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Effect of supplementation of L-carnitine during *in vitro* maturation on embryo production in Sahiwal cows

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ABSTRACT

Embryo transfer technology enables rapid dissemination of superior genetics and *in vitro* produced embryos have gained more consideration than the *in vivo* derived embryos due to higher yield of embryos with respect to time. The primary limitation impacting *in vitro* derived embryos is the success of *in vitro* maturation as *in vitro* derived oocytes contain more lipid content contributing to increased oxidative stress and compromised developmental competence. In the present study, lipid modulator, L-carnitine at 1 mM and 3mM was supplemented in the maturation media to decrease lipid content and subsequent oxidative stress in the oocytes undergoing maturation. A total of 326 follicles from overy were aspirated out of which 228 cumulus oocyte complexes (COCs) were collected with a recovery rate of 69.93%. The *in vitro* maturation of the COC's supplementation with 3 mM L-Carnitine exhibited significantly ($p<0.05$) lower complete expansion of cumulus cells in comparison to maturation media supplemented with 1 mM L-carnitine (48, 75.00% vs 58, 90.62%). The cleavage rate of the COC's matured with supplementation of 3 mM L-Carnitine displayed considerably ($p<0.05$) less cleavage in comparison to group supplemented with 1 mM L-Carnitine (7.67±0.33,75.40% vs 8.67±0.21,83.87%). The blastocyst rate of the COC's matured with supplementation of 3 mM L-Carnitine showed significantly ($p<0.05$) lower embryo production (4.17± 0.31,40.98% vs 5.83± 0.80,56.45%) in comparison to group supplemented with 1 mM L-Carnitine. So, the supplementation of 1 mM L-carnitine is suitable for Sahiwal oocytes during *in vitro* maturation for higher embryo production.

Keywords: Blastocyst, *In vitro* maturation, Lipid, L-carnitine, Ovum pick-up, Sahiwal

One of the most important tools for the rapid proliferation of genetically superior dairy cattle is embryo transfer technology. *In vitro* and *in vivo* embryo production are the two main techniques for producing embryos. *In vitro* embryo production (IVEP) is generally considered more efficient in terms of the number of embryos produced per unit time compared to traditional superovulation-based methods. The first and most pivotal step in IVEP is *in vitro* maturation (IVM) of oocytes (Singh *et al.* 2025a). Therefore, refining IVM culture conditions to more closely mimic the *in vivo* environment is vital for advancing IVEP and expanding its practical applications.

The lipid content of *in vitro* recovered oocytes is one of the important parameters that affect oocyte developmental competence. The lipid content of *in vitro* matured oocytes is higher than that of *in vivo* matured oocytes (Collado *et al.* 2017). Lipid beta-oxidation seems to be crucial for the nuclear maturation of oocytes, the ejection of the first polar body, and meiotic maturation to metaphase II stage. The lipid droplets serve as essential energy reservoirs during oocyte maturation and also contribute to membrane biosynthesis (Sturmey *et al.* 2009). Lipid droplets also positively influence developmental competence during *in vitro* culture (Aardema *et al.* 2011). Oocytes retrieved from slaughter house ovaries of Korean native cows, having higher lipid droplets were associated with higher embryo production rates (Jeong *et al.* 2009).

Beta-oxidation produces reactive oxygen species (ROS), which impair oocyte competency (Grupen, 2014). ROS are controlled by both enzymatic and non-enzymatic antioxidants. Oxidative stress results when ROS generation exceeds a cell's antioxidant capability, causing lipid peroxidation, DNA damage, and protein degradation. It has been demonstrated that when oocytes mature *in vitro*, they produce more ROS than when they mature *in vivo* (Zeng *et al.* 2014). Furthermore, compared to oocytes grown *in vivo*, those matured *in vitro* might be less able to mitigate the harmful consequences of elevated ROS (Uppangala *et al.* 2015). So, this could explain why IVM-derived oocytes have lower developmental competence.

Various approaches have been utilized to influence lipid accumulation, yet the outcomes have been variable

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(Sudano *et al.* 2013). Lipid modulators are compounds that impact lipid content, and incorporating lipid modulators such as L-carnitine, phenazine ethosulfate (PES), forskolin, and fatty acids (oleic acid, linoleic acid, and linolenic acid) into maturation media is anticipated to produce favourable results.

L-carnitine is recognized as a beneficial modulator of lipids, as research has shown that its supplementation can decrease lipid levels (Verma *et al.* 2018) and ROS in cattle (Ghanem *et al.* 2014, Kolodziejczyk *et al.* 2011). This quaternary amine plays a crucial role in cellular energy production by transporting long-chain free fatty acids from the cytosol to the mitochondria, which aids in their oxidation and production of adenosine triphosphate (Spricigo *et al.* 2017). *In vitro* assays have demonstrated the ability of L-carnitine to act as an effective scavenger of superoxide anions, hydrogen peroxide and inhibit lipid peroxidation (Gulcin, 2006). Cumulus cells and oocytes do not have the capacity to biosynthesize L-carnitine from precursor amino acids as they lack the metabolic machinery (Montjean*et al.* 2012).

The L-carnitine had been supplemented in maturation media with variable results but the earlier researches were conducted mostly on exotic breeds from the oocytes recovered from slaughterhouse ovaries but the research based on oocytes collected from Sahiwal cows via ovum pick-up is limited. The current research sought to assess the impact of L-Carnitine supplementation during *in vitro* maturation on the rate of blastocyst formation.

MATERIALS AND METHODS

The study was conducted on Sahiwal cows (n=3) maintained at Dairy Farm, Directorate Livestock Farms, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. The cows were screened for the normal genitalia and regular oestrus cycles. The chemicals utilized in this research were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA), unless indicated differently.

Ovum Pick-up (OPU): OPU was carried out under posterior epidural anaesthesia, which was accomplished with 3–4 ml of 2% lignocaine hydrochloride. Before commencing OPU, perineum region was cleaned thoroughly using soap and mopped with clean towel. An ultrasound machine (GE Healthcare) with a 7.5 MHz trans-vaginal sector transducer, encased in a non-metallic casing with a needle guiding system, was utilized to perform OPU. A 70 mm Hg vacuum pressure was produced using a Mini tube OPU vacuum pump (flow rate of 10–15 ml/min; Singh *et al.* 2016). An 18-gauge, 1.2 X 75 mm sterile needle, which was linked to a 50 ml centrifuge tube via 150 cm of 1 mm diameter silicon tubing, was used to aspirate the follicles. The needle was kept in a needle guider that was installed in a probe holder.

Ensuing follicle visualization and alignment in the needle line on the ultrasound machine's monitor, suction was used, and at the same time, the needle was pushed into surface follicles, aspirating all visible surface follicles ≥3 mm. The follicular fluid from the punctured follicles was collected in a 50 ml tube (one tube per animal) and kept in a thermostatically controlled digital dry bath that was kept at 37°C. Aspirated follicular contents were promptly transferred to the lab for oocyte screening, assessment, and additional processing as soon as they were collected. By keeping the tube in the WTA Pocket heater, proper precautions were taken to reduce temperature variations of the contents during handling. Within ten minutes, the aspirated follicular contents of each animal were filtered through WTA small filters, the contents of which were then transferred into a 90 mm petri dish and examined for oocytes using a Nikon SMZ-800N stereomicroscope. The retrieved oocytes were classified as A, B, C, and D based on the appearance of the cumulus mass and cytoplasm (Neglia *et al.* 2003).

In vitro maturation (IVM): The base IVM medium was freshly prepared (10 ml) and consisted of TCM-199 (9.5 ml), 5% (0.5 ml) fetal calf serum (FCS), 50 µg/10 ml luteinizing hormone (LH), 12.5 µg/10 mL follicle-stimulating hormone (FSH), 500µg/10 ml gentamicin with supplementation of 1 mM L-carnitine, for group 1. The maturation media for group 2 contained L-carnitine at a concentration of 3 mM along with other constituents mentioned in group 1. The group 3, acted as control in this experiment and the base medium was prepared similarly like group 1, without L- Carnitine supplementation. The freshly prepared media were filtered using syringe filter (0.2µm; Corning). Selected COCs were cultured in sterile petri plates (35mm) at the rate of 5µl maturation media per oocyte. A total of 6 replicates of 10-12 oocytes each for each group were executed in this study. The oocytes were cultured in a benchtop incubator (MIRI, ESCO Medical) maintained at 38.5^{0}C, with 6% CO_2 and high relative humidity for 22-24hours.

Maturation rate assessment: The degree to which the cumulus cell mass expanded was used to measure maturation. The oocytes were evaluated after maturity and categorized in accordance with Singh *et al.* (2021), as follows:

- Full cumulus expansion (Expanded)- fully expanded cumulus, loosened matrix three time away from zona pellucida
- Moderate cumulus expansion (Intermediate)- Cells are loosen, but not to that extant and two times diameter away from zona pellucida
- No expansion (Compact)- Cells are closely attached to zona and not loosen and no expansion. There is no evident change visible when compared with pre maturated oocyte

In vitro fertilization (IVF): The IVF was done with conventional semen samples having at least 50 percent progressive motility and 2 million/ml concentration per IVF drop. The sperm concentration was measured using Makler Chamber and the amount of sperm suspension to be added was calculated. Meanwhile when semen was being

prepared, the matured oocytes were washed in 5 drops of 100 µl pre-equilibrated IVF media (Stroebech, Prod. No. 1.06.020) and transferred into the pre-equilibrated IVF media drops. Once the sperms were ready and concentration was determined, the decided amount of sperm suspension was added in each fertilization drop quickly and IVF petri dish was kept back in CO_2 incubator maintained at maintained at 38.5^{0}C, with 5 per cent CO_2 and high relative humidity for *in vitro* fertilization for 18-20 hours.

Cleavage rate assessment: After 72 hours of IVF, the presumptive zygotes were checked for cleavage. The zygotes of all the drops were checked quickly under stereo zoom microscope (Nikon SMZ800N) for cleavage.

In vitro culture (IVC): The IVC drop of 50 µl IVC media for each group were prepared in 35x10 mm petri dish (Thermo Scientific) covered under mineral oil. *In vitro* culture (IVC; Stroebech, Prod. No. 1.07.020) media was equilibrated in mixed gas (5% CO_2, 5% O_2, 90% N_2, 38.5°C temperature and ≥90% RH) bench top incubator (Miri, ESCO-MEDICAL) overnight or at least 2 hours before IVC for stabilization of pH.

IVC was performed 18-20 hours after IVF. The 135 µm denuding pipette (Gynetics Medical Products N V, Rembert Dodoensstraat 513920 Lommel, Belgium) was fixed in denuding handle (EZ Grip,). Two drops of 200 µl and 400 µl of IVC media (Stroebech) were prepared in 60 mm petri dish and oocytes were placed in 200 µl drop from IVF drop. The presumptive zygotes were transferred from 200 to 400 µl drop and denuded slowly to remove all the cumulus cells. After all of the zygotes had been denuded, they were rinsed with 100 µl of wash media and then rinsed with five drops of IVC media that had been pre-equilibrated. Following washing, the zygotes were moved into IVC medium drops that had already been equilibrated, and the culture plate was put in a benchtop, mixed gas incubator.

Denuding was performed slowly and carefully to avoid rupture of zona pellucida due to pressure created by the denuding pipette. After 72 hours of IVC, 20 µl IVC media was withdrawn from each drop and freshly equilibrated 20 µl of IVC media was added to each drop.

Evaluation of embryos: The developing embryos were evaluated for stage on day 7 from IVF. The evaluation of stage of embryos was performed as per International Embryo Transfer Society (IETS) guidelines (Manual of the IETS, 5th Edition).

Statistical analysis: The statistical analysis was carried out in MS Office Professional plus with data analysis tool using one-way analysis of variance (ANOVA) followed by post hoc analysis. When ANOVA revealed significant differences (p-value < 0.05), values were compared by pairwise multiple comparison post hoc analysis. Bonferroni correction was applied to reduce the risk of false positive. The data was presented as mean ± standard error and p <0.05 was considered significant.

RESULTS AND DISCUSSION

A total of 326 follicles (Mean± Std. Error; 18.11±0.71 per OPU session) were aspirated out of which 228 (12.67±0.53 per OPU session) COCs were collected at a recovery rate of 69.93%. Bhardwaj (2022) aspirated 20.40±2.25 follicles and retrieved 13.06±2.52 COC's per OPU session at a recovery rate of 64.01% in Sahiwal cattle. The oocyte recovery rate in Sahiwal cattle was found to be 67.43% by Singh *et al.* (2025b) and 72% by Preeti *et al.* (2025). Gutierrez *et al.* (2021), visualized and punctured 12.3±1.14 follicles and 7.09±0.72 oocyte complexes were retrieved and corresponded to 68.3% recovery rate in dairy cattle. A higher oocyte recovery rate of 74.9% had been reported in Murrah buffaloes (Verma *et al.* 2026).

Out of 228 COCs, 48 (21.05%) were of Grade A, 55 (24.12%) were of Grade B, 85 (37.28%) were of Grade C and 40 (17.54%) were of Grade D. COCs belonging to Grade D were discarded and were not included in the study. Singh *et al.* (2025b), using a vacuum of 70 mmHg, observed a very similar distribution, with 15.85% Grade A, 22.26% Grade B, the majority in Grade C, 39.62% and Grade D, 22.26% in Sahiwal cattle. Preeti *et al.* (2025) reported 77.42% culturable and 22.58% non-culturable oocytes in Sahiwal cows. Similar to our results, Sandhu *et al.* (2025) reported a discard rate of 22.58% in Sahiwal cattle oocytes.

Table 1, shows how the maturation rate of bovine oocytes (Fig. 1) was affected by the addition of 1 mM and 3 mM L-carnitine to maturation media. According to the findings, *in vitro* maturation of the COC's supplementation with 3mM L-Carnitine showed (n, %) significantly (p<0.05)

Table 1. Cumulus expansion of the COCs matured in media supplemented with 1mM L-carnitine, 3 mM L-carnitine and control

Group	Replicates	Total COCs (n)	Cumulus Cell Expansion		
			A Full (n) (%)	B Partial (n) (%)	C No (n) (%)
Group 1 (L-carnitine 1 mM)	6	64	58 (90.62)^{a}	4 (6.25)^{b}	2 (3.12)^{b}
Group 2 (L-carnitine 3 mM)	6	64	48 (75.00)^{b}	10 (15.62)^{a}	6 (9.37)^{a}
Group 3 (Control)	6	60	53 (88.33)^{ab}	5 (8.33)^{b}	2 (3.33)^{b}

Values marked with superscript (^{ab}) in a same column differ significantly at 5% level.

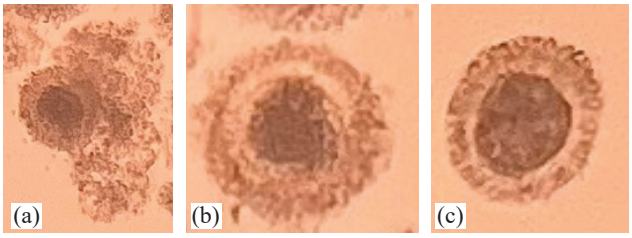

Fig. 1. Representative images of cumulus–oocyte complexes (COCs) after *in vitro* maturation, showing full expansion [1(a)], partial expansion [1(b)], and no expansion [1(c)] under a stereo zoom microscope at 80× magnification.

lower complete expansion (Fig 1a) of cumulus cells (48, 75.00) in comparison to maturation media supplemented with 1 mM L-carnitine (58, 90.62). Complete cumulus cell expansion was found to be similar in 1 mM L-carnitine group and the control group. *In vitro* maturation of COC's supplemented with 3 mM L-Carnitine revealed significantly reduced complete cumulus cell expansion (8.00±0.36 vs 10.10±0.94) compared to control in Sahiwal cattle oocytes (Singh *et al.* 2025c).

COC's showing intermediate expansion (Fig. 1b) were significantly higher ($p<0.05$) in maturation media (n, %) supplemented with 3mM L-Carnitine (10, 15.62) in comparison to 1 mM L-carnitine group (4, 6.25) and the control group (5, 8.33). Comparing the 3 mM L-carnitine group (6, 9.37) to the 1 mM L-carnitine (2, 3.12) and control group (2, 3.33), COCs exhibiting no expansion (Fig 1c) were also considerably high ($p<0.05$). It showed that in maturation medium supplemented with 3 mM L-Carnitine, a notably high number of COCs were unable to achieve full cumulus expansion. In comparison to control, COCs exhibiting intermediate expansion were significantly higher in maturation media supplemented with 3 mM L-Carnitine in Sahiwal cattle oocytes and a significant number of COCs were unable to achieve complete cumulus expansion in maturation media supplemented with 3 mM L-Carnitine (Singh *et al.* 2025c). Sandhu *et al.* (2025) reported similar expansion rate (5.48%) of COCs in the group supplemented with 5% FCS, used as a control in our study.

L-carnitine supplementation in maturation media has produced a range of outcomes. According to Chankitisakul *et al.* (2013), adding 0.6 mg/ml (3.03 mM) L-carnitine to maturation media had no effect on the maturation rate (metaphase II) in bovines. In another study, L-carnitine (3.8 mM) was supplemented in maturation media for the maturation of bovine oocytes retrieved from slaughterhouse ovaries and it was observed that the cumulus cell expansion was significantly higher in 3.8 mM L-carnitine group in comparison to the control group (Gonzalez *et al.* 2019). A significantly higher maturation rate of bovine oocytes retrieved from slaughter house ovaries was obtained for the groups supplemented with 0.3 (1.8 mM) and 0.6 (3.8 mM) mg/mL L- carnitine in comparison to control (Phongnimitr *et al.* 2013).

The effect of supplementation of 1 mM and 3mM L-Carnitine in maturation media on the cleavage and blastocyst rate is presented in Table 2. The cleavage rate (Fig. 2) of the COC's matured with supplementation of 3mM L-Carnitine showed significantly ($p<0.05$) lower cleavage (7.67±0.33, 75.40 %) in comparison to group supplemented with 1 mM L-Carnitine (8.67 ±0.21, 83.87 %). The control group's cleavage rate was found to be similar to that of the 1 mM and 3 mM L-carnitine groups. The blastocyst rate (Fig. 3) of the COC's matured with supplementation of 3 mM L-Carnitine showed significantly ($p<0.05$) lower embryo production (4.17±0.31, 40.98) in

Table 2. Cleavage and Blastocyst rate of oocytes matured in media supplemented with 1 mM L-carnitine, 3 mM L-carnitine and control in Sahiwal cows

Group	Replicates	COCs Matured (a) (n) (Mean±SE)	Oocytes Fertilized (IVF) (b) (n) (Mean±SE)	Oocytes Cultured (IVC) (c) (n) (Mean±SE)	Cleavage Rate (d) (n) (Mean±SE) (%)	Blastocyst rate (Day 7) (e) (n) (Mean±SE) (%)
Group 1 (L-carnitine 1 mM)	6	64 (10.67± 0.21)	63 (10.50± 0.22)	62 (10.33± 0.21)	52 (8.67± 0.21) (83.87) [a]	35 (5.83± 0.80) (56.45) [a]
Group 2 (L-carnitine 3 mM)	6	64 (10.67± 0.21)	63 (10.50± 0.22)	61 (10.16± 0.17)	46 (7.67± 0.33) (75.40) [b]	25 (4.17± 0.31) (40.98) [b]
Group 3 (Control)	6	60 (10.00± 0.00)	60 (10.00± 0.00)	59 (9.83± 0.17)	48 (8.00± 0.26) (81.35) [ab]	31 (5.17± 0.40) (52.54) [ab]

a= Total COCs matured in each group; b= COCs fertilized out of a; c=COCs Cultured out of b; d=Cleavage Rate out of c (%= n/c*100); e= Blastocyst rate (n/c*100); Values marked with superscript ([ab]) in a same column differ significantly at 5% level; Note: Values in parenthesis are percentages

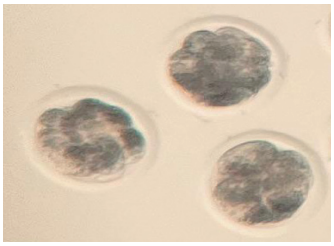

Fig. 2. Representative image of cleaved oocytes after *in vitro* fertilization

Fig. 3. Representative image of blastocyst

comparison to group supplemented with 1 mM L-Carnitine (5.83±0.80, 56.45). It was found that the control group's blastocyst rate was comparable to that of the 1 mM and 3 mM L-carnitine groups.

The cleavage rate and the blastocyst rate were found to be similar in 3.8 mM L-carnitine supplemented group and the control group in bovines (Gonzalez *et al.* 2019). In bovine oocytes, L-carnitine supplementation (3.03 mM) during maturation had no influence on embryo development (Zolini *et al.* 2019). When 3 mM L-Carnitine was added to the maturation media, the cleavage rate decreased non significantly (7.90±0.41 vs 9.00±0.88) compared to the control in Sahiwal cattle oocytes (Singh *et al.* 2025c).

In contrast, swamp buffalo oocytes supplemented with 3.8 mM L-carnitine yielded significantly higher embryos compared to control (Liang *et al.* 2020). According to the findings of Knitlova *et al.* (2017), supplementing oocytes recovered from slaughtered Holstein dairy cows with L-carnitine (2.5 mM) during maturation enhances their development to blastocyst formation. Blastocyst rate was significantly higher in 1.5mM L- carnitine group than 1.0 mM group and control in buffalo oocytes obtained from slaughter house ovaries (Verma *et al.* 2018). Significantly, higher blastocyst rate was observed in buffalo oocytes with maturation media containing 1 mM L-carnitine compared to control (Sokary *et al.* 2021). Limited study is present on Sahiwal cow oocytes developmental competence with the supplementation of L-carnitine in the maturation media.

In comparison to supplementing with 1 mM L-carnitine, it can be determined that adding 3 mM L-carnitine to the maturation media results in a notable reduction in embryo formation. So, the supplementation of 1 mM L-carnitine is suitable for Sahiwal oocytes during *in vitro* maturation for higher embryo production.

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