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Revised

Utilisation of endogenous fatty acid stores for energy production in bovine pre-implantation embryos

ML Sutton-McDowall*, D Feil, RL Robker, JG Thompson & KR Dunning

The Robinson Institute, Research Centre for Reproductive Health, School of Paediatrics and Reproductive Health, The University of Adelaide, Adelaide SA 5005, Australia

*Corresponding author;

E-mail: melanie.mcdowall@adelaide.edu.au

Telephone: +61 8 8313 1013

Facsimile: + 61 8 8303 8177

E-Mail Addresses

D Feil: deanne.feil@adelaide.edu.au

RL Robker: rebecca.robker@adelaide.edu.au

JG Thompson: jeremy.thompson@adelaide.edu.au

KR Dunning: kylie.dunning@adelaide.edu.au

Abstract

Current embryo culture media are based on the carbohydrate metabolism of embryos. However, little is known about the metabolism of endogenous lipids. L-carnitine is a ß-oxidation co-factor absent in most culture media. The aim of this study was to investigate the influence of L-carnitine supplementation on bovine embryo development. Abattoir-derived cattle cumulus oocyte complexes were cultured and fertilised. Post-fertilisation, presumptive zygotes were transferred into a basic cleavage medium ±carbohydrates (glucose, lactate and pyruvate) ±5 mM L-carnitine and cultured for 4 days in vitro. The absence of carbohydrates during culture resulted in embryos arresting at the 2- and 4-cell stages. Remarkably, +L-carnitine significantly increased development to the morula stage compared to +carbohydrates alone ($P < 0.001$). The beneficial effects of L-carnitine were further demonstrated by inclusion of carbohydrates, with 14-fold more embryos reaching the morula stage after culture in the +carbohydrates +L-carnitine group compared to the +carbohydrates group ($P < 0.05$). While there was a trend for +L-carnitine to increase ATP levels ($P = 0.09$), ADP levels were higher and ATP:ADP ratio were 1.9-fold lower (main effect, $P < 0.05$) compared to embryos cultured in –L-carnitine. This indicates +L-carnitine embryos were more metabolically active, with higher rates of ATP-ADP conversion. In conclusion we found L-carnitine supplementation supports pre-compaction embryo development and there is an additive effect of +L-carnitine +carbohydrate on early embryo development, most likely through increased ß-oxidation levels within embryos.

Keywords

In vitro embryo production; lipids; ß-oxidation; bovine

Abbreviations

COC = cumulus oocyte complex; CPT1B = carnitine palmitoyl transferase 1B; DAPI = 4',6-diamidino-2-phenylindole; ICM = inner cell mass; IVP = *in vitro* embryo production; ROS reactive oxygen species

1. Introduction

Over the past few decades, there has been a vast improvement in the success rate of *in vitro* embryo production (IVP) attributed to the creation of culture media based on the metabolic requirements of the embryo and the composition of oviductal and uterine fluids. The oocyte and embryo has differential energy requirements during development and the energy substrate preferences of gametes and embryos change [1,2], cumulus-oocyte complexes (COCs) preferentially utilise glucose [3-5]; pre-compaction embryos utilise pyruvate and lactate [6], and return to a preference for glucose by post-compaction [7-9]. Changes in metabolic requirements during this period is reflected in commonly used IVP media including synthetic oviduct fluid [10], human tubal fluid [11], mouse tubal fluid [12] and G1/G2 [13]. The composition of embryo culture media is historically based on the carbohydrate requirements of the embryo and while much research has focused on the metabolism of carbohydrates, little is known about lipid metabolism within pre-implantation embryos.

The cytoplasm of oocytes and embryos of some mammalian species (such as cattle and pig; [14]) are rich with lipids, whereas others are not (human and mouse; [15]). This variation in intracellular lipid density of oocytes and embryos between species is hypothesised to be related to the length of time between ovulation and implantation ("Time to attachment" theory, reviewed by [16]). For example, ruminant oocytes have higher lipid densities compared mouse and human, with cattle and sheep oocytes containing 15 ng/nL and 21 ng/nL lipid respectively, compared to mouse oocytes which contain 6.25 ng/nL lipids [16]. This is associated with~20-30 days between ovulation and implantation in cattle [17] compared to ~ 4.5-6 days in the mouse [18].

Lipids and fatty acids are precursors for prostaglandin and steroid hormone biosynthesis, membrane biosynthesis and substrates for energy production. The utilisation and density of lipids change during pre-implantation development with significant decreases in triglyceride concentrations in mature pig oocytes compared to immature oocytes [19] and lipase activity increases with the progression of cattle oocyte maturation [20]. Post-fertilisation there are minimal changes in lipid density until the 8-cell stage [19]. Conversely, lipid accumulation within embryos is related to poor embryo development. *In vitro* produced oocytes and embryos cultured in the presence of serum have higher intracellular lipid content compared to *in vivo* produced embryos [21] and serum-free culture systems [22]. Furthermore, sheep embryos cultured in the presence of serum have higher lipid content, longer gestation length and higher birth weight post-transfer [23]. The cause of higher lipid concentrations and poor developmental potential of *in vitro* produced pre-implantation embryos is unknown and might be an artefact of incomplete culture conditions compared to the *in vivo* environment.

Triglycerides are the major type of lipid in the bovine oocyte [24,25], with palmitic and oleic acid accounting for 32% and 25% (w/w) respectively, of total fatty acids in the bovine oocyte [14]. Considering the metabolism of 1 M palmitic acid produces ~106 M ATP compared to 1 M glucose producing ~27-31 M ATP, intracellular lipids could be used as a potential and more economical energy source for pre-implantation embryos than exogenous energy sources such as carbohydrates. Fatty acids derived from triglycerides are converted to acetyl-CoA and ATP via ß-oxidation (**Fig. 1**). Intracellular fatty acids (in the form of acyl-CoA) are translocated into in the mitochondria by carnitine palmitoyl transferase 1B (CPT1B), a process facilitated by acyl-CoA binding to L-carnitine. ß-oxidation results in the conversion of acyl-CoA to acetyl-CoA. Acetyl-CoA can then be metabolised further via tricarboxylic acid cycle and oxidative phosphorylation. The rate-limiting enzyme of ß-oxidation is CPT1B and requires L-carnitine as a co-factor (**Fig 1**). To date, the concentration of L-carnitine in reproductive tract fluids such as

uterine and oviduct fluid, has not been measured. However, plasma L-carnitine levels in cattle range from 270-700 μM [7].

Currently, media preparations for *in vitro* embryo production do not contain L-carnitine. We recently demonstrated that L-carnitine supplementation during mouse *in vitro* follicle culture and oocyte maturation (IVM) significantly improves oocyte developmental competence, demonstrated by increased blastocyst development and inner cellular mass (ICM) numbers [26,27]. Furthermore, *Cpt1b* mRNA expression in mouse COCs increases during maturation and inhibition of ß-oxidation during IVM decreased oocyte developmental competence.

Considering bovine embryos have high concentrations of intracellular lipids relative to mouse embryos, we hypothesized that pre-compaction bovine embryos utilise endogenous lipid pools more efficiently for energy production when L-carnitine alone is supplemented to the culture medium. The aim of this study was to determine if L-carnitine supplementation could facilitate the utilisation of endogenous lipid stores for energy production in the pre-compaction embryo when cultured in a simple culture environment in the absence or presence of well characterised exogenous energy substrates, glucose, lactate and pyruvate.

2. Materials and methods

Unless stated, all chemicals were purchased from Sigma Aldrich (St Louis, MO, USA). IVM medium was VitroMat (IVF Vet Solutions; Adelaide, SA, Australia), supplemented with 4 mg/mL low fatty acid BSA (ICPbio Ltd, Auckland, New Zealand) + 0.1 IU/mL FSH (Puregon; Organon, Oss, Netherlands). Working *in vitro* fertilisation (IVF) medium was VitroFert (IVF Vet Solutions) + 4 mg/mL fatty acid free BSA + 10 IU/mL heparin. The composition of cleavage media is described in **Table 1**. Two base media were prepared ± carbohydrates (glucose,

lactate and pyruvate) and both media did not contain amino acids. Either 1 or 5 mM L-carnitine was added (see experimental design).

2.1.Oocyte collection and culture

Abattoir derived cow ovaries were transported to the laboratory in warm saline (30-35°C). Antral follicles greater than 3 mm in diameter were aspirated using an 18-gauge needle attached to a 10 mL syringe. Intact cumulus oocyte complexes (COCs) with greater than 3 compact, cell layers were selected in follicular fluid and washed twice in IVM medium. Groups of 40-50 COCs were transferred into 500 µL of pre-equilibrated IVM medium overlaid with mineral oil and were cultured for 24 h at 38.5°C in 6% CO_2 in humidified air.

2.2.In vitro fertilisation and embryo culture

Mature COCs were washed once in wash medium (Vitro Wash, IVF Vet Solutions; supplemented with 4 mg/ml low fatty acid BSA), once in fertilisation medium and groups of 40-50 COCs were transferred into pre-equilibrated IVF wells containing 500 µL of IVF medium overlaid with mineral oil. Thawed sperm from a single sire with proven fertility were prepared using a discontinuous Percoll gradient (45%: 90%) and added to IVF wells with COCs at a final concentration of 1 x 10^6 sperm/mL as per [28]. After 24 h of culture (Day 1), presumptive zygotes were mechanically denuded of cumulus cells using a finely pulled glass pipette and transferred into cleavage medium. Groups of five presumptive zygotes were transferred into 20 µL of the corresponding cleavage medium (see experimental design) and cultured at 38.5°C in 6% CO_2, 7% O_2, nitrogen balance.

2.3.Cell numbers

The number of cells in pre-compaction embryos were confirmed using 4',6-diamidino-2-phenylindole (DAPI) nuclear stain. Fresh embryos were incubated with 15 μM DAPI for 15 mins at room temperature and in the dark. Embryos were washed twice in PBS + 0.01% BSA, mounted on microscope slides with 1:1 PBS:Prolong anti-fade (Invitrogen, Carlsbad, CA, USA) and visualised on a fluorescent microscope using a UV filter (absorption: 358 nm, emission: 461 nm).

2.4.Experiment 1: Carbohydrates or lipids? Pre-compaction embryo development in the presence of carbohydrates ± L-carnitine

To determine if pre-compaction embryos preferentially utilise carbohydrates or endogenous lipid stores for energy production, on Day 1 presumptive zygotes were transferred into cleavage media + carbohydrates ± 5 mM L-carnitine. Embryo development was assessed on Day 5. Three replicate experiments were performed with 40-50 presumptive zygotes used per treatment group within each replicate.

2.5.Experiment 2: Pre-compaction embryo development in the presence of L-carnitine and absence of carbohydrates.

The aim of this experiment was to determine if endogenous lipid stores could be used as an alternative energy source in the absence of carbohydrates when cultures were

177 supplemented with L-carnitine. From Day 1, presumptive zygotes were transferred into the
178 following media; 1) – carbohydrates (cleavage media – carbohydrates); 2) + carbohydrates
179 (cleavage media + carbohydrates); 3) 1 mM L-carnitine (cleavage media – carbohydrates + 1
180 mM L-carnitine) and 4) 5 mM L-carnitine (cleavage media – carbohydrates + 5 mM L-carnitine).
181 Embryo development was assessed on Day 5 and cell numbers were confirmed with DAPI
182 staining. Three replicate experiments were performed with 40-50 presumptive zygotes used per
183 treatment group within each replicate.
184

185 ***2.6.Experiment 3: Inhibition of ß-oxidation reverses the positive effects of***
186 ***carnitine supplementation***
187

188 Etomoxir is a non-reversible inhibitor of CPT1IB, the rate-limiting enzyme of ß-oxidation;
189 hence the aim of this experiment is to determine if the effects of L-carnitine supplementation
190 are mediated via ß-oxidation. From Day 1, presumptive zygotes were cultured in the following
191 media; 1) Control (cleavage media), 2) + etomoxir (cleavage media + 50 µM etomoxir), 3) + L-
192 carnitine (cleavage media + 5 mM L-carnitine) and 4) + L-carnitine + etomoxir (cleavage media
193 + 5 mM L-carnitine + 50 µM extomoxir). All media contained carbohydrates. Embryo
194 development was assessed on Day 5 and cell numbers were confirmed using DAPI staining.
195 Four experimental replicates were performed with 40-50 presumptive zygotes used per
196 treatment group within each replicate.
197

198 ***2.7.Experiment 4: ATP and ADP content following L-carnitine supplementation***
199

We hypothesis that L-carnitine supplementation causes increased energy production, in the form of ATP. On Day 1, presumptive zygotes were transferred into the following cleavage media; 1) –carbohydrates (cleavage media – carbohydrates); 2) + carbohydrates (cleavage media + carbohydrates); 3) + L-carnitine (cleavage media – carbohydrates + 5 mM L-carnitine) and 4) + L-carnitine + carbohydrates (cleavage media + carbohydrates + 5 mM L-carnitine). On Day 2, embryos were transferred into a 96-well plate wells (5 embryos per well) and stored at -80°C. ATP and ADP levels were determined using an ApoSENSOR ADP/ATP Ratio assay kit (Biovision; Mountain View, CA, USA), as per manufacturer's instructions. Five replicate experiments were performed per treatment (5 embryos per well).

2.8.Experiment 5: Lipid content of embryos cultured in the presence of carnitine

The influence of L-carnitine supplementation on lipid content within the pre-compaction embryo was determined. On Day 1, presumptive zygotes were transferred into cleavage media – carbohydrates ± 5 mM L-carnitine. Embryo development was assessed on Day 2, 4-8 cell embryos were fixed in 4% paraformaldehyde overnight at room temperature and transferred into PBS. Lipid specific lipophilic dye BODIPY 493/503 (Invitrogen, Carlsbad CA USA) was dissolved in 100% ethanol to a working concentration of 1 mg/mL and further diluted in 1 mg/mL polyvinylpyrrolidone in PBS (PBS/PVP) to a working concentration of 1 µg/mL. Embryos were incubated overnight at room temperature in the dark, washed once in PBS/PVP and mounted on cover slips in fluorescent mounting media (DAKO, Carpenteria CA USA). Images were visualised using a Leica SP5 spectral scanning confocal microscope (Leica Microsystems GmbH; Wetzlar, Germany), using identical magnification and gain settings for all imaging. The mean intensity per pixel was calculated from the pixel intensity measured in each

cell of the embryo (avoiding the nucleus) using Adobe Photoshop CS3 (Adobe; San Jose, CA USA). Two replicate experiments were performed with 30 zygote cultured per treatment group per replicate.

2.9.Statistical analyses

Proportion data (embryo development) were arcsine transformed and differences between groups were tested using a mixed model, followed by Bonferroni post-hoc test. Standard curves for the ATP/ADP assay were generated using known concentrations of ATP and linear regression analyses. Standard curves were used if the r^2 >0.95. P-Values less than 0.05 were deemed statistically significant ($P < 0.05$). All statistical analyses were performed using SPSS statistical software (Version 17, SPSS, Chicago, IL, USA) and SigmaPlot graphical software (Version 11, SPSS).

3. Results

3.1.Experiment 1: Carbohydrates or lipids? Pre-compaction embryo development in the presence of carbohydrates ± L-carnitine

To determine whether L-carnitine could improve embryo development when both exogenous carbohydrates and endogenous lipid stores are available for energy production presumptive zygotes were cultured in media containing carbohydrates ± 5 mM L-carnitine between Days 1-5. On Day 5, there were no significant differences in cleavage rates or the proportion of embryos reaching the 4- and 8-cell stages. However, in the presence of L-carnitine, significantly more embryos reached the greater than 8-cell stage (3-fold, **Table 2**) and

morula stage (14-fold, **Table 2**) than embryos cultured in medium without L-carnitine ($P < 0.05$). Furthermore, early blastocyst stage embryos were seen in the L-carnitine supplemented group (0 mM = 0 ± 0%, vs. 5 mM L-carnitine = 13.6 ± 7%, **Table 2**).

3.2. Experiment 2: Pre-compaction embryo development in the presence of carnitine and absence of carbohydrates.

Following fertilisation, bovine presumptive zygotes were transferred into cleavage media ± carbohydrates (glucose, lactate and pyruvate) ± L-carnitine. On Day 5, there were no differences in cleavage or 4-cell rates (**Table 3**). However, in the absence of carbohydrate, embryos arrested at the 2- or 4-cell stage (**Table 3**). Supplementation of cleavage media - carbohydrates with 1 mM or 5 mM L-carnitine recovered embryo development to the 8-cell stage comparable to the control group (+carbohydrates). The addition of 1 mM L-carnitine to media without carbohydrates supported embryo compaction at a rate similar to embryos cultured in the presence of carbohydrates (**Table 3**). Furthermore, a higher dose of L-carnitine further promoted embryo development with 4.3-fold more embryos reaching the morula stage when cultured in the presence of 5 mM L-carnitine (-carbohydrates) compared to cultures containing carbohydrates alone (+ carbohydrates = 12.2 ± 6.9% vs. – carbohydrates + 5 mM L-carnitine = 39.6 ± 3.3% morulae; $P < 0.001$; **Table 3**).

3.3. Experiment 3: Inhibition of ß-oxidation reverses the positive effects of L-carnitine supplementation

ß-oxidation was inhibited by supplementing cultures with etomoxir, a non-reversible inhibitor of CPT1B (rate-limiting enzyme). Preliminary experiments were conducted to determine the effective inhibitory dose of etomoxir (data not shown). Presumptive zygotes were cultured in media containing carbohydrates, ± 50 μM etomoxir and ± 5 mM L-carnitine and embryo development assessed on Day 5. L-Carnitine and/or etomoxir supplementation did not affect the proportion of embryos reaching the 2-, 4- and 8-cell stages (**Table 4**). However, both L-carnitine and etomoxir supplementation significantly influenced embryo development to the greater than 8-cell stage (main effects, $P < 0.05$), with etomoxir +L-carnitine reversing the beneficial effects of L-carnitine alone on embryo development (**Table 4**).

3.4.Experiment 4: ATP and ADP content following L-carnitine supplementation

Presumptive zygotes were transferred into media containing ± carbohydrates ± 5 mM L-carnitine for 24 h and ATP/ADP content and ATP:ADP ratios were determined. In regards to ATP concentrations, the combination of carbohydrates and L-carnitine supplementation did not have any additive effects on ATP levels compared to carbohydrates alone (Interaction, $P = 0.156$, **Fig 2A**). However, there was significantly more ATP produced in embryos exposed to carbohydrates (main effects; - carbohydrates = 0.26 ± 0.05 vs. + carbohydrates = 0.45 ± 0.05 pmol/embryo; $P = 0.009$) and there was a trend for the presence L-carnitine to increase ATP levels (main effects, $P = 0.09$). L-Carnitine supplementation alone did not significantly increase ATP levels compared to cultures – carbohydrates – L-carnitine (**Fig 2A**).

Both L-carnitine and carbohydrate supplementation for 24h resulted in significantly higher ADP levels in embryos (main effects, $P < 0.005$). Specifically, the combination of carbohydrates and L-carnitine resulted in 6.6-fold higher ADP levels compared to embryos cultured in the absence of carbohydrates and L-carnitine (**Fig 2B**, $P < 0.005$).

While there were no significant interactions between L-carnitine and carbohydrate supplementation, the ATP:ADP ratio was significantly higher in the absence of L-carnitine (main effect, $P < 0.05$) and there was a trend for the absence of carbohydrates to also increase the ATP:ADP ratio (main effect, $P = 0.110$). Lower ATP:ADP ratios in the +carbohydrate + L-carnitine group compared to the –carbohydrate – L-carnitine group (**Fig 2C**, $P < 0.05$) indicate that embryos are more active in the presence of L-carnitine and carbohydrates, resulting the conversion of a significant proportion of ATP to ADP.

3.5. Experiment 5: Lipid content of embryos cultured in the presence of L-carnitine

Presumptive zygotes were cultured in the presence and absence of 5 mM L-carnitine and the absence of carbohydrates for 24h and lipid content of embryos were determined using BODIPY 493/503 stain. After 24h, L-carnitine supplementation significantly reduced the intensity of lipid staining within embryo by 2.8-fold compared to embryos cultured in media without L-carnitine (**Fig 3**).

4. Discussion

Carbohydrate metabolism (glucose, lactate and pyruvate) by the embryo has been widely studied to understand the importance of these substrates in supporting early embryo development. Media formulations are often designed to reflect the *in vivo* environment within the oviduct and uterus and sequential culture systems have been developed to accommodate the changing metabolism requirements of the pre-implantation embryo [2]. However, the metabolism of endogenous lipids by the embryo is largely unknown. Endogenous lipids are a

potential energy source within the embryo via ß-oxidation but require transportation of fatty acids into the mitochondria by CPT1 (**Fig 1**). However, current media formulations do not contain factors to assist in ß-oxidation, such as L-carnitine, a co-factor of CPT1. The aim of the current study was to determine if the pre-compaction development of bovine embryos could be enhanced by potentially utilising endogenous lipid stores when cultured in the presence of L-carnitine.

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 result in higher lipid content within embryos [23,29]. It is plausible that many factors that promote embryo development are related to lipid metabolism, such as increased production of ATP and conversion of lipids to steroids and hormones. Hence, the absence of co-factors to facilitate lipid metabolism in media, such as L-carnitine, may be a major contributing factor of compromised embryo development during *in vitro* culture when compared to *in vivo* development.

While there was a positive, additive effect of L-carnitine and carbohydrate supplementation on embryo development to the 8-cell and morula stages of development, there was no interaction between carbohydrates and L-carnitine supplementation in regards to ATP production. The beneficial effects of L-carnitine supplementation were in part, due to increases in ß-oxidation, indicated by the reduction in embryo development to beyond the 8-cell stage in the presence of etomoxir (a specific inhibitor of the rate-limiting enzyme of ß-oxidation). However, increased ß-oxidation was probably not the only mechanism through which L-carnitine supplementation improved development. As with lipids, carbohydrates are also required for other cellular functions. Glucose in particular is a substrate for nucleic acid synthesis, extracellular matrix components and cell signalling via post-translational

345 modifications [30]. The provision of L-carnitine during culture may have freed carbohydrates for
346 functions other than energy production.
347 In addition to involvement in lipid metabolism, L-carnitine also plays a role in reducing
348 oxidative stress by enhancing the activity of numerous antioxidant enzymes such as
349 superoxide dismutase, catalase and glutathione peroxidase [31]. *In vitro* cultured embryos
350 produce higher levels of reactive oxygen species (ROS) through increased oxidative
351 phosphorylation via environmental factors such as higher oxygen and glucose concentrations
352 [32]. It has recently been demonstrated that supplementing porcine IVM cultures with L-
353 carnitine increase gluthathione and decreased ROS levels in oocytes [33]. Considering the
354 deleterious effects of ROS on embryo development [34], the beneficial effects of L-carnitine
355 supplementation in this current study may also be through the reduction of oxidative stress.
356 Lipid content and the rate of ß-oxidation change throughout pre-implantation embryo
357 development. Lipid density decreases through increased ß-oxidation during oocyte maturation
358 [26] and from the 8-cell to late blastocyst stages of development [35]. However, lipid density
359 and oxidation remains relatively quiescent between the zygote and 8-cell stages [35]. A similar
360 pattern of metabolism is seen for carbohydrates; with glucose, pyruvate and oxygen
361 consumption remaining constant between the 1-cell and 8-cell stages [6]. Relatively low levels
362 of energy are required for cell division compared to oocyte maturation, embryo compaction and
363 blastulation and this explains why in the absence of either carbohydrates or L-carnitine, most
364 embryos arrested at the 4- and 8-cell stages in the current study.
365 Regardless of possible differences in energy metabolism by *in vivo* and *in vitro* produced
366 embryos, L-carnitine supplementation during mouse *in vitro* follicle culture and IVM [26,27] and
367 early bovine cleavage cultures (current study) improves embryo development. There is also a
368 dose effect as in the absence of carbohydrates, embryos cultured in the presence of 1 mM L-
369 carnitine had similar development to embryos cultured in +carbohydrates and 5 mM L-carnitine

increased development beyond that observed in +carbohydrates conditions. Further studies are required to investigate the influence of L-carnitine supplementation in more complex media, such as in the presence of amino acids. Also it is likely that different concentrations of L-carnitine could be required at different stages of embryo development, similarly to sequential cultures containing varying glucose, pyruvate and lactate levels in current culture media. Considering endogenous lipids are required for other cell functions such as membrane biosynthesis and hormone production, too high L-carnitine concentrations may deplete lipid density to levels detrimental to embryo development.

In conclusion, we have demonstrated that supplementing early bovine embryo culture media with L-carnitine, a co-factor of ß-oxidation, improves embryo development in the absence of carbohydrates. Embryo development was further increased by L-carnitine in the presence of carbohydrates. Improved embryo development was in part due to increased utilisation of endogenous lipid stored via ß-oxidation and cell activity, indicated by increased ADP production and decreased ATP:ADP ratios. The addition of L-carnitine supplementation during bovine *in vitro* embryo production has the potential to decrease the disparities in development between *in vivo* and *in vitro* embryo production.

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488 **Figure captions**

489

490 **Figure 1.** Energy production within the pre-implantation embryo. **A)** Carbohydrates such
491 as glucose, lactate and pyruvate are preferentially metabolised for energy (in the form of ATP)
492 via glycolysis within the cytoplasm, followed by tricarboxylic acid cycle (TCA) and oxidative
493 phosphorylation within the mitochondria. **B)** Intracellular fatty acids (acyl-CoA) can also be
494 utilised for energy production, in particular in the absence of exogenous carbohydrates. Acyl-
495 CoA binds to L-carnitine (catalysed by CPTIB), allowing translocation into the mitochondria and
496 allowing for ß-oxidation, to form acetyl-CoA. Acetyl-CoA can then be metabolised further via
497 TCA cycle and oxidative phosphorylation. CPTIB = carnitine palmitoyl transferase IB, CPTII =
498 carnitine palmitoyl transferase II.

499

500 *Colour for web, black and white for print*

501

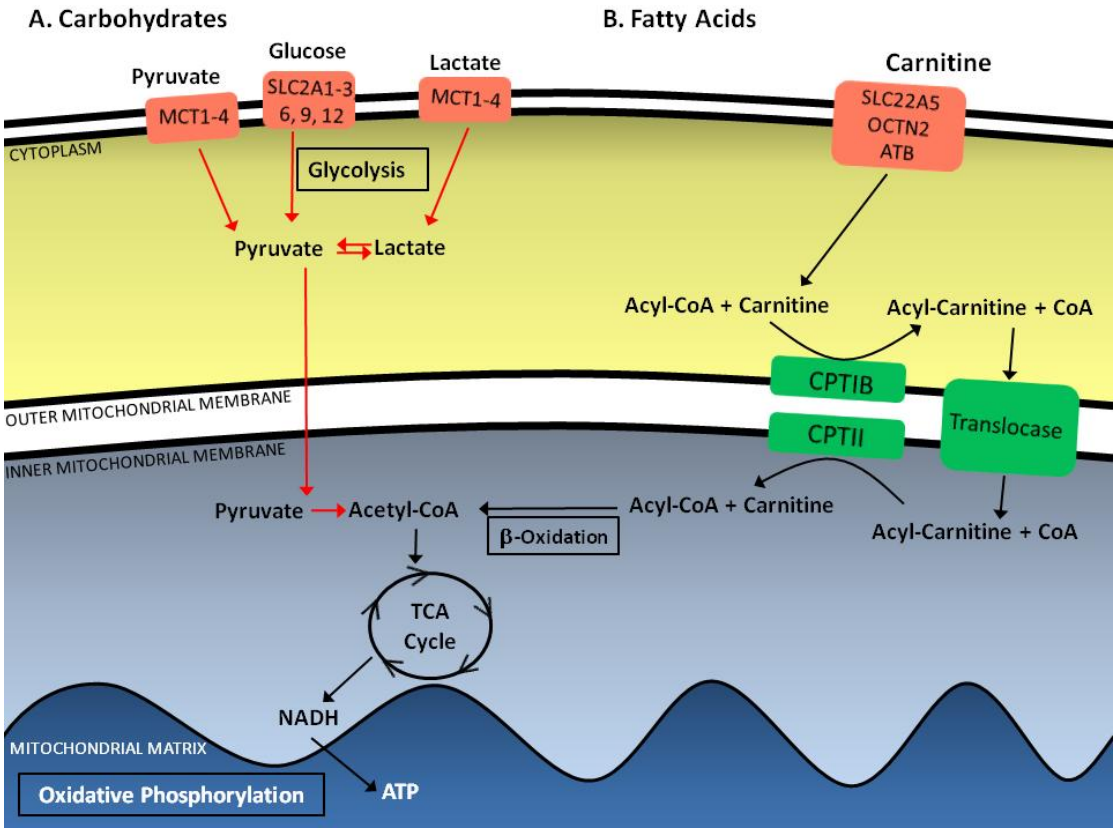
502 **Figure 2.** A) ATP, B) ADP and C) ATP:ADP ratios of embryos after culture in the
503 presence or absence of carbohydrates and L-carnitine. Bars represent mean + SEM and
504 different superscripts indicate significant differences (^{ab} $P < 0.05$).

505

506 **Figure 3.** Fluorescence intensity of lipid staining (BODIPY 493/503) within embryos
507 following culture ± 5 mM L-carnitine. A) average intensity of staining and representative images
508 of embryos cultured in the absence (B) or presence (C) of 5 mM L-carnitine. Data are
509 presented as means + SEM and asterisks indicate significant differences (* $P < 0.001$). The
510 scale bar represents 50 μM.

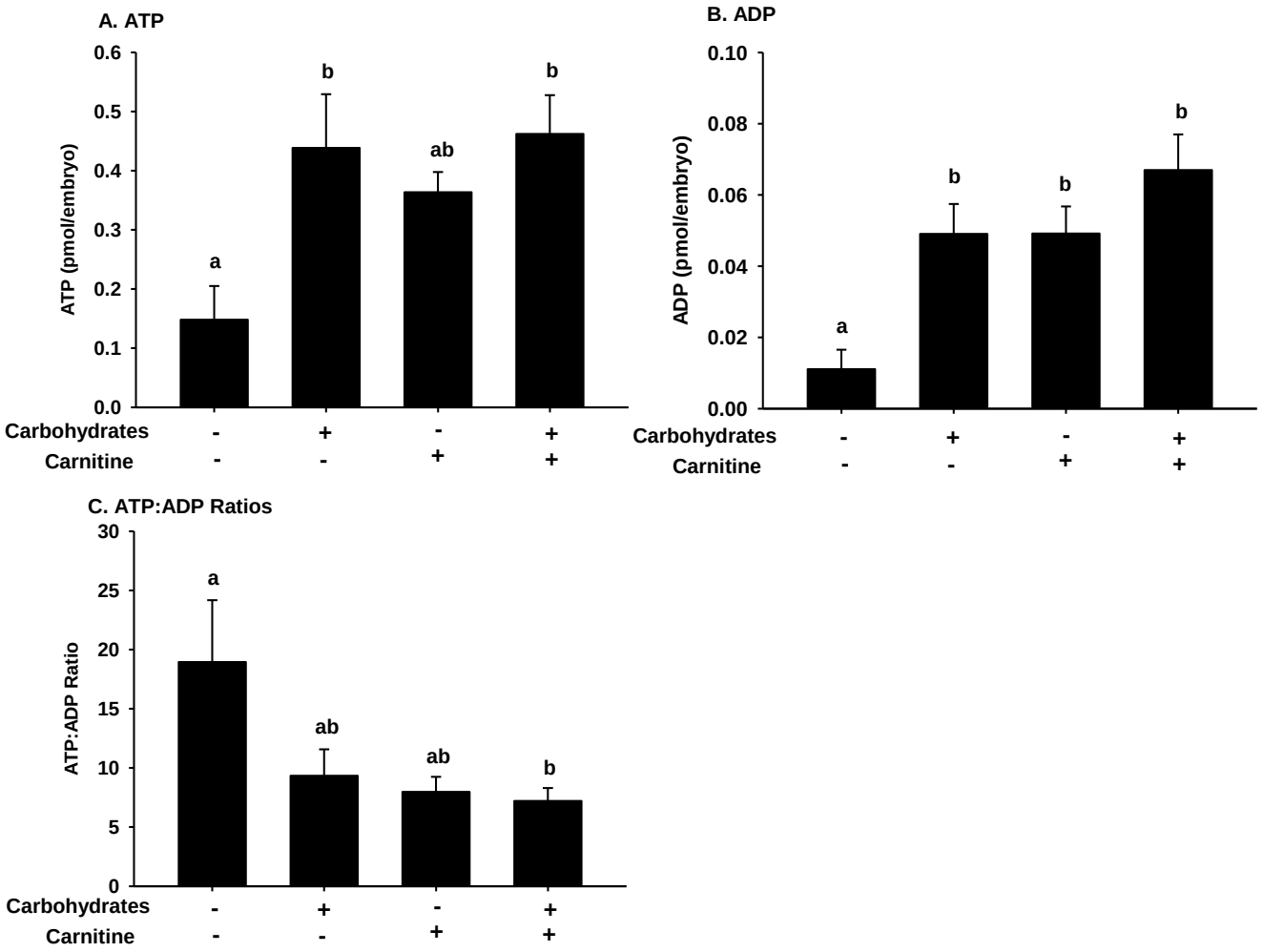
511 *Colour for online and black and white for print

512 Figure 1.



513

514 Figure 2.



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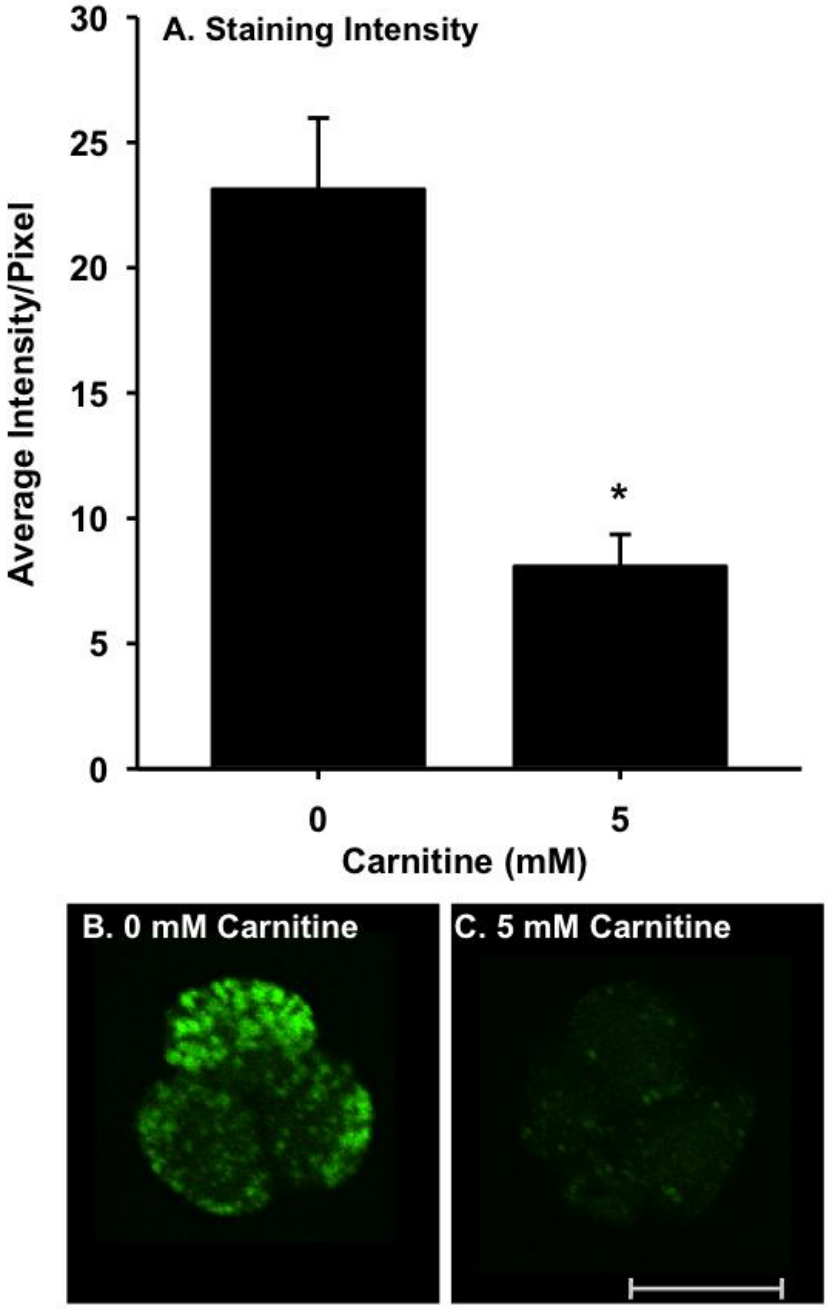
516 Figure 3.



517

Table 1. The composition of cleavage media

Component (mM)	- Carbohydrates	+ Carbohydrates
NaCl	115	105
KCl	5.5	5.5
$MgSO_4$ $7H_2O$	1	1
NaH_2PO_4	0.25	0.25
$NaHCO_3$	25	25
$CaCl_2$	1.8	1.8
Glucose	-	0.5
Na Pyruvate	-	0.35
Na Lactate	-	10.5
BSA (mg/ml)	4	4
Osmolarity	275	276

Table 2. Mean (± SEM) influence of 5 mM L-carnitine supplementation, in the presence of carbohydrates, on pre-compaction development of bovine embryos

L-Carnitine (mM)	Embryos (n)	Cleaved /Total (%)	Embryo Development/Cleaved (%)				
			4-Cells	8-Cells	> 8-Cells	Morula	Blastocyst
0	145	72.9 ± 3.9	75.9 ± 3	39.9 ± 0.4	14 ± 5.9	3.1 ± 1.9	0 ± 0
5	142	64.5 ± 3.9	67.2 ± 13.1	62.4 ± 10	47.2 ± 9.3 *	43.8 ± 9.1 *	13.6 ± 7

[a, b] Within a column, values without a common superscript differed (P < 0.05).

Table 3. Mean (± SEM) influence of L-carnitine supplement in the absence of carbohydrates on pre-compaction development of bovine embryos

Carbohydrates	L-Carnitine (mM)	Embryos (n)	Cleaved /Total (%)	Embryo Development/Cleaved (%)			
				4-Cell	8-Cell	> 8-Cells	Morula
-	-	127	62.1 ± 16.3	35.6 ± 24.1	1.6 ± 1.6 [a]	0 ± 0 [a]	0 ± 0 [a]
+	-	119	78.7 ± 4.6	79 ± 11.4	39.9 ± 12.2 [ab]	12.2 ± 6.9 [a]	4.7± 2.5 [a]
-	1	133	85.3 ± 2.8	93.1 ± 3.9	56.7 ± 7.4 [b]	25.1 ± 5.7 [ab]	5.3 ± 1.5 [a]
-	5	128	85.8 ± 2.1	83 ± 0.8	63.1 ± 1.6 [b]	39.6 ± 3.3 [b]	20.4 ± 3 [b]

[a, b] P Within a column, values without a common superscript differed (P < 0.005).

Table 4. Mean (± SEM) development of bovine embryos following culture ± L-carnitine ± etomoxir (an inhibitor of ß-oxidation).

L-Carnitine (5 mM)	Etomoxir (50 µM)	Embryos (n)	Cleaved/ Total (%)	Embryo Development/Cleaved (%)			
				4-Cells	8-Cells	> 8-Cells	Morula
-	-	198	83.9 ± 4.8	89.7 ± 4.1	56.9 ± 10	15.1 ± 2.9 [ab]	6.5 ± 1.8
-	+	197	75.8 ± 6.8	90.9 ± 3	58.1 ± 8.1	9.0 ± 2.4 [a]	2.5 ± 1.1
+	-	186	77.7 ± 3.2	93.5 ± 2.5	82.8 ± 5.1	33.9 ± 8 [b]	14.8 ± 6.6
+	+	206	68.1 ± 3.2	88.9 ± 2.2	59.0 ± 7.7	19.2 ± 4.9 [a]	4.2 ± 2.1

[a, b] Within a column, values without a common superscript differed (P < 0.05).