromatin detection, respectively. The intensity of fluorescence was recorded in equatorial sections of each oocyte and processed by NIS-ELEMENTS AR 3.0 software. The lipid content per oocyte was expressed as a mean intensity (± SEM) of total lipid droplet fluorescence (Fig. 2).

2.5. Oocyte fertilization

The L-carnitine-treated and untreated oocytes of each category were inseminated with spermatozoa isolated from the same batch of frozen-thawed semen of a ~~standard~~ bull tested in IVF system. Motile spermatozoa were isolated by the swim-up method. Briefly, semen (25 µL) was placed under a layer of SP-TALP medium (1 000 µL) in each of eight tubes and incubated in an atmosphere with 5% CO_2 at 38.8 ^{o}C for 1 h. The upper fraction from each tube (850 µL) was collected and centrifuged twice (200 × g, 10 min). The pellet was resuspended in modified Tyrode's medium (IVF-TALP). Fertilization was carried out in IVF-TALP medium containing 1×10^6/mL spermatozoa and 10 µg/mL heparin at a spermatozoa/oocyte ratio of 10.000:1. The oocytes were co-cultured with the spermatozoa for 19 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air. ~~Adequate numbers~~ Aliquot parts of L-carnitine treated and untreated oocytes in each category were examined to determine the efficiency of fertilization.

2.6. Fertilization assessment

The inseminated oocytes were fixed overnight in 2.5% aqueous glutaraldehyde solution at 4 C and stained with bisbenzimide Hoechst 33258 (Serva, Heidelberg, Germany) in citrate buffer for 10 min at room temperature. After they were rinsed in PBS-Dulbecco, wet mounts were prepared in 5 µL glycerin buffer and subsequently examined by epifluorescence at 400 x

magnification. Oocytes with two polar bodies, either one male and one female pronucleus or at the syngamy stage were considered to be normally fertilized (Fig. 3). Oocytes with two polar bodies - that is, defined by both a male and a female pronucleus or their fusion - were considered to be normally fertilized (Fig. 3). Fertilization efficiency was determined by the proportion of fertilized to inseminated oocytes as well as the proportion of oocytes at syngamy to fertilized oocytes. Oocytes with two polar bodies - that is, defined by both a male and a female pronucleus or their fusion - were considered to be normally fertilized (Fig. 3). Fertilization efficiency was determined by the proportion of fertilized to inseminated oocytes as well as the proportion of oocytes at syngamy to fertilized oocytes.

2.7. Embryo ~~cultivation~~ culture and development assessment

The remaining presumptive zygotes were transferred to a Buffalo rat liver cell line monolayer (BRL cell line, ATCC, Rockville, MD, USA) and cultured in Menezo B2 medium with 10% ECS. The cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 38.8 °C. Embryo development efficiency was recorded on Day 2 (D2), Day 7 (D7) and Day 8 (D8) after oocyte fertilization (Day 0 = day of IVF). It was expressed as proportions of D2 cleaved oocytes, D7 early blastocysts and D8 blastocysts developed from the inseminated oocytes. The proportion of D8 expanded blastocysts in relation to the total number of blastocysts was also assessed. Aliquot of the blastocysts that developed from MLC oocytes was used for blastocyst differentiation assessment.

2.8. Blastocyst assessment

The immunohistochemi~~stry~~ cal method based on the detection of specific markers and described by Wydooghe et al. [22] was modified for trophectoderm (TE) cell staining. Briefly, blastocysts were fixed for 60 min in 3.7% paraformaldehyde at room temperature and stored in PBS containing 0.4% bovine serum albumin (PBS-BSA) at 4 C. They were incubated overnight in 0.5% Triton X-100 and 0.05% Tween 20 in PBS at 4 C. Subsequently, the blastocysts were washed three-times for 2 min in PBS-BSA and incubated overnight in blocking solution containing 10% goat serum and 0.05% Tween 20 in PBS at 4 C. They were washed and incubated overnight with ready-to-use mouse monoclonal anti-CDX2 primary antibody (BioGenex; Fremont, USA) at 4 C. After washing, the blastocyts were incubated with secondary goat anti-mouse IgG antibody (1:100) conjugated with Alexa Fluor 488 (Jackson ImmunoResearch, Inc; West Grove, PA, USA) for 60 min at room temperature. Subsequently they were washed and mounted onto slides in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) with 1 µM of the DNA dye TO-PRO-3 (Invitrogen, Molecular Probes) to visualize TE and inner cell mass (ICM) nuclei. The total cell number (TCN) and the number of TE cells were evaluated using confocal microscopy (Fig. 4). The 488 nm excitation band and a 510 to 560 nm detector and the 633 nm excitation band and a 650 to 690 nm detector were used for visualization of TE cells and all blastocyst nuclei, respectively. Micrographs were evaluated using NIS-Elements AR 3.00 software.

2.9. Statistical analysis

At least ~~three~~ four replicas were carried out for each oocyte category and each assessment. ~~All~~ Data for maturation, fertilization and embryo development, and blastocyst differentiation were analyzed by one-way ANOVA SPSS version 11.5 for Windows (SPSS, INC. IL, USA). The significance of differences among the values was evaluated by the Chi-square test. Data for lipid content were subjected to one-way ANOVA, and the significance of

differences among means was evaluated by Fisher's least significant difference test (StatSoft, Inc. 2011, STATISTICA, version 10). Differences at $P < 0.05$ were considered statistically significant.

3. Results

The impact of L-carnitine on meiotically more and less competent bovine oocytes was characterized in terms of the efficiency ~~and kinetics~~ of maturation, fertilization and embryo development. The kinetics of fertilization and that of embryo development were also assessed on the basis of syngamy onset and blastocyst formation, respectively.

3.1. Effect of L-carnitine on oocyte maturation

The maturation efficiency of oocytes with different meiotic competence matured in the presence or absence of L-carnitine is shown in Table 1. The yield of MII oocytes from the treated MMC oocytes ~~did not differ~~ was significantly higher ($P < 0.05$) ~~between~~ than that from the treated ~~and~~ MLC oocytes, however, this was not due to ~~regardless of~~ L-carnitine treatment. The proportion of MII oocytes with mitochondrial clusters was significantly higher ($P < 0.05$) in the L-carnitine-treated MLC oocytes than in those that were untreated. No such difference was observed in the case of the MMC oocytes.

Lipid content in the MLC oocytes matured with or without L- carnitine is presented in Figure ~~1~~ 5. The mean (± SEM) lipid content was significantly lower ($P < 0.05$) in the MLC oocytes matured with L-carnitine than in those matured without it.

3.2. Effect of L-carnitine on oocyte fertilization

The efficiency of fertilization and pronuclear syngamy in MMC and MLC oocytes matured in the presence or absence of L-carnitine are shown in Table 2. Significantly higher ($P < 0.05$) proportions of fertilized oocytes and oocytes at the syngamy stage were found in the MLC oocytes matured with L-carnitine than in those matured without it. In the MMC category, no significant difference in the proportion of fertilized oocytes was observed, but a significantly lower proportion of oocytes at syngamy was found in the L-carnitine-treated oocytes than in those that were untreated.

3.3. Effect of L-carnitine on embryo development

No significant differences in the cleavage rate on Day 2 were found between the MMC and MLC categories, regardless of L-carnitine treatment. In the MLC category, the proportion of Day 7 early blastocysts developed from oocytes matured with L-carnitine was significantly higher ($P < 0.05$) than the proportion of those developed from the oocytes matured without it (Table 3). Similarly, a significantly higher ($P < 0.05$) proportion of Day 8 blastocysts developed from the MLC oocytes matured with L-carnitine than from those matured without it (Table 4). In the MMC category, no significant differences in proportions of Day 7 early blastocysts and Day 8 blastocysts were found between the L-carnitine-treated and those that were untreated. However, the proportion of Day 8 expanded blastocysts ~~from~~ in relation to the total number of blastocysts ~~MMC oocytes~~ was significantly higher ($P < 0.05$) in the treated than in the untreated oocytes.

3.4. Effect of L-carnitine on blastocyst differentiation

Differentiation assessment carried out on the MLC oocytes showed no significant differences in the total number of cells, TE and ICM cells per blastocyst, or ICM/TE and ICM/TCN ratios between the D 8 blastocysts derived from treated oocytes and those obtained from untreated oocytes (Table 5).

4. Discussion

The developmental competence of mammalian oocytes and embryos is closely associated with their metabolic activity [23]. Apart from carbohydrates, lipids are important energy sources for oocyte maturation and embryonic development. ATP production from lipids processed by mitochondria involves L-carnitine. Its presence significantly increases β-oxidation and ATP production essential for oocyte maturation and fertilization, and for embryo development [24,25]. In addition to its role in metabolism, L-carnitine participates in reducing the ROS level in both oocytes and embryos [26,27]. A significantly lower level of intracellular H_2O_2 has been detected in MII oocytes matured in the presence of L-carnitine in comparison with controls [18]. Up to now the influence of L-carnitine on bovine [15,28,29], porcine [18] and mouse [20] oocytes and embryos cultured under *in vitro* conditions has been investigated with promising results.

It has been generally accepted that bovine oocytes undergo maturation changes as follicles grow, stagnate and regress but, only recently, specific changes in the number and distribution of mitochondria, lipid droplets and the smooth endoplasmic reticulum have been reported [7]. In this study, the effect of L-carnitine on bovine oocytes with different meiotic and developmental competence collected from differently sized follicles in the growth, stagnation and regression phases is presented for the first time. To confirm our hypothesis that the effect of L-carnitine could differ in oocytes with different meiotic competence, we collected more and less competent oocytes and matured them in the presence or absence of L-carnitine, fertilized them with spermatozoa of proved bull and cultured them into the blastocyst stage. During these experiments we assessed the efficiency of each step, i.e maturation, fertilization and embryonic development rates.

No significant effect of L-carnitine treatment on nuclear maturation of bovine oocytes, as compared with untreated oocytes, has been reported by Chankitisakul et al. [28]. On the other hand, L-carnitine significantly improved nuclear maturation of porcine oocytes in relation to the dose used [18]. In L-carnitine-treated MII porcine oocytes, active mitochondria were found in high densities in both the peripheral and the central area; in the untreated oocytes, mitochondria were distributed mainly in the peripheral cytoplasm [18]. Mitochondrial and lipid droplet volume did not change significantly in L-carnitine-treated lamb oocytes but the cytoplasm increased in volume compared with the untreated oocytes [16]. In mice, L-carnitine accelerated nuclear and cytoplasmic maturation and improved normal spindle configuration and mitochondrial distribution in MII oocytes [20,21].

In this study, the effect of L-carnitine on maturation of bovine oocytes was assessed on the basis of the frequency of MII oocytes with mitochondrial clusters, because a relationship between mitochondrial cluster formation and ATP content has been found in our previous study [10]. A correlation between large mitochondrial cluster formation and ATP content has also been described in mouse oocytes [30].

In our study, nuclear maturation tended to be enhanced in the presence of L-carnitine in both MMC and MLC oocytes; however, the differences in MII oocyte frequency between L-carnitine-treated and untreated oocytes within category were not significant. Regarding the frequency of MII oocytes with mitochondrial clusters, this was improved after L-carnitine treatment in both MMC and MLC oocytes, in MLC oocytes the improvement was statistically

significant. Abundant mitochondrial activity and lipid utilization are associated with cytoplasmic maturation of oocytes. In our experiments, a significantly lower lipid content was detected in MLC oocytes after their maturation with L-carnitine. A significantly reduced density of lipids in porcine oocytes matured with L-carnitine, as compared with those matured without it, has been described by Somfai et al. [18]. The authors have observed that L-carnitine-treated oocytes have lipid droplets distributed near the central area and under the oolemma while untreated oocytes showed most of the lipid droplets located in the peripheral area. Bovine oocytes matured with L-carnitine also redistributed lipid droplets from the peripheral area to the inner cytoplasm and increased their ATP content [28].

There is very little information on the influence of L-carnitine on the maturation of bovine and porcine oocytes in relation to their IVF outcomes. It has been reported that L-carnitine significantly increases intracellular glutathione level [21] and stimulates formation of male pronuclei in fertilized oocytes [31,32]. No significant differences in penetration and pronuclear syngamy in association with L-carnitine treatment have been observed in porcine oocytes. Monospermic fertilization rates did not differ significantly between L-carnitine-treated and untreated oocytes. On the other hand, the proportion of zygotes with two polar bodies was significantly higher in L-carnitine-treated oocytes than in untreated control oocytes [18].

In our experiments with bovine oocytes, in the MMC category, the normal fertilization rate of L-carnitine-treated oocytes was similar to that of untreated oocytes but, in the MLC category, it was significantly higher in treated than in untreated oocytes. It was unexpected to find that L-carnitine had a different effect on the kinetics of pronuclear fusion, slowed down syngamy onset in MMC oocytes, but did not affect syngamy formation in MLC oocytes. It can be speculated that fertilized MMC oocytes are, for a certain period, occupied with the expression of genes and synthesis of proteins manifested by a faster embryo development at the blastocyst stage. This assumption is supported by differences in the levels of mRNA transcripts found between bovine embryos derived from more competent oocytes and those developed from less competent oocytes in our earlier study [33].

From the majority of studies mentioned here, it is evident that supplementation of medium with L-carnitine during maturation or cultivation improves the development of early embryos into the blastocyst stage and positively influences blastocyst quality. However, the results are not so clear-cut. Apparently, there are some differences in the effect of L-carnitine on oocytes and embryos in different animal species. Significantly higher blastocyst rates were achieved in sheep [3,34] and mouse [21] oocytes matured with L-carnitine in comparison with conltrols. In bovine [12,15] and porcine [18,35] embryos cultured with L-carnitine, no improvement in development was observed although mitochondrial density was increased, cytoplasmic lipid content was reduced and expression of some lipid regulating genes was altered. An increase in the expression of lipid metabolism-related genes and ATP content in bovine embryos developed with L-carnitine has also been described by Takahashi et al. [29]. In these embryos, the gene expression remained enhanced until the blastocyst stage, but the ATP content was significantly increased only up to the 2-cell stage. The authors suggest that the metabolism-activating effect of L-carnitine decreases during embryo cultivation.

The cultivation of bovine embryos into blastocysts in the presence of L-carnitine significantly increases the total number of cells and the number of TE cells per blastocyst [12,29]. In our experiments, the total number of cells and the number of TE cells per blastocyst also increased, but not significantly. Similar results in bovine embryos derived from oocytes matured with L-carnitine have been reported by Chankitisakul et al. [28] and Phongnimitr et al. [15].

The present study shows that the impact of L-carnitine on bovine oocytes during maturation differs in relation to meiotic and developmental competences of oocytes. While

the meiotically less competent oocytes utilize L-carnitine to promote their cytoplasmic maturation and improve embryo development, the meiotically more competent oocytes use L-carnitine to accelerate blastocyst formation and expansion.

In conclusion, this study confirmed a positive influence of L-carnitine on the maturation and development of mammalian oocytes and embryos, as reported previously. A new finding of this study is that, in bovine oocytes and embryos, the effect of L-carnitine is specific and depends on oocyte meiotic and developmental competence. Our results may contribute to a better understanding of cytoplasmic maturation in mammalian oocytes and the development of more effective systems for *in vitro* embryo production in cattle.

Acknowledgements

This study was supported by Grants COST CZ-LD14104 of the Ministry of Education, Youth and Sports and QJ1510138 and RO0516 of the Ministry of Agriculture of the Czech Republic.

References

[1] Ferreira EM, Vireque AA, Adona PR, Meirelles FV, Ferriani RA, Navarro PAAS. Cytoplasmic maturation of bovine oocytes: Structural and biochemical modifications and acquisition of developmental competence. Theriogenology 2009; 71: 836–48.

[2] de Paz P, Sanchez AJ, De la Fuente J, Chamorro CA, Alvarez M, Anel E, et al. Ultrastructural and cytochemical comparison between calf and cow oocytes. Theriogenology 2001; 55: 1107–16.

[3] Reader KL, Cox NR, Stanton JA, Juengel JL. Mitochondria and vesicles differ between adult and prepubertal sheep oocytes during IVM. Reprod Fertil Dev 2015; 27: 513–22.

[4] Hagemann LJ. Influence of the dominant follicle on oocytes from subordinate follicles. Theriogenology 1999; 51: 449–59.

[5] Hendriksen PJ, Vos PL, Steenweg WN, Bevers MM, Dieleman SJ. Bovine follicular development and its effect on the in vitro competence of oocytes. Theriogenology 2000; 53: 11–20.

[6] Feng WG, Sui HS, Han ZB, Chang ZL, Zhou P, Liu DJ, et al. Effects of follicular atresia and size on the developmental competence of bovine oocytes: A study using the well-in-drop culture system. Theriogenology 2007; 67: 1339–50.

[7] Dadarwal D, Adams GP, Hyttel P, Brogliatti GM, Caldwell S, Singh J. Organelle reorganization in bovine oocytes during dominant follicle growth and regression. Reprod Biol Endocrinol RBE 2015; 13: 124.

[8] Van Blerkom J. Mitochondrial function in the human oocyte and embryo and their role in developmental competence. Mitochondrion 2011; 11: 797–813.

[9] Marcovina SM, Sirtori C, Peracino A, Gheorghiade M, Borum P, Remuzzi G, et al. Translating the basic knowledge of mitochondrial functions to metabolic therapy: role of L-carnitine. Transl Res J Lab Clin Med 2013; 161: 73–84.

[10] Machatkova M, Jeseta M, Hulinska P, Knitlova D, Nemcova L, Kanka J. Characteristics of bovine oocytes with different meiotic competence in terms of their mitochondrial status and expression of nuclear-encoded factors. Reprod Domest Anim 2012; 47: 806–14.

[11] Jeseta M, Ctvrtlikova Knitlova D, Hanzalova K, Hulinska P, Hanulakova S, Milakovic I, et al. Mitochondrial patterns in bovine oocytes with different meiotic competence related to their in vitro maturation. Reprod Domest Anim 2014; 49: 469–75.

[12] Ghanem N, Ha AN, Fakruzzaman M, Bang JI, Lee SC, Kong IK. Differential expression of selected candidate genes in bovine embryos produced in vitro and cultured with chemicals modulating lipid metabolism. Theriogenology 2014; 82: 238–50.

[13] Spricigo JF, Morato R, Arcarons N, Yeste M, Dode MA, Lopez-Bejar M, et al. Assessment of the effect of adding L-carnitine and/or resveratrol to maturation medium before vitrification on in vitro-matured calf oocytes. Theriogenology 2017; 89: 47–57.

[14] Yamada T, Imai H, Yamada M. Beneficial effects of acetyl-L-carnitine treatment during IVM on post-fertilization development of bovine oocytes in vitro. Reprod Fertil Dev 2006; 18: 280–1.

[15] Phongnimitr T, Liang Y, Srirattana K, Panyawai K, Sripunya N, Treetampinich C, et al. Effect of L-carnitine on maturation, cryo-tolerance and embryo developmental competence of bovine oocytes. Anim Sci J 2013; 84: 719–25.

[16] Reader KL, Cox NR, Stanton JAL, Juengel JL. Effects of acetyl-L-carnitine on lamb oocyte blastocyst rate, ultrastructure, and mitochondrial DNA copy number. Theriogenology 2015; 83: 1484–92.

[17] Men H, Agca Y, Riley LK, Critser JK. Improved survival of vitrified porcine embryos after partial delipation through chemically stimulated lipolysis and inhibition of apoptosis. Theriogenology 2006; 66: 2008–16.

[18] Somfai T, Kaneda M, Akagi S, Watanabe S, Haraguchi S, Mizutani E, et al. Enhancement of lipid metabolism with L-carnitine during in vitro maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes. Reprod Fertil Dev 2011; 23: 912–20.

[19] Abdelrazik H, Sharma R, Mahfouz R, Agarwal A. L-carnitine decreases DNA damage and improves the in vitro blastocyst development rate in mouse embryos. Fertil Steril 2009; 91: 589–96.

[20] Moawad AR, Xu B, Tan SL, Taketo T. L-carnitine supplementation during vitrification of mouse germinal vesicle stage-oocytes and their subsequent in vitro maturation improves meiotic spindle configuration and mitochondrial distribution in metaphase II oocytes. Hum Reprod 2014; 29: 2256–68.

[21] Zare Z, Farahani RM, Salehi M, Piryaei A, Novin MG, Fathabadi FF, et al. Effect of L-carnitine supplementation on maturation and early embryo development of immature mouse oocytes selected by brilliant cresyle blue staining. J Assist Reprod Genet 2015; 32: 635–43.

[22] Wydooghe E, Vandaele L, Beek J, Favoreel H, Heindryckx B, De Sutter P, et al. Differential apoptotic staining of mammalian blastocysts based on double immunofluorescent CDX2 and active caspase-3 staining. Anal Biochem 2011; 416: 228–30.

[23] Thompson JG, Lane M, Gilchrist RB. Metabolism of the bovine cumulus oocyte complex and influence on subsequent developmental competence. Soc Reprod Fertil Suppl 2007; 64: 179–90.

[24] Dunning KR, Cashman K, Russell DL, Thompson JG, Norman RJ, Robker RL. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. Biol Reprod 2010; 83: 909–18.

[25] Dunning KR, Akison LK, Russell DL, Norman RJ, Robker RL. Increased beta-oxidation and improved oocyte developmental competence in response to L-carnitine during ovarian in vitro follicle development in mice. Biol Reprod 2011; 85: 548–55.

[26] Mansour G, Abdelrazik H, Sharma RK, Radwan E, Falcone T, Agarwal A. L-carnitine supplementation reduces oocyte cytoskeleton damage and embryo apoptosis induced by incubation in peritoneal fluid from patients with endometriosis. Fertil Steril 2009; 91: 2079–86.

[27] Wu GQ, Jia BY, Li JJ, Fu XW, Zhou GB, Hou YP, et al. L-carnitine enhances oocyte maturation and development of parthenogenetic embryos in pigs. Theriogenology 2011; 76: 785–93.

[28] 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 2013; 79: 590–8.

[29] Takahashi T, Inaba Y, Somfai T, Kaneda M, Geshi M, Nagai T, et al. Supplementation of culture medium with L-carnitine improves development and cryotolerance of bovine embryos produced in vitro. Reprod Fertil Dev 2013; 25: 589–99.

[30] Yu Y, Dumollard R, Rossbach A, Lai FA, Swann K. Redistribution of mitochondria leads to bursts of ATP production during spontaneous mouse oocyte maturation. J Cell Physiol 2010; 224: 672–80.

[31] Yoshida M, Ishigaki K, Nagai T, Chikyu M, Pursel VG. Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. Biol Reprod 1993; 49: 89–94.

[32] Luberda Z. The role of glutathione in mammalian gametes. Reprod Biol 2005; 5: 5–17.

[33] Nemcova L, Jansova D, Vodickova-Kepkova K, Vodicka P, Jeseta M, Machatkova M, et al. Detection of genes associated with developmental competence of bovine oocytes. Anim Reprod Sci 2016; 166: 58–71.

[34] Mishra A, Reddy IJ, Gupta PS, Mondal S. L-carnitine mediated reduction in oxidative stress and alteration in transcript level of antioxidant enzymes in sheep embryos produced in vitro. Reprod Domest Anim 2016; 51: 311–21.

[35] You J, Lee J, Hyun SH, 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 2012; 78: 235–43.

Table 1
Effect of L-carnitine on bovine oocyte maturation.

Oocyte category	IVM	MII stage / matured		Mitochondrial clusters / MII stage	
		n	%	n	%
MMC	L-carnitine	57/59	96.6[a]	51/57	89.5[a]
	Control	56/63	88.9[ab]	43/56	76.8[ab]
MLC	L-carnitine	85/103	82.5[bc]	58/85	68.2[b]
	Control	79/105	75.2[c]	41/79	51.8[c]

Values with different superscripts are significantly different (Chi-square test; $P < 0.05$). Replica: n = 5.
Abbreviations: MMC = meiotically more competent oocytes derived from medium follicles (6–10 mm); MLC = meiotically less competent oocytes derived from small follicles (< 5 mm).

Table 2
Effect of L-carnitine on bovine oocyte fertilization.

Oocyte category	IVM	Fertilized / Inseminated		Syngamy stage / Fertilized	
		n	%	n	%
MMC	L-carnitine	198/214	92.5^{ab}	72/198	36.4^{a}
	Control	191/222	86.0^{ab}	101/191	52.9^{bc}
MLC	L-carnitine	393/432	91.0^{a}	216/393	55.0^{c}
	Control	379/441	85.9^{b}	177/379	46.7^{ab}

Values with different superscripts are significantly different (Chi-square test; $P < 0.05$). Replica: n = 5.
Abbreviations: MMC = meiotically more competent oocytes derived from medium follicles (6–10 mm); MLC = meiotically less competent oocytes derived from small follicles (< 5 mm).

Table 3
Effect of L-carnitine on bovine embryo development.

Oocyte category	IVM	Cleaved on D2 / Inseminated		Early blastocysts on D7/ Inseminated	
		n	%	n	%
MMC	L-carnitine	90/97	92.8[a]	49/97	50.5[a]
	Control	58/68	85.3[a]	32/68	47.1[a]
MLC	L-carnitine	262/303	86.5[a]	96/303	31.7[b]
	Control	226/264	85.6[a]	61/264	23.1[c]

Values with different superscripts are significantly different (Chi-square test; P < 0.05). Replica: n = 4.
Abbreviations: MMC = meiotically more competent oocytes derived from medium follicles (6–10 mm); MLC = meiotically less competent oocytes derived from small follicles (< 5 mm); D2 = 48h after oocyte insemination; D7 = 168h after oocyte insemination.

Table 4
Effect of L-carnitine on bovine blastocyst formation.

Oocyte category	IVM	Total blastocysts on D8/ Inseminated		Expanded/Total blastocysts	
		n	%	n	%
MMC	L-carnitine	44/97	45.4[a]	32/44	72.7[a]
	Control	27/68	39.7[ab]	16/27	59.3[b]
MLC	L-carnitine	101/303	33.3[b]	64/101	63.4[ab]
	Control	68/264	25.8[c]	47/68	69.1[ab]

Values with different superscripts are significantly different (Chi-square test; $P < 0.05$). Replica: n = 4.
Abbreviations: MMC = meiotically more competent oocytes derived from medium follicles (6–10 mm); MLC = meiotically less competent oocytes derived from small follicles (< 5 mm); D8 = 192h after oocyte insemination.

Table 5
Effect of L-carnitine on bovine blastocyst differentiation.

Oocyte category	IVM	Blastocysts examined (n)	Cell number per blastocyst (mean ± SEM)			Ratio (mean ± SEM)	
			Total (n)	TE (n)	ICM (n)	ICM/TE	ICM /TCN (%)
MLC	L-carnitine	44	135.7 ± 26.6 [a]	94.2 ± 18.7 [a]	41.5 ± 10.5 [a]	0.44 ± 0.4	30.6 ± 4.8
	Control	47	129.0 ± 24.3 [a]	89.2 ± 15.4 [a]	39.8 ± 9.6 [a]	0.45 ± 0.3	31.0 ± 3.3

Values are not significantly different (Chi-square test; P > 0.05). Replica: n = 4.

MLC = meiotically less competent oocytes derived from small follicles (< 5 mm); TCN = total cell number per blastocyst.

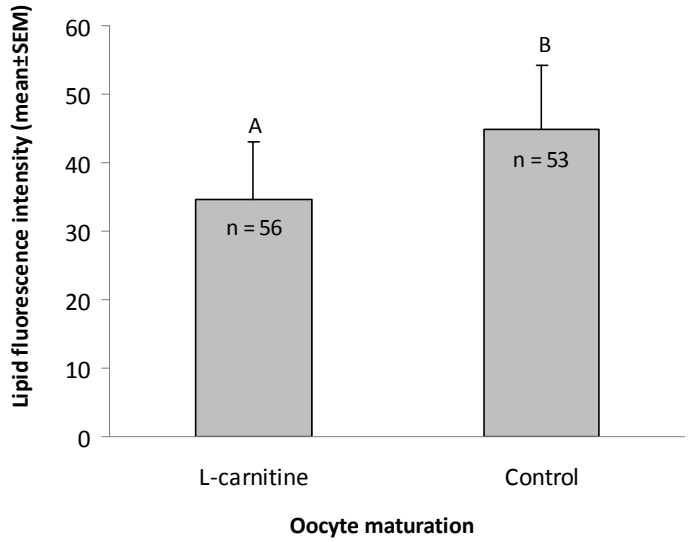

Fig. 5. Lipid content in MLC bovine oocytes after maturation with and without L-carnitine. Different letters indicate signifficant differences (Fisher's test; $P < 0.05$). Replica = 5. MLC = meiotically less competent oocytes derived from small follicles (< 5 mm).

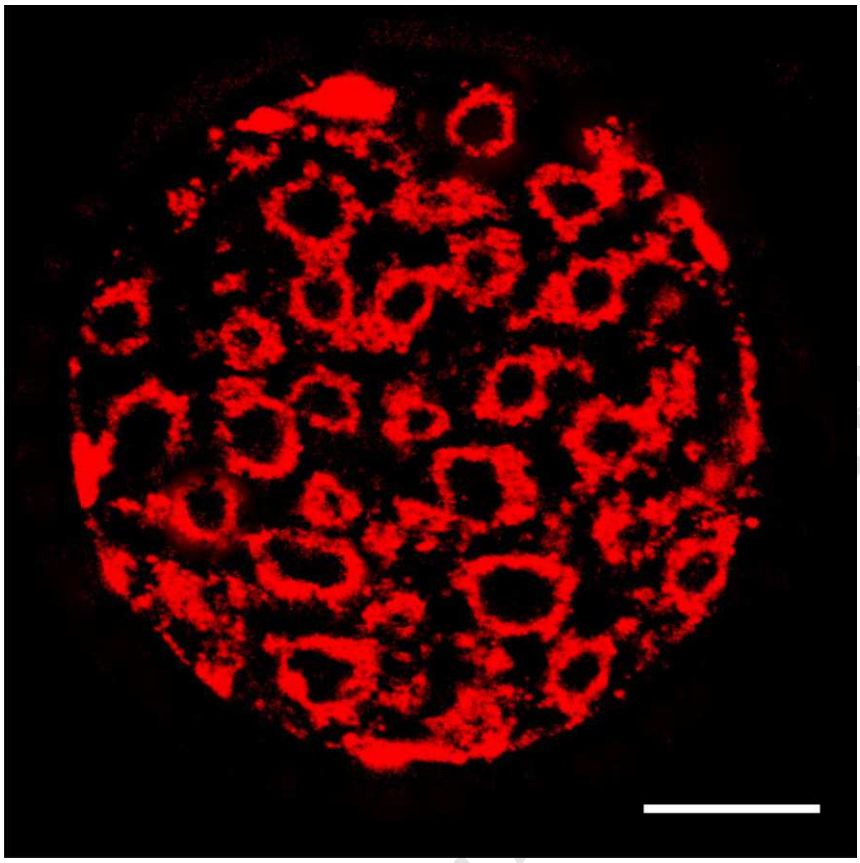

Fig. 1. Representative image of bovine oocyte with mitochondrial clusters after 24 h-maturation. Mitochondria were stained with MitoTracker Orange CMTMRos and oocytes were examined by confocal microscopy. Scale bar = 30 µm.

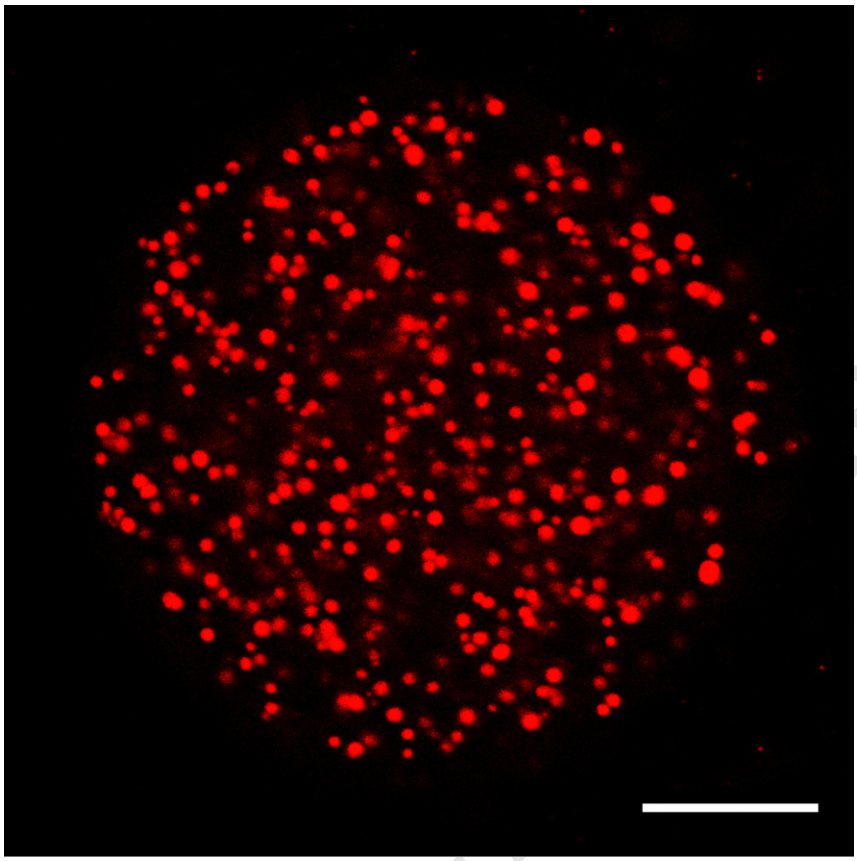

Fig. 2. Representative image of bovine oocyte with lipid droplets after 24 h-maturation. Lipid droplets were stained with Nile red and oocytes were examined by confocal microscopy. Scale bar = 30 µm.

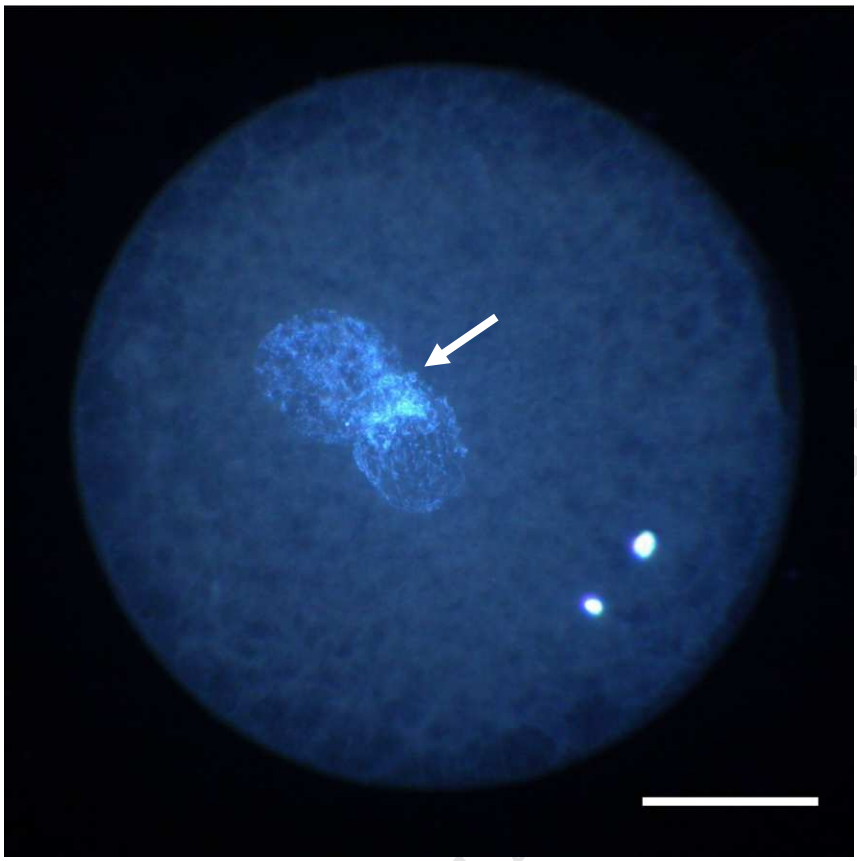

Fig. 3. Representative image of bovine oocyte with male and female pronuclei at the syngamy stage (arrow) at 19 h after insemination. Two extruded polar bodies are also visible. Chromatin was stained with Hoechst-33258 and oocytes were examined by fluorescent microscopy. Scale bar = 30 µm.

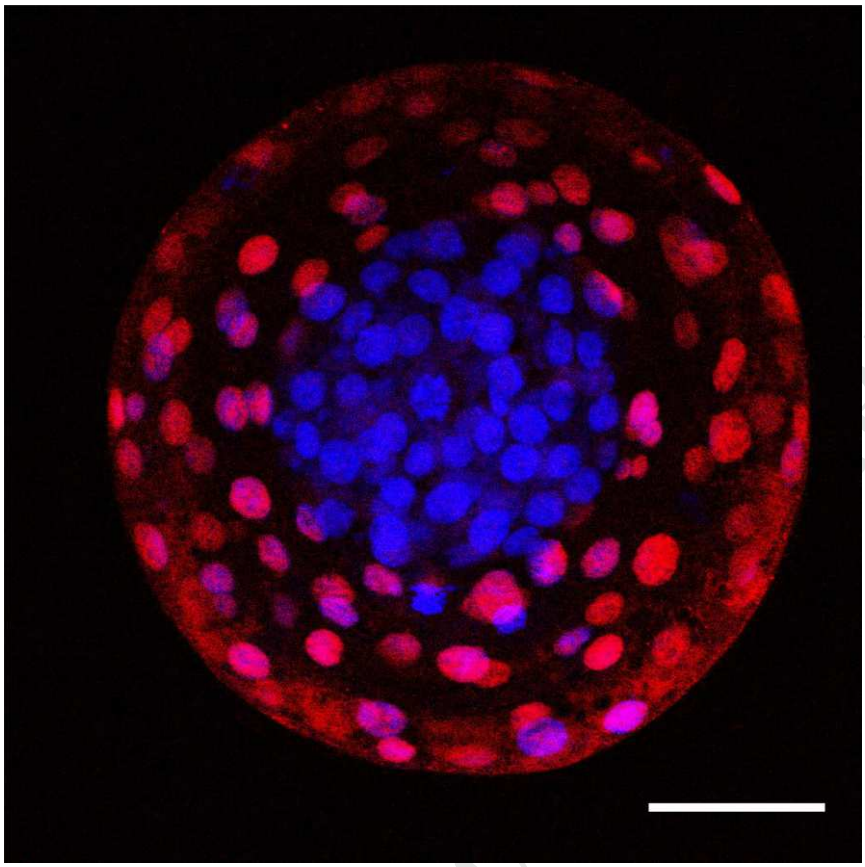

Fig. 4. Representative image of bovine expanded blastocyst at Day 8. Nuclei were visualized by differential staining of ICM (blue colour; TO-PRO-3) and TE (pink colour; anti CDX2 protein-IgG antibody conjugated with Alexa Fluor 488) and blastocysts were examined by confocal microscopy. Scale bar = 50 µm.

Highlights:

- The effect of L-carnitine during maturation of bovine oocytes was assessed.
- The impact of L-carnitine on oocytes depends on their meiotic competence.
- Maturation with L-carnitine increases development of embryos from less competent oocytes.
- Expansion of blastocysts from more competent oocytes matured with L-carnitine is accelerated.