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—Original Article—

Cryotolerance of porcine blastocysts is improved by treating *in vitro* matured oocytes with L-carnitine prior to fertilization

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Abstract. Sufficient generation of adenosine triphosphate (ATP) by oocytes is critical for fertilization and embryo development. The objective of this study was to determine the effects of supplementing media with L-carnitine, a co-factor required for the metabolism of fatty acids, during the peri-fertilization period on embryo development and energy generation. Firstly, *in vitro* matured (IVM) porcine oocytes were co-incubated with sperm in IVF medium supplemented with 0–24 mM L-carnitine. The blastocyst formation rate of the control group was greater than those of the L-carnitine groups ($P < 0.05$), except for the 3 mM L-carnitine group. Subsequently, oocytes and/or sperm were treated without or with 3 mM L-carnitine for either the 1 h pre-IVF oocyte incubation; the pre-IVF sperm preparation; the first 30 min of IVF; or the entire 5.5 h of IVF. Despite similar fertilization rates among the groups, the cleavage rate of the pre-IVF oocyte group was significantly greater than those of the other groups, except for the pre-IVF sperm group. Additionally, the oocyte ATP content and the cryotolerance of the resulting blastocysts were examined following the pre-IVF oocyte treatment. Oocyte ATP content was also similar among the groups ($P > 0.05$). Following vitrification, the post-warming survival rate of blastocysts derived from L-carnitine-treated oocytes was greater than that of blastocysts derived from untreated oocytes (42.4% *vs.* 24.9%; $P < 0.05$). In conclusion, a 1 h oocyte exposure to 3 mM L-carnitine immediately prior to insemination enhanced cleavage and improved the cryotolerance of resulting blastocysts. While the findings are suggestive of a lipolytic action, further studies are required to clarify the contributions of lipid metabolism and oxidative mechanisms to the observed effects of the L-carnitine treatment.
Key words: *In vitro* fertilization (IVF), Lipid metabolism, Pig embryo, Vitrification

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At the time of fertilization, there is an increased adenosine triphosphate (ATP) requirement to support calcium oscillations and calcium homeostasis [1]. In aged oocytes, noted for having poor developmental outcomes, deficient ATP content at fertilization is believed to lead to altered calcium oscillation patterns and homeostasis [2]. Sufficient ATP is also required to support cellular events triggered by fertilization, including polymerization of microtubules, cell cycle regulation, segregation of chromosomes and membrane biosynthesis [3]. ATP production and accumulation during oocyte maturation is likely to be an important factor for fertilization. Dysfunctional or low numbers of mitochondria, resulting in lower ATP content of oocytes at fertilization, has been shown to lead to a higher incidence of fertilization failure [3−5]. Cattle oocytes morphologically graded as being of higher quality had a higher ATP content following *in vitro* maturation (IVM) and had increased blastocyst development following *in vitro* fertilization (IVF) [6]. Further, the ATP content of both mouse and human oocytes has been positively correlated with embryo viability [5, 7]. In mouse oocytes, ATP content peaks at the time of extrusion of the first polar body, with metaphase II (MII) stage oocytes consuming greater amounts of ATP than those arrested at the germinal vesicle stage [8]. Further, in porcine MII stage oocytes that were aged, the electron density of lipid droplets decreased over time [9]. This higher consumption of ATP by MII stage oocytes, coupled with decreasing lipid stores, suggests that oocytes may be utilizing lipid substrates during MII arrest prior to fertilization.

In toad eggs, fertilization triggers a decrease in triglycerides, hypothesized to be due to metabolic breakdown of lipid substrates for ATP production to meet increased energy requirements [10]. Porcine oocytes contain large amounts of endogenous lipid [11], and although they differ physiologically from toad eggs, this lipid fraction may play a similar role. Also, following sperm penetration of porcine oocytes, the electron density of lipid droplets decreases, implying a reduction of lipid content at this time [12]. Given that lipids are a very efficient fuel source, it may be that the β-oxidation pathway is utilized during fertilization.

The impact of supplementing IVF medium with metabolic regulators is poorly understood. L-carnitine is a key co-factor involved in the carnitine shuttle, with this being the rate-limiting step in the entry of activated fatty acids to the mitochondrial matrix for metabolic breakdown, and is also known to have strong antioxidant properties. L-carnitine has also been shown to have an effect on *in vitro* sperm parameters. The addition of 1.76 mM L-carnitine to testicular mouse sperm increased motility and chromatin quality following 30–180 min incubations at room temperature [13]. Further, inclusion of 3.1 mM L-carnitine led to higher human sperm motility after 2 h incubation at 37ºC, although there was no effect on DNA oxidation or sperm viability [14]. The positive effects on sperm are believed

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to be due to the antioxidant activity of L-carnitine. Accumulation of reactive oxygen species (ROS) in sperm causes ATP depletion, lipid peroxidation and axonemal phosphorylation [15], affecting sperm motility.

Upregulation of the β-oxidation pathway by supplementing IVM medium with L-carnitine has been shown to improve oocyte maturation in mice [16], cattle [17] and pigs [18, 19]. Also, inclusion of L-carnitine in embryo culture medium decreased lipid content and increased cryosurvival in cattle embryos [20]. It is believed that by stimulating lipid metabolism, greater amounts of lipid stores are used to generate ATP to support development while also improving cryotolerance due to decreased lipid content. Potentially, stimulating lipid metabolism during fertilization may increase ATP levels to support the energy requirements of the oocyte. Any beneficial effects seen from the inclusion of L-carnitine during IVF would need to be clarified as affecting sperm and/or oocytes.

The aim of this study was to determine if L-carnitine supplementation either during or immediately prior to fertilization of IVM porcine oocytes enhances fertilization and developmental outcomes. Further, this study examined the effect of supplementing IVF medium with L-carnitine on the cryotolerance of the resulting blastocysts.

Materials and Methods

Chemicals and media

All chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Washing and preparation of oocytes were carried out using HEPES-buffered Porcine X Medium (PXM; 108 mM NaCl, 10 mM KCl, 0.35 mM KH_2PO_4, 0.40 mM $MgSO_4$, 5.0 mM $NaHCO_3$, 25 mM HEPES, 0.2 mM sodium pyruvate, 2.0 mM calcium lactate, 3.0 mg/ml polyvinyl alcohol (PVA); [21]). IVM was performed using Porcine Oocyte Medium (POM; 108 mM NaCl, 10 mM KCl, 0.35 mM KH_2PO_4, 0.4 mM $MgSO_4$, 25 mM $NaHCO_3$, 4.0 mM glucose, 0.2 mM sodium pyruvate, 2.0 mM calcium lactate, 2.0 mM glutamine, 5.0 mM hypotaurine, 0.1 mM cysteamine, Minimum Essential Medium (MEM) amino acids (Gibco 11130051, Grand Island, NY, USA), MEM non-essential amino acids (Gibco 11140050), 65 µg/ml penicillin G, 50 µg/ml streptomycin sulfate; [21]) supplemented with 3.0 mg/ml fatty acid-free bovine serum albumin (BSA; IVP grade gamma irradiated, MP Biomedicals, Auckland, New Zealand), 10 ng/ml epidermal growth factor, 10 IU/ml equine chorionic gonadotropin (eCG; Pregnecol; Bioniche Animal Health Pty Ltd, Armidale, NSW, Australia) and 10 IU/ml human chorionic gonadotropin (hCG; Chorulon; Intervet Australia Pty Ltd, Bendigo East, VIC, Australia). Tyrode's albumin lactate pyruvate-polyvinyl alcohol (TALP-PVA) medium [22] supplemented with 3.0 mM calcium lactate, 2.0 mM caffeine-sodium benzoate and 3.0 mg/ml BSA [23] was used for IVF. The sperm preparation medium consisted of TALP-PVA medium supplemented with 0.5 mg/ml BSA (SpermTALP). As per the experimental design, supplementation of the TALP-PVA and SpermTALP media with L-carnitine (L-carnitine hydrochloride; Sigma-Aldrich C0283) involved adding a quantity of the L-carnitine powder directly to a volume of the medium to achieve the desired final concentration. Once the powder had completely dissolved, the pH of the medium at room temperature (22ºC) was adjusted to that of the corresponding non-supplemented, non-equilibrated medium (pH 7.55) and then filter sterilized. All L-carnitine supplemented media were freshly prepared prior to use in each experimental replication. Porcine Zygote Medium-3 (PZM-3; [24]) was used for embryo *in vitro* culture (IVC). All droplets and wells of media were covered with embryo tested mineral oil and equilibrated in a humidified atmosphere of 6% CO_2 in air at 38.5ºC for at least 3 h prior to use.

In vitro maturation

Ovaries from prepubertal gilts were collected immediately after slaughter at a local abattoir and transported to the laboratory within 1 h at 34–38ºC in 0.9% NaCl (Baxter Healthcare, Deerfield, IL, USA) supplemented with an antibiotic/antimycotic solution (100 IU/ml penicillin G, 0.25 µg/ml streptomycin sulphate and 0.85% amphotericin B; Gibco). Cumulus oocyte complexes (COCs) were aspirated from antral follicles 3–6 mm in diameter using a 21-gauge needle through which constant suction (1 l/min) was applied, and collected in a vacutainer tube. Collected COCs were washed twice in PXM. Oocytes with an evenly granulated cytoplasm and at least three complete layers of compact cumulus cells were selected and washed in POM. Washed COCs were transferred to 4-well dishes (~50 COCs per well; Nunc, Roskilde, Denmark) containing POM (500 µl per well), and cultured in a humidified atmosphere of 6% CO_2 in air at 38.5ºC.

In vitro fertilization

After 44 h of IVM, oocytes were partially denuded of cumulus cells by gentle pipetting in PXM after brief exposure (< 1 min) to 0.5 mg/ml hyaluronidase. Oocytes were washed and transferred to 4-well dishes containing IVF medium (500 µl per well). Meanwhile, boar semen frozen in a 0.25 ml straw was thawed immediately upon retrieval from liquid nitrogen storage by agitating the straw in a water bath at 42ºC for 20 sec. Sperm were purified by density gradient centrifugation at 720 *g* for 10 min using a two-layer (45% and 90%) PureSperm (Nidacon Laboratories AB, Gothenburg, Sweden) discontinuous gradient prepared with SpermTALP medium. Following this, the pellet was gently aspirated, made up to 1 ml in volume with SpermTALP medium and centrifuged at 310 *g* for 5 min. The supernatant was removed, and the pellet was gently resuspended in 400 µl of SpermTALP medium. Sperm motility and concentration were then assessed. Sperm were added to the insemination wells (approximately 50 oocytes per well) at a concentration of 200 motile sperm/oocyte. Gametes were co-incubated for 30 min at 38.5ºC in 6% CO_2 in air, after which the oocytes and zona-bound sperm were carefully transferred to a second well containing fresh IVF medium (500 µl) and incubated for a further 5 h, resulting in a total IVF co-incubation of 5.5 h [25, 26]. For experiments with a pre-IVF incubation period, oocytes were held in IVF medium without (control) or with 3.0 mM L-carnitine for 1 h, and then transferred to IVF medium without (control) or with 3.0 mM L-carnitine (as per the experimental design) immediately prior to insemination.

In vitro culture and embryo assessment

At the completion of IVF, presumptive zygotes were denuded of remaining cumulus cells and loosely bound sperm, washed in PZM-3, placed in 50 µl droplets of PZM-3 (maximum of 15 zygotes/droplet), and incubated in 6% CO_2, 5% O_2 and 89% N_2 at 38.5ºC. On Day 4

of IVC, cleavage was assessed and the PZM-3 was supplemented with 10% fetal calf serum (FCS; heat inactivated, Australian origin; Gibco 10100139) by adding 5 µl of equilibrated FCS to each 50 µl droplet. Blastocyst formation was assessed on Day 7 of IVC. Blastocysts used to determine total cell numbers were washed in PXM and transferred to absolute ethanol containing 0.3 mg/ml Hoechst 33342. After staining for 30 min, the blastocysts were transferred to absolute ethanol, fixed overnight in the dark at 4ºC, and then slide mounted. The stained nuclei were visualized using fluorescence microscopy (Olympus BX61; Olympus, Tokyo, Japan) and counted using ImageJ software (v. 1.46r, National Institutes of Health, USA).

To assess fertilization, presumptive zygotes were fixed in acetic acid:ethanol (1:3) 12 h after insemination for a minimum of 3 days. Following fixation, zygotes were slide mounted and stained with 1% (w/v) orcein in 45% (v/v) acetic acid for 30 min, destained with glycerol:acetic acid:water (1:1:3) and examined using phase-contrast microscopy at 400 × magnification. Zygotes were classified as unfertilized (meiotic spindle and one polar body present), polyspermic (multiple pronuclei and/or multiple decondensed sperm heads present), penetrated (one pronucleus and/or decondensed sperm head present), or fertilized normally (two pronuclei and two polar bodies present).

Measurement of oocyte ATP concentration

The ATP concentration of oocytes was measured using a commercial assay kit (FL-ASC; Sigma). Briefly, oocytes were completely denuded of cumulus cells, and washed three times in PBS supplemented with 3 mg/ml polyvinylpyrrolidone. Oocytes were transferred in groups of 10 to Eppendorf tubes containing 50 µl ice-cold PBS and stored at −80ºC until analysis. For analysis, samples were thawed on ice protected from light. Ice-cold somatic cell reagent (100 µl) was added to each tube, briefly centrifuged, and incubated on ice for 5 min. Next, ice-cold assay mix solution (diluted 1:25 with ATP assay mix dilution buffer; 100 µl) was added, and each tube was briefly centrifuged and incubated on ice for 5 min. Sample luminescence was measured in a flat bottom 96-well plate (Greiner Bio-One, Frickenhausen, Germany) using a microplate reader with a luminescence optical system (POLARstar Optima; BMG Labtech, Ortenburg, Germany). A seven-point standard curve (0–60 pM/tube) was included in each assay to enable the unknown ATP concentrations to be determined. The ATP concentration for each sample was then divided by the number of oocytes in the tube to obtain the final value (expressed as pM ATP/oocyte).

Blastocyst vitrification and warming

On Day 7 of IVC, blastocysts were vitrified as previously described [25, 27]. Blastocysts were classified morphologically as either A, B or C grade, according to the criteria of the Society for Assisted Reproductive Technology (SART) grading system (A = good, B = fair, C = poor; [28]). Only blastocysts of grades A and B were vitrified. Briefly, groups of up to 10 blastocysts were washed in pre-warmed HEPES-buffered Medium 199 (Gibco 12340030) supplemented with 20% FCS (H199-FCS; 38.5ºC) for 5 min. Blastocysts were then transferred to equilibration medium (H199-FCS supplemented with 7.5% ethylene glycol and 7.5% DMSO; 22ºC) and held for 3 min before being transferred to vitrification medium (H199-FCS supplemented with 17% ethylene glycol, 17% DMSO and 0.4 M sucrose; 22ºC) for 45 sec. Embryos were loaded into a super-fine open-pulled straw (SOPS; Minitube, Tiefenbach, Germany) within the 45 sec period and immediately plunged into liquid nitrogen. The equilibration and vitrification media were used at room temperature (22ºC). To warm, the open end of the SOPS was placed directly into pre-warmed Thaw Medium 1 (H199-FCS supplemented with 0.14 M sucrose; 38.5ºC) immediately upon retrieval from liquid nitrogen. Blastocysts were held in this medium for 6 min before a 5 min hold in pre-warmed Thaw Medium 2 (H199-FCS supplemented with 0.075 M sucrose; 38.5ºC) and a final 5 min hold in pre-warmed H199-FCS (38.5ºC). The warming media were used at 38.5ºC. The blastocysts were then washed in PZM-3 supplemented with 20% FCS, transferred to 50 µl droplets of PZM-3 supplemented with 20% FCS, and cultured in 6% CO_2, 5% O_2 and 89% N_2 at 38.5ºC. Cryosurvival was assessed after 24 h of post-warming culture, with surviving blastocysts classified as those in which blastocoele re-expansion had clearly occurred.

Experimental design

Experiment 1: Dose response effect of L-carnitine supplementation during IVF

The medium used for the entire 5.5 h duration of IVF was supplemented with 0, 3, 6, 12 or 24 mM L-carnitine. The presumptive zygotes were cultured, cleavage and blastocyst formation were recorded, and blastocyst cell numbers were determined. The experiment was replicated four times with 30 to 45 presumptive zygotes per treatment group in each replicate.

Experiment 2: Effect of L-carnitine supplementation during pre-IVF and IVF

IVF was conducted with the addition of 3 mM L-carnitine for defined periods (Table 1). This dose was selected based on the results of Experiment 1. Oocytes were subjected to a 1 h pre-IVF incubation in IVF medium without (control) or with 3 mM L-carnitine (pre-IVF oocytes). Sperm were prepared in SpermTALP medium without (control) or with 3 mM L-carnitine (pre-IVF sperm). Oocytes and sperm were co-incubated using the 2-step IVF method described previously [25, 26], without (control) or with 3 mM L-carnitine supplementation for either the first 30 min only (IVF brief), or the entire 5.5 h interval (IVF entire). Approximately 15 presumptive zygotes per treatment group were fixed 12 h after insemination, and stained to assess fertilization. The remaining zygotes were cultured, cleavage and blastocyst formation were recorded, and blastocyst cell numbers were determined. The experiment was replicated four times with 30 to 45 presumptive zygotes per treatment group in each replicate.

Experiment 3: Effect of L-carnitine treatment on ATP content of oocytes and post-warming survival of resulting blastocysts

Immediately prior to IVF, oocytes were incubated for 1 h in IVF medium without or with 3 mM L-carnitine (i.e. the 1 h pre-IVF incubation described in Experiment 2). The co-incubation of oocytes and sperm was performed in standard IVF medium without L-carnitine supplementation. A cohort of oocytes was assayed to determine their ATP content after 44 h maturation, and again following the 1 h pre-IVF incubation. The remaining oocytes were subjected to IVF and IVC, and cleavage and blastocyst formation were recorded. Blastocysts of grade A and B [28] were vitrified on Day 7. Cryosurvival was assessed 24 h after warming. The experiment was replicated four times. For

Table 1. Design of Experiment 2[a)]

Treatment group	Sperm preparation [b)]	Pre-IVF [c)] (1 h)	IVF step 1 [c)] (30 min)	IVF step 2 [c)] (5 h)
Control (untreated)	–	–	–	–
Pre-IVF sperm	LC	–	–	–
Pre-IVF oocytes	–	LC	–	–
IVF brief	–	–	LC	–
IVF entire	–	LC	LC	LC

[a)] LC: medium was supplemented with 3 mM L-carnitine. [b)] Sperm preparation procedures were performed using SpermTALP medium and took a maximum of 1 h to complete. [c)] All pre-IVF oocyte incubations and IVF gamete co-incubations were performed using TALP-PVA medium.

the ATP measurements, 10 oocytes were pooled at each time point per group in each replicate. For the post-warming survival assessment, a mean of 9 blastocysts were vitrified per group in each replicate.

Statistical analysis

Analyses were performed using GenStat 16th Edition (VSN International Ltd, Hemel Hempstead, UK). Percentage data were arcsine transformed prior to analysis. Oocyte ATP content, cleavage, blastocyst formation and post-warming survival data were analyzed by one-way analysis of variance (ANOVA), blocking by replicate. Fisher's protected LSD post-hoc test was used when significant differences were detected. Blastocyst cell number data were analyzed using the Student's *t*-test. All data are expressed as the mean ± the standard error of the mean (SEM). A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Experiment 1: Dose response effects of L-carnitine supplementation during IVF

The dose response effects of L-carnitine supplementation on cleavage and blastocyst formation are shown in Fig. 1. The cleavage rates were similar among the treatment groups ($P > 0.05$). The blastocyst formation rates of the 6, 12 and 24 mM L-carnitine groups were lower than that of the control group ($P < 0.05$). Supplementation with 3 mM L-carnitine did not reduce the blastocyst formation rate compared with the control ($P > 0.05$). The mean blastocyst cell number of the 24 mM L-carnitine group (32.0 ± 6.7 cells) was lower than that of the 3 mM L-carnitine group (71.9 ± 12.4 cells; $P < 0.05$), but did not differ significantly from that of the control group (70.8 ± 15.9 cells) and the other L-carnitine groups (6 mM: 43.4 ± 7.0 cells; 12 mM: 47.8 ± 11.2 cells).

The 3 mM L-carnitine dose was selected for use in the subsequent experiments, as the higher L-carnitine doses had detrimental effects on blastocyst development.

Experiment 2: Effects of L-carnitine supplementation during pre-IVF and IVF

The period of L-carnitine supplementation during pre-IVF and/or IVF did not significantly affect the percentages of penetrated oocytes (54.9 ± 9.3% to 65.4 ± 11.6%) or normally fertilized, monospermic oocytes (10.4 ± 4.6% to 30.4 ± 7.2%). The incidence of male pronuclear formation (84.4 ± 5.5% to 91.1 ± 3.2%) was also similar among the groups ($P > 0.05$). The effects of L-carnitine supplementation on cleavage and blastocyst formation are shown in Fig. 2. The cleavage rate of oocytes exposed to L-carnitine during the 1 h pre-IVF period was higher than those of control oocytes (no

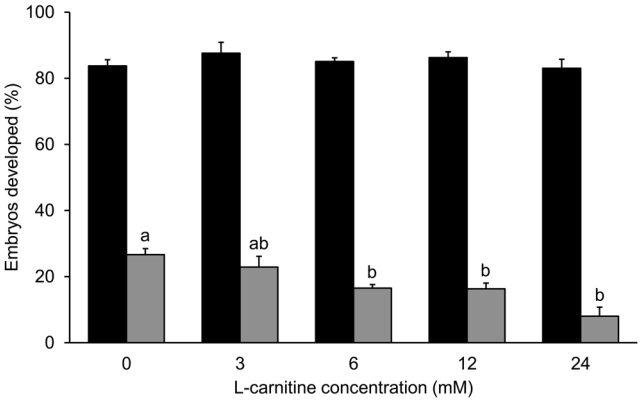

Fig. 1. The effects of L-carnitine dose during IVF on the development of porcine embryos. The IVF medium was supplemented with 0, 3, 6, 12 and 24 mM L-carnitine and used for the duration of gamete co-incubation as described for the 2-step IVF procedure [25, 26]. Data are expressed as the percentages (mean ± SEM) of total oocytes that cleaved (black bars) and cleaved embryos that formed blastocysts (gray bars). Bars without a common letter differ significantly ($P < 0.05$).

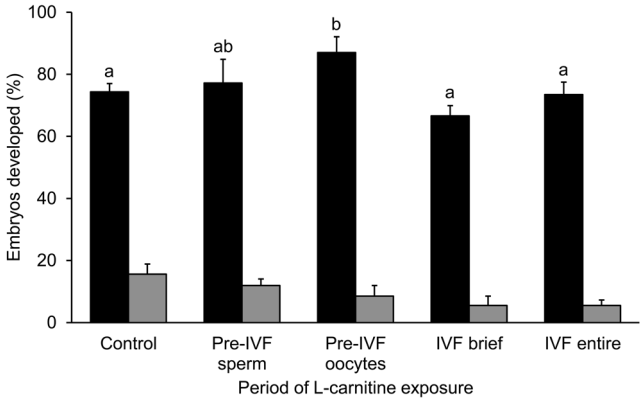

Fig. 2. The effects of supplementing 3 mM L-carnitine to media used for gamete IVF preparation and co-incubation on the development of porcine embryos. L-carnitine supplementation of the sperm preparation medium (SpermTALP medium) and IVF medium (TALP-PVA medium) used for each treatment group is detailed in Table 1. L-carnitine was either completely absent (Control) or present in the SpermTALP medium used to prepare the sperm for IVF (pre-IVF sperm); the IVF medium used to incubate the matured, denuded oocytes for 1 h prior to IVF (pre-IVF oocytes); the IVF medium used for the initial 30 min of gamete co-incubation (IVF brief); or the IVF medium used for the entire 5.5 h of gamete co-incubation (IVF entire). Data are expressed as the percentages (mean ± SEM) of total oocytes that cleaved (black bars) and cleaved embryos that formed blastocysts (gray bars). Bars without a common letter differ significantly ($P < 0.05$).

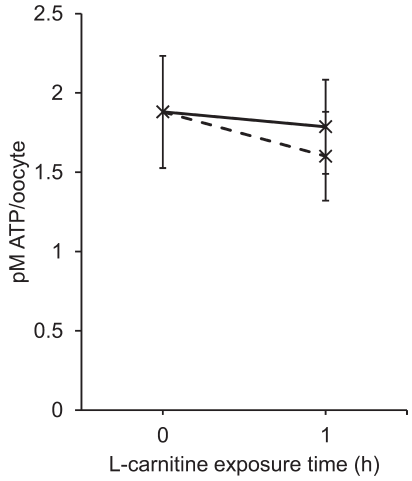

Fig. 3. The effect of adding L-carnitine to the medium used during the pre-IVF period on the concentration (mean ± SEM) of ATP in porcine oocytes. After 44 h of IVM, oocytes were denuded of cumulus cells, selected for the presence of a polar body, and sampled either immediately (0 h) or after incubation (1 h) in IVF medium without (solid line) or with (dashed line) 3 mM L-carnitine.

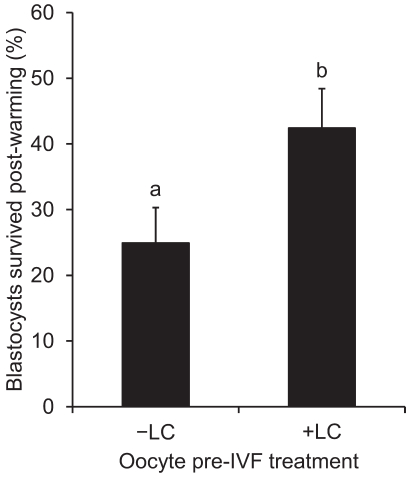

Fig. 4. The effect of adding L-carnitine to the medium used during the pre-IVF period on the post-warming survival of vitrified blastocysts. Following IVM, oocytes were partially denuded of cumulus cells and cultured in TALP-PVA medium without (−LC) or with (+LC) 3 mM L-carnitine for 1 h, immediately prior to gamete co-incubation in TALP-PVA medium without L-carnitine. After IVF and IVC, Day 7 blastocysts of grade A and B quality were vitrified and their cryosurvival was assessed 24 h post-warming. Data are expressed as the percentages (mean ± SEM) of vitrified-warmed blastocysts that formed a blastocoele. Bars without a common letter differ significantly ($P < 0.05$).

L-carnitine exposure) and oocytes exposed to L-carnitine during the entire IVF period ($P < 0.05$). The cleavage rate of oocytes fertilized using sperm exposed to L-carnitine during the pre-IVF period did not differ from those of the other groups ($P < 0.05$). The blastocyst formation rates of all the groups did not differ ($P > 0.05$). Likewise, the blastocyst cell numbers of all the groups (67.1 ± 20.7 cells to 103.0 ± 19.4 cells) did not differ significantly (67.1 ± 20.7 cells *vs.* 103.0 ± 19.4 cells; $P = 0.112$).

Experiment 3: Effects of L-carnitine treatment on ATP content of oocytes and post-warming survival of resulting blastocysts

The oocyte ATP content was similar among groups before and after incubation ($P > 0.05$; Fig. 3). There was no difference in the ATP content of oocytes following incubation with or without L-carnitine ($P > 0.05$; Fig. 3). Consistent with the results of Experiment 2, treatment of oocytes with L-carnitine prior to IVF (i.e. the 1 h pre-IVF period) increased the cleavage rate compared with the control (86.6 ± 1.3% *vs.* 79.1 ± 2.9%; $P < 0.05$). The blastocyst formation rates did not differ significantly (L-carnitine: 14.0 ± 4.1%; control: 13.2 ± 3.7%). Also, the proportions of Day 7 blastocysts that were classified as A or B grade, and were therefore vitrified, did not differ significantly (L-carnitine: 40 vitrified of 44 total; control: 34 vitrified of 38 total). The post-warming survival rate of blastocysts derived from the L-carnitine-treated oocytes was greater than that of blastocysts derived from the untreated control oocytes (42.4 ± 6.0% *vs.* 24.9 ± 5.4%; $P < 0.05$; Fig. 4).

Discussion

This study examined the potential of L-carnitine treatments immediately prior to and during IVF to elevate the ATP concentration of porcine oocytes and thereby improve fertilization outcomes. The capacity of oocytes and early stage embryos to generate ATP has been correlated with developmental competence and embryo development in cattle, mice and humans [5–7, 29]. L-carnitine treatments during *in vitro* maturation have previously been found to increase ATP levels in mouse oocytes [16]. However, the results of this study indicate that the relatively brief L-carnitine treatment did not alter ATP levels significantly or improve fertilization. Rather, when included for the duration of gamete co-culture, supplementation of 6–24 mM L-carnitine exerted detrimental effects on subsequent development. Though the inhibition of fatty acid β-oxidation and the concomitant upregulation of glucose metabolism do not appear to be detrimental to pig embryo development [30], the results suggest that adjustments to glucose metabolism induced by L-carnitine treatments during IVF may hamper post-fertilization events.

Incubation of oocytes in fertilization medium supplemented with L-carnitine for 1 h prior to gamete co-incubation increased the post-warming survival of vitrified Day 7 blastocysts. Porcine oocytes and embryos have large amounts of intracellular lipid droplets [11, 31], which is known to be the reason for their poor cryotolerance [32]. Previous studies have shown that inclusion of L-carnitine during bovine embryo culture decreased lipid content [20] and increased cryosurvival rates [33, 34]. The results of this study show that only a short exposure to L-carnitine significantly improved the cryotolerance of resulting blastocysts. This remarkable finding suggests that even though stimulation of lipid metabolism during IVF may be detrimental to some aspects of embryo development, any reduction in cytoplasmic lipid content ultimately enhances the capacity of

porcine embryos to survive vitrification. The increased post-warming survival rate seen with L-carnitine supplementation was not due to a difference in the morphological characteristics of the blastocysts, as the proportions of A and B grade expanded and hatching blastocysts vitrified in the untreated control and L-carnitine-treated groups were similar. Clearly, the effectiveness of morphological assessment for evaluating the cryotolerance of porcine blastocysts is limited, and further studies are needed to characterize the apparent variations in the cytoplasmic lipid content of embryos treated with L-carnitine.

Embryo cleavage rates were increased following IVF of oocytes incubated with L-carnitine for 1 h prior to the addition of sperm. Mitochondrial ATP is essential for sustaining calcium oscillations triggered by fertilization events, which then subsequently upregulate ATP production to satisfy energy demands [1, 35]. In humans, total carnitine content of follicular fluid was not correlated with embryo developmental outcomes following IVF, although enzymes involved in β-oxidation were strongly expressed in mature oocytes and the expression of enzymes involved in carnitine synthesis was not detected [36]. Given this, the addition of exogenous L-carnitine may aid in upregulating β-oxidation rates in the oocyte. Higher ATP content in mature oocytes has been correlated with greater developmental success [5, 6], with the majority of ATP derived from oxidative phosphorylation [1, 2, 8]. Acetyl-CoA, produced via β-oxidation, is metabolized through the tricarboxylic acid (TCA) cycle to produce NADH and $FADH_2$, both of which are then fully metabolized via oxidative phosphorylation to produce ATP. However, in this study, there was no associated increase in the ATP content of oocytes following incubation in medium supplemented with L-carnitine. This does not preclude the possibility that the L-carnitine treatment induced an increase in ATP production, but this increase was accompanied by an increase in ATP consumption, resulting in no net gain, which may account for the improved oocyte and blastocyst quality.

Other studies have found no correlation between ATP content of porcine oocytes and developmental competence [37, 38]. In some homozygous mouse strains, excessive ATP production and increased cytoplasmic lipid deposition were associated with early cleavage arrest [39]. It is possible that rather than a critical level of ATP being required to support fertilization, the higher ATP content is indicative of a healthy, functional mitochondria population necessary for embryo development. Current media formulations do not contain co-factors to support β-oxidation. In humans, IVF patients with higher reproductive potential have decreased levels of total carnitine in serum and follicular fluid, suggesting upregulation of lipolytic pathways and depletion of carnitine stores in these patients [40]. Expression of carnitine synthesis enzymes is low in human oocytes and early embryos, while β-oxidation enzymes are strongly expressed [36], supporting a role for exogenous carnitine in embryo culture media. However, inappropriate lipolytic activity induced by L-carnitine treatment during the entire IVF period may explain the reduced developmental potential of oocytes observed at the higher doses.

Alternatively, the effects of L-carnitine may be attributed to its strong antioxidant properties. Markers of oxidative stress in human follicular fluid have been correlated to fertilization and pregnancy outcomes following IVF [41, 42], with higher fertilization success seen in oocytes recovered from follicular fluid with higher antioxidant levels [41]. Addition of L-carnitine to medium for the duration of porcine oocyte maturation decreased levels of intracellular ROS, hydrogen peroxide and lipid peroxides, reduced apoptosis, and increased glutathione concentrations [18, 19, 43–45]. Oxidative stress is proposed to be the catalyst for post-ovulatory oocyte aging [46], which is associated with reduced fertilization rates and poor embryo quality. Certain compounds with antioxidant activity have been shown to delay signs of post-ovulatory oocyte aging [47, 48]. The antioxidant properties of L-carnitine may have assisted in maintaining oocyte developmental competence prior to fertilization, resulting in the observed increase in cleavage rate and improvement in blastocyst cryotolerance. Culture in antioxidant-containing medium following IVF has previously been found to reduce ROS levels in porcine embryos and improve the cryotolerance of the resulting blastocysts, without any apparent enhancement of blastocyst yield or quality [49]. Here, the improved blastocyst cryotolerance was achieved by exposure to L-carnitine for only 1 h immediately prior to IVF. If the effect of this L-carnitine exposure was indeed via an antioxidant effect, it seems incredible that such a brief treatment can elicit such a long-term benefit. Similar to the findings of the present study, at the higher concentrations of L-carnitine tested, high concentrations of antioxidants included in medium during bovine IVF decreased blastocyst formation rates [50]. Perhaps the findings together highlight the importance of reducing the ROS levels in oocytes and zygotes, while antioxidant exposure during gamete co-incubation is not ideal. Reactive oxygen species are required for fertilization [51, 52], playing a role in sperm capacitation, hyperactivation and the acrosome reaction [53]. This indicates that higher doses or longer exposure to L-carnitine may be detrimental during IVF due to its antioxidant properties suppressing the concentrations of vital ROS. Further research is required to determine the optimal concentration and incubation time to maximize the beneficial effects of L-carnitine supplementation at fertilization.

Although there were no beneficial effects on fertilization, cleavage or blastocyst formation rates when the sperm preparation medium was supplemented with L-carnitine, prior evidence suggests that this is an area for future research. Spermatozoa have a low antioxidant defense capacity and are particularly susceptible to oxidative damage due to a high lipid content of membranes [54]. Incubation in L-carnitine has been shown to increase the motility and chromatin quality of testicular mouse sperm [13], as well as the motility of human sperm [14]. However, given that cleavage rates were increased in oocytes incubated with L-carnitine prior to fertilization, with sperm prepared in the absence of L-carnitine, there was clearly a beneficial effect of L-carnitine on oocytes. The individual effects of L-carnitine on male and female gametes needs to be further clarified in future studies.

In conclusion, incubation of oocytes in fertilization medium supplemented with 3 mM L-carnitine for 1 h prior to co-incubation with sperm increased the capacity of oocytes to undergo cleavage and improved the cryotolerance of the resulting blastocysts. Inclusion of 3 mM L-carnitine for any of the examined periods of gamete co-culture had no significant effects on fertilization status or the development of blastocysts. Also, the fertilizing capacity of sperm appeared to be unaffected by exposure to L-carnitine during sperm preparation. Although the oocyte ATP levels were not significantly elevated by the brief pre-IVF treatment, the metabolism of lipids and other energy substrates may have been altered by L-carnitine supplementation.

Alternatively, the antioxidant properties of L-carnitine may have provided benefit for the maintenance of oocyte viability during the pre-insemination period. However, given that such a brief exposure of oocytes to an antioxidant seems unlikely to be of benefit to blastocysts that form 7 days later, we propose that the improvement in blastocyst cryotolerance was due to a reduction in oocyte lipid content. More detailed *in vitro* assessments are needed to determine the influence of the brief L-carnitine treatment on sperm functionality and oocyte and blastocyst quality.

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