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# Supplementing α-Linolenic acid in the *in vitro* maturation media improves nuclear maturation rate of oocytes and early embryonic development in the Nili Ravi buffalo

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## Abstract

The present study was conducted to investigate the effect of omega-3 poly unsaturated fatty acid (PUFA), α-linolenic acid (ALA; 18:3 n-3) on the *in vitro* maturation (IVM) of buffalo oocytes and subsequent embryonic development. Buffalo cumulus-oocyte complexes (COCs; n = 2282) were *in vitro* matured in TCM-199 (0.6% fatty acid free bovine serum albumin, 0.02 Units/ml FSH, 1 µg/ml 17-β-estradiol, 10 µg/ml epidermal growth factor, 50 µg/ml gentamicin) supplemented with 0 (control), 25, 50, 100, 150 or 300 µm ALA under an atmosphere of 5% $CO_2$ in air at 38.5ºC for 22-24 h. The matured oocytes were then fertilized in Tyrode's Albumin Lactate Pyruvate (TALP) medium and cultured in synthetic oviductal fluid (SOF) medium. Concentrations up to 100 µm ALA improves ($P \leq 0.05$) the cumulus expansion compared to control. Higher percentage of oocytes reaching MII stage was observed at 50 µm and 100 µm of ALA compared to control ($P \leq 0.05$). Concentrations of 150 and 300 µm ALA were detrimental both for cumulus expansion and nuclear maturation rate of buffalo oocytes. Moreover, supplementation with 100 µm ALA improved ($P \leq 0.05$) cleavage rate compared to control and treatment with 50 and 100 µm ALA yielded significantly higher morulae compared to control. The results of present study indicate that the supplementation with 100 µm ALA to the IVM medium improves nuclear maturation rate of buffalo oocytes and subsequent early embryonic development.

**Keywords**: α-linolenic acid, buffalo, embryonic development, nuclear maturation, omega-3 PUFAs.

## Introduction

The Nili-Ravi buffalo is considered to be one of the best milk producers among other breeds of buffaloes in the world (Warriach *et al*., 2008). Although, this breed has the potential of producing more than 5000 L of milk/lactation (Bilal *et al*., 2006), the average milk yield is quite low, which raises the opportunities of genetic improvement through assisted reproductive technologies like artificial insemination, and multiple ovulation and embryo transfer (MOET). However, the implementation of these technologies is hampered due to some inherent reproductive problems in buffalo such as fewer primordial follicles, which results in smaller number of recruitable follicles, high level of atresia, poor estrus behavior detection and a poor response to superovulation protocols (Madan, 1990). Recently, the emphasis has now been shifted towards the *in vitro* production (IVP) of buffalo embryos (Hansen, 2006) utilizing germ plasm of both male and female animals simultaneously (Barakat *et al*., 2012).

*In vitro* maturation (IVM) is a key step during which oocytes undergo all the necessary changes required for successful fertilization and further embryonic development (Wang *et al*., 1997). Therefore, the maturation medium is crucial, not only for the maturation of oocyte itself but also for its further competence after IVF (Bavister *et al*., 1992; Kharche *et al*., 2006). Previously, several studies have evaluated different types of media (Totey *et al*., 1993; Abdoon *et al*., 2001; Zicarelli *et al*., 2003), and its supplements including different sources of protein (Chauhan *et al*., 1998), hormones (Totey *et al*., 1993), thiol compounds (Zicarelli *et al*., 2005; Gasparrini *et al*., 2006), growth factors (Purohit *et al*., 2005) and antioxidants (Ullah *et al*., 2006) for *in vitro* maturation of buffalo oocytes with variable success rates and blastocyst production rate of no more than 10-20% (Kumar and Anand, 2012) which is relatively lower when compared to 30-40% blastocyst production rate in cattle (Rizos *et al*., 2008).

Fatty acids are essential for numerous physiological functions (Wathes *et al*., 2007), such as membrane biosynthesis (Sturmey *et al*., 2009), signal transduction and gene expression (Sampath and Ntambi, 2005). They also provide a potent energy source and prevent the lipotoxic effects result in from increased cellular contents of saturated fatty acids through lipid storage and β-oxidation (Dunning *et al*., 2014). Fatty acids are also precursors for prostaglandins and progesterone synthesis and therefore play an important role in the regulation of normal reproductive function (Abayasekara and Wathes, 1999; Mattos *et al*., 2000). Polyunsaturated fatty acids (PUFAs) play a significant role in increasing the number (Lucy *et al*., 1991) and size of ovarian follicles (Zeron *et al*., 2002), level of LH (Lucy *et al*., 1991) and progesterone in follicular fluid

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(Ryan *et al*., 1992), oocyte quality (Zeron *et al*., 2002), regulation of ovulation, CL function(Abayasekara and Wathes, 1999; Mattos *et al*., 2000) and pregnancy rate (Bellows *et al*., 1999).

PUFAs must be provided by diet, since their *in vivo* synthesis is not possible due to absence of proper enzymes (Gurr *et al*., 2002). Change in the composition of dietary fatty acids not only modifies fatty acid composition in the blood plasma but also of the reproductive tissues including, follicular fluid, cumulus cells and the oocytes (Ferguson and Leese, 1999; Zeron *et al*., 2002; Bilby *et al*., 2006; Childs *et al*., 2008; Fouladi-Nashta *et al*., 2009; Wonnacott *et al*., 2010), which can directly influence the competence of oocytes for further development and/or fertility (Armstrong *et al*., 1990; Burke *et al*., 1997; Petit *et al*., 2001; Wonnacott *et al*., 2010). For instance, dietary fish oil supplementation has been shown to alter the specific n-3 and n-6 fatty acids in follicular fluid, cumulus cells and oocytes in both beef heifers (Childs *et al*., 2008) and ewes (Zeron *et al*., 2002; Wonnacott *et al*., 2010). Such changes in the fatty acid profile may improve oocyte maturation, which is essential for successful fertilization and further embryo development (Marei *et al*., 2009) Fatty acids supplemented into IVM medium yielded positive effects on oocyte maturation, fertilization, and embryonic development in the rat (Khandoker and Tsujii, 1999), goat, sheep (Veshkini *et al*., 2015) and cattle (Kim *et al*., 2001).

Alpha-linolenic acid (ALA; C18:3) is the dietary precursor for the long-chain omega-3 PUFAs (Brenna *et al*., 2009). It plays an important role in folliculogenesis, developmental competence of oocyte (Moallem *et al*., 2013), fertilization rate and embryo quality (Thangavelu *et al*., 2007). As ALA is produced by the ovarian follicles and the amount of ALA increases as the ovarian follicles enlarge (Veshkini *et al*., 2015); a role of specific unsaturated fatty acids like ALA may be speculated in the oocyte maturation and/or follicular growth. In fact, ALA has been shown to improve the fertility rate in both cattle and sheep by improving folliculogenesis and fertilization rate *in vivo* (Moallem *et al*., 2013). When supplemented in the *in vitro* maturation medium it was reported to regulate the molecular mechanism leading to increased number of MII stage oocytes and improved their subsequent development into early embryos in both cattle (Fouladi-Nashta *et al*., 2009; Marei *et al*., 2009) and sheep (Ghaffarilaleh *et al*., 2014). However, the role of ALA supplementation of IVM media for Nili Ravi buffalo oocytes has not been studied yet. In this study we hypothesized that the supplementation of ALA in IVM media improves the quality of *in vitro* matured buffalo oocytes and their subsequent development post-fertilization. The main objective of the study was to evaluate the effects of ALA supplementation of the IVM media on the *in vitro* oocyte maturation and subsequent embryonic development in buffalo.

## Materials and Methods

*Collection of oocytes from buffalo ovaries*

Buffalo ovaries (n = 2500) were collected from the local abattoir and within two hours of collection transferred to the laboratory in a thermos containing sterilized phosphate buffered saline (PBS) kept at 33-35°C. Fresh PBS was used to wash the ovaries immediately after arrival. Sterile disposable plastic syringe (10 ml) fitted with 18 gauge needle was used to aspire cumulus-oocyte complexes (COCs) from antral follicles (2-8 mm). Searching for COCs was done under stereomicroscope in PBS and was classified as grade A, B, C and D, on the basis of their cumulus investment and ooplasm homogeneity (Sabasthin *et al*., 2013). Grade A COCs had four or more than four compact cumulus layers with homogenous ooplasm, while grade B oocytes had two to three layers of compact cells with homogenous ooplasm. Grade C oocytes had only one layer of less compact cells with irregular ooplasm, while the oocytes having irregular dark ooplasm and highly expanded cumulus cells and denuded oocytes were considered as grade D (Sabasthin *et al*., 2013). COCs (n = 2282) with homogenous ooplasm and more than two compact layers of cumulus cells were selected for the experiments.

In vitro *maturation (IVM) of oocytes*

COCs were *in vitro* matured in 100 µl drops covered with sterile mineral oil for 24 h at 38.5ºC in 5% $CO_2$ in air with 95% humidity. All the media and culture dishes were equilibrated at 38°C in $CO_2$ incubator for at least 1-2 h before experiment.

*Assessment of cumulus cell expansion*

After 24 h of maturation, cumulus cell expansion was assessed by visual assessment using stereomicroscope as 1) not expanded (no expansion observed in cumulus cells), 2) partially expanded (expansion of outer layers of cumulus cells only), or 3) fully expanded (expansion of all layers of cumulus cells; Azam *et al*., 2017).

*Oocyte staining and determination of stage of nuclear maturation*

For determination of nuclear stage in meiosis, COCs were completely denuded. Denuded oocytes were washed twice in PBS and fixed overnight in aceto-ethanol. Oocytes were placed on a grease free glass slide and covered with a cover slip. Oocytes were slightly compressed onto the glass slide with a needle until drop containing oocytes touched the cover slip. The oocytes were then stained with 1% aceto-orcein and de-stained with aceto-glycerol as described by Azam *et al*. (2017). Oocyte's nuclear maturation was evaluated with a phase contrast microscope at 200X to 400X magnification.

Based on morphology, nuclear maturation was categorized as described by Azam *et al.* (2017). Oocyte nucleus with a nucleolus and filamentous chromatin is at germinal vesicle (GV) stage. Oocyte without nucleolus, nuclear membrane and shortening of chromosomes is at germinal vesicle breakdown (GVBD) stage. At MI stage chromosomes look as thick

dots arranged at metaphase plate. The segregation of chromosomes start at anaphase-I and at Telophase-I, complete segregation of chromosomal sets occur. First polar body is released at M-II.

In vitro *fertilization (IVF) of oocytes*

Frozen semen was used for *in vitro* fertilization (IVF). Three 0.5 ml straws of cryopreserved buffalo semen were thawed in water bath at 37°C for 30 sec. Thawed semen was placed in a 15 ml conical tube. Spermatozoa with maximum motility were collected by swim up technique (Parrish *et al*., 1986). About 250 µl of thawed semen was deposited at the bottom of four 15 ml tubes containing 3 ml of pre warmed sperm wash medium (TALP: modified calcium-free Tyrode's Albumin Lactate Pyruvate with 6 mg/ml BSA fraction-V). Tubes were incubated at 45° angle for 30 min. Supernatant from each tube was removed and transferred into another 15 ml conical tube and centrifuged at 1600 rpm for 10 min. The pellet obtained after centrifugation of supernatant was assessed for sperm motility, and concentration was determined in an improved Neubauer counting chamber. Subsequently, sperm suspention was resuspended in pre-warmed fertilization TALP (supplemented with 0.1mM hypotaurine, 0.2mM penicillamine, 0.01mM epinephrine, 10 µg/ml heparin) to a final concentration of 2 x $10^6$ cells $ml^{-1}$.

After 24 h of IVM, buffalo oocytes were washed in fertilization media and were placed in fertilization droplets (5 COCs/50 µl droplet) of pre warmed fertilization media under mineral oil. Oocytes and spermatozoa were co-incubated for 20 h at 38.5°C under 5% $CO_2$ with maximum humidity (Gasparrini *et al*., 2008). The day of fertilization was defined as day 0.

In vitro *culture (IVC) of embryos*

Following fertilization, presumptive zygotes were denuded by vigorous pipetting in PBS and transferred to 25 µl drops containing IVC media (SOF) supplemented with BME, MEM and 5% fetal calf serum (FCS). Embryo culture was performed at 38.5°C in a humidified incubator with 5% $CO_2$ in air. On day 2 the cleavage rate (number of oocytes cleaved/total COCs incubated × 100) was evaluated. Further developmental stages (4-8 cell stage, >8 cell stage and morula) were evaluated and recorded every other day.

*Experimental design*

Linolenic acid (ALA; Stock solution in DMSO) was added at different levels (0 µm (control), 25, 50, 100, 150, 300 µm) to the maturation medium (TCM-199) supplemented with 0.6% fatty acid free-bovine serum albumin (FAF-BSA), 0.02 IU/ml FSH, 1 µg/ml estradiol-17β (E2), 10 µg/mL epidermal growth factor and 50 µg/ml gentamicin.

*Experiment 1*

Oocytes (1200) were randomly allocated to the experimental groups as described: 1) 0 µm ALA (control); 2) 25 µm ALA (25); 3) 50 µm ALA (50); 4) 100 µm ALA (100); 5) 150 µm ALA (150); or 6) 300 µm ALA (300). The experiment was replicated for seven times. Cumulus cells expansion and nuclear maturation status of oocytes after 24 h of IVM were evaluated.

*Experiment 2*

Oocytes (1082) were matured as in the experiment 1. Matured oocytes were fertilized and cultured. The experiment was replicated seven times. Embryonic development was assessed as cleaved, 4-8 cell stage, >8 cell stage and morula formation.

*Statistical analysis*

The data on the effect of different doses of ALA on COCs expansion, MII stage, cleavage rate and developmental stages (2 cell stage, 4-8 cell stage, >8 cell stage and morula) were analyzed by one-way analysis of variance (ANOVA) after square root transformation ($Y = \sqrt{X+0.5}$) and presented as Mean ± SEM. Duncan's multiple range test (DMRT) was used to compare the treatment means. Results were considered significant at $P < 0.05$.

## Results

*Experiment 1*

The data on the effect of ALA supplementation of maturation media on cumulus expansion are shown in Fig. 1 and 2. The addition of 25 µm ALA in maturation media did not show improvement ($P > 0.05$) in cumulus expansion compared to control. However, supplementation with 50 and 100 µm ALA resulted in increased rate of cumulus expansion ($P \leq 0.05$ ) compared to control. The higher levels of ALA, *i.e.,* 150 and 300 µm resulted in significant decrease ($P < 0.05$) in expansion of cumulus cells compared to other groups (Fig. 1 and 2).

Data of the effects of ALA supplementation in maturation media on percentage of oocytes at different stages of nuclear maturation are shown in Fig. 3. The higher percentage of oocytes reaching MII stage ($P \leq 0.05$) was observed with 50 µm ALA (67.0% ± 2.2) compared to control (57.4% ± 2.5) and 25 µm ALA (60.3% ± 1.9) in IVM medium. Further improvement ($P \leq 0.05$) was observed with 100 µm ALA (76.3% ± 2.5). The higher concentrations of ALA i.e., 150 µm (51.3% ± 1.8) and 300 µm (29.8% ± 2.3) resulted in a dose dependent decrease in percentage of oocytes reaching MII stage compared to 100 µm ALA. The percentage of oocytes remaining at GVBD and MI stage were recorded higher in 300 µm ALA (26.2% ± 2.2 and 39.1% ± 1.4) compared to control (17.7% ± 3.1 and 20.7% ± 2.4), respectively. Percentage of degenerative oocytes was higher ($P < 0.05$) with 300 µm ALA (4.9% ± 2.5) compared to control, 25 µm ALA and 150 µm ALA (1.9% ± 1.2, 1.8% ± 1.2, 1.0% ± 1.0) respectively. No degenerative oocytes were observed with 50 µm ALA and 100 µm ALA.

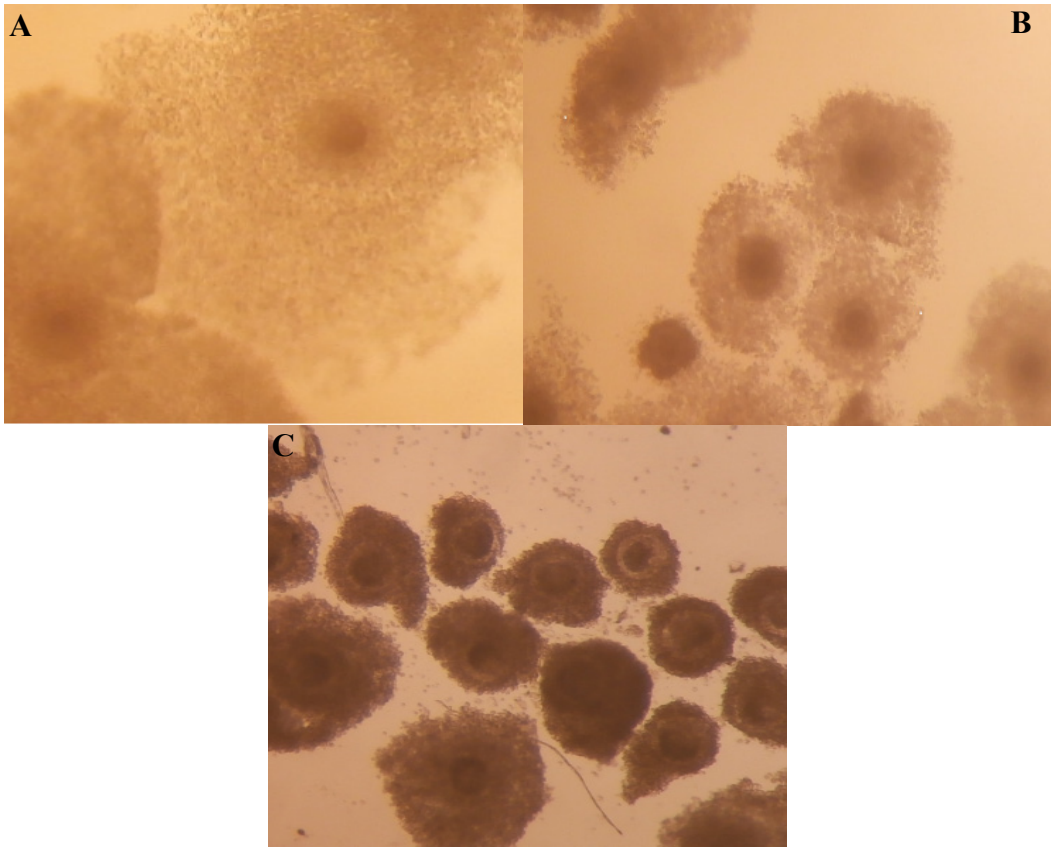


Figure 1. Representative pictures of COCs (A: fully expanded, B: partially expanded, C: not expanded) after 24 h of maturation.

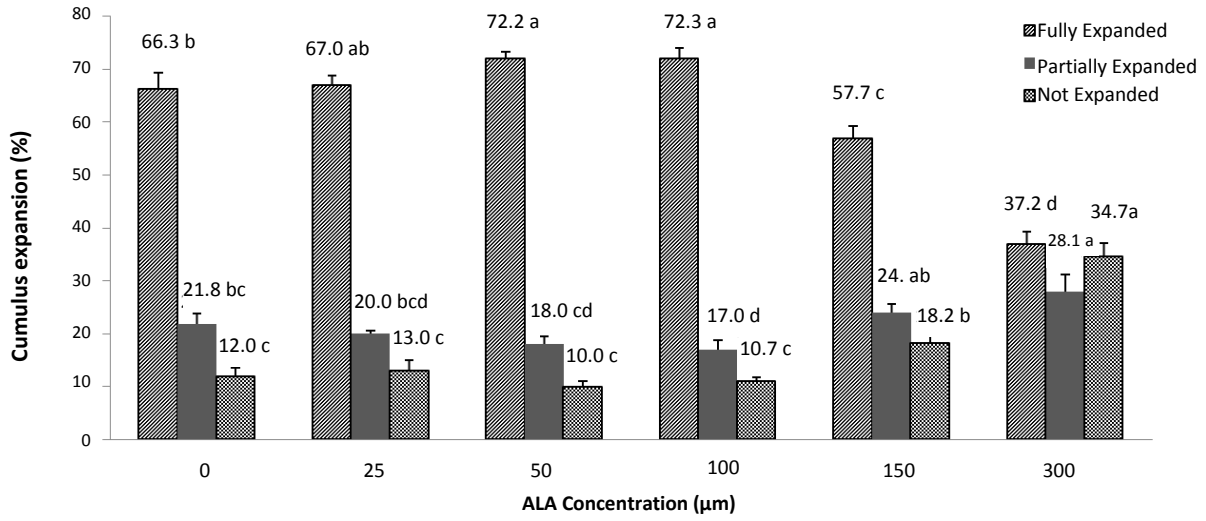


Figure 2. Cumulus expansion rate of buffalo COCs after 24 h of maturation in IVM medium supplemented with different concentrations of ALA. Data are shown as Mean percentage ± SEM. Bars with different superscripts differ significantly ($P < 0.05$). 0 (Control), 25 (supplementation with 25 µm ALA in the maturation media), 50 (supplementation with 50 µm ALA in the maturation media), 100 (supplementation with 100 µm ALA in the maturation media), 150 (supplementation with 150 µm ALA in the maturation media), 300 (supplementation with 300 µm ALA in the maturation media).

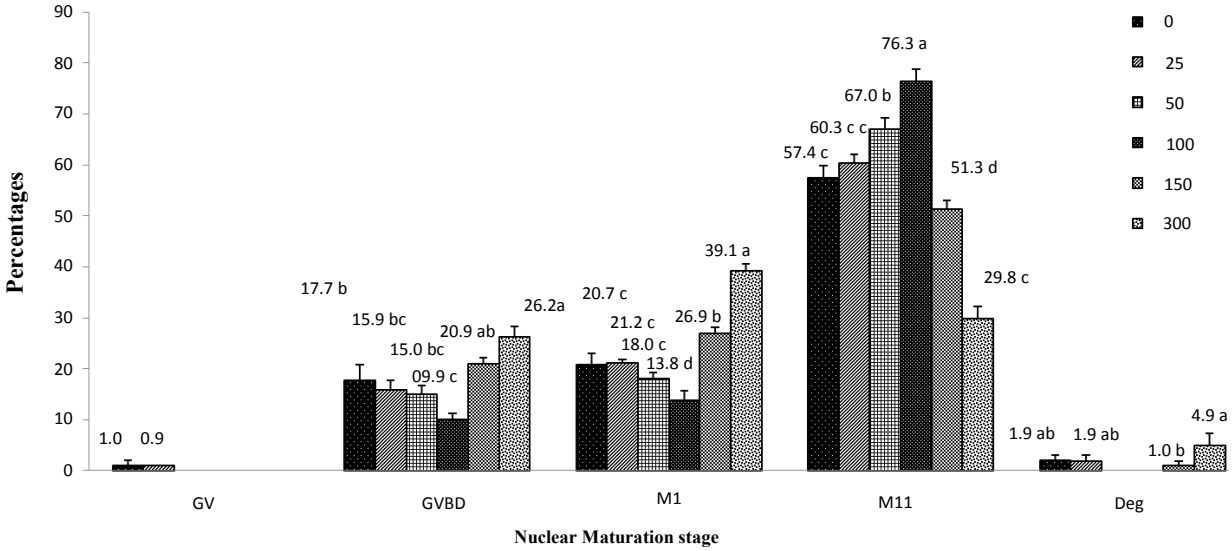


Figure 3**.** Nuclear maturation of buffalo oocytes after 24 h of maturation in IVM medium supplemented with different concentrations of ALA. Data are shown as Mean percentage ± SEM. Bars with different superscripts differ significantly ($P < 0.05$). GV: germinal vesicle, GVBD: germinal vesicle breakdown, MI: metaphase I, MII: metaphase II, Deg: degenerated.

*Experiment 2*

Data on the effect of ALA supplementation on percentage of cleaved, 4-8 cells, >8 cells and morula are presented in Table 1. Supplementation with 25, 50 and 150 µm ALA (49.7 to 56.9%) did not improve ($P > 0.05$) the cleavage rate compared to the controls (50.4% ± 2.0). The addition of 100 µm ALA (57.8% ± 1.0) raised ($P < 0.05$) the cleavage rate compared to the controls (50.4% ± 2.0) and 150 µm ALA (49.7% ± 2.8), but did not do so when compared to 25 (51.1% ± 2.5) and 50 µm ALA (56.9% ± 2.0). A significant decrease ($P < 0.05$) in cleavage rate was recorded when maturation media was supplemented with 300 µm ALA (21.0% ± 2.5).

Embryos reaching 4-8 cell stage were higher ($P \leq 0.05$ ) when the oocytes were matured in the presence of ALA at 100 µm (47.0% ± 2.9) compared to control (34.0% ± 1.1) and to 25 µm ALA (36.6% ± 3.8). At 50 µm ALA (42.3% ± 1.5) embryos developed up to 4-8 cell stage differ significantly ($P < 0.05$) with control and to 150 µm ALA (31.2% ± 0.8), while did not differ ($P > 0.05$) from 25 and 100 µm. The higher concentration used in this study, 300 µm (14.3% ± 1.8) resulted in the worst decrease ($P \leq 0.05$) on number of 4-8 cell embryos.

Recorded number of embryos that reached more than 8 cell stage was higher ($P \leq 0.05$) when the oocytes were matured in media supplemented with 100 µm ALA (38.0% ± 2.2) followed by 50 µm ALA (31.1% ± 1.6) and by control (22.2% ± 2.9) and 25 µm ALA (23.1% ± 2.4). The increase in ALA supplementation up to 150 µm (14.4% ± 1.8) and 300 µm (1.2% ± 1.2) compromised ($P \leq 0.05$) the embryo development to >8 cell embryos compared to other groups.

The percentage of morulae was increased ($P < 0.05$) with the supplementation of 50 µm ALA (17.8% ± 2.3) and 100 µm (21.0% ± 2.6) compared to control (11.2% ± 2.6) and 25 µm ALA (12.1% ± 2.1), while 150 µm ALA (7.8% ± 0.2) in maturation media resulted in decreased ($P \leq 0.05$ ) number of morulae. None of the embryos developed to morula stage ($P > 0.0$) when the oocytes were matured with 300 µm ALA.

Table 1. Effect of supplementation of IVM medium with different concentrations of ALA on the cleavage rate and subsequent embryo development of buffalo oocytes after *in vitro* fertilization.

| Treatments | Developmental stages (Mean percentage ± SEM) | | | | |
|---|---|---|---|---|---|
| | No. of COCs | Cleavage % ± SEM | -8 cell embryos % ± SEM | >8 cell embryos % ± SEM | Morulae % ± SEM |
| 0 | 182 | 92 (50.4 ± 2.0)$^{b}$ | 62 (34.0 ± 1.1)$^{c}$ | 40 (22.2 ± 2.9)$^{c}$ | 20 (11.2 ±2.6)$^{b}$ |
| 25 | 180 | 92 (51.1 ± 2.5)$^{ab}$ | 66 (36.6 ± 3.8)$^{bc}$ | 42 (23.1 ± 2.4)$^{c}$ | 22 (12.1 ± 2.1)$^{b}$ |
| 50 | 180 | 102 (56.9 ± 2.0)$^{ab}$ | 76 (42.3 ± 1.5)$^{ab}$ | 56 (31.1 ± 1.6)$^{b}$ | 32 (17.8 ± 2.3)$^{a}$ |
| 100 | 180 | 104 (57.8 ± 1.0)$^{a}$ | 84 (47.0 ± 2.9)$^{a}$ | 68 (38.0 ± 2.2)$^{a}$ | 38 (21.0 ± 2.6)$^{a}$ |
| 150 | 180 | 90 (49.7 ± 2.8)$^{b}$ | 56 (31.2 ± 0.8)$^{c}$ | 26 (14.4 ± 1.8)$^{d}$ | 14 (7.8 ± 0.2)$^{b}$ |
| 300 | 180 | 38 (21.0 ± 2.5)$^{c}$ | 26 (14.3 ± 1.8)$^{d}$ | 2 (1.2± 1.2)$^{e}$ | 0 (0.0 ± 0.0)$^{c}$ |

Data were collected in seven independent replicates. $^{a,b,c,d,e}$The values with different superscripts in the same column differ significantly ($P < 0.05$). 0 (Control), 25 (supplementation with 25 µM ALA in the maturation media), 50 (supplementation with 50 µm ALA in the maturation media), 100 (supplementation with 100 µm ALA in the maturation media), 150 (supplementation with 150 µm ALA in the maturation media), 300 (supplementation with 300 µm ALA in the maturation media).

## Discussion

The fatty acid content of developing oocytes changes depending on the environment in which they develop (Wakefield *et al*., 2008). Changing the micro-environment of oocyte also changes intra-cytoplasmic concentration of lipids. Therefore, PUFAs in the micro-environment may replace saturated FAs in the oocyte cytoplasm and make the oocyte membrane comparatively more permeable for sperm entry and increase fertilization rate and developmental competence of the oocyte. Present study shows that supplementation of maturation media with 100 µm ALA increased the number of fully expanded oocytes. Supporting our current findings, a previous study (Marei *et al*., 2009) demonstrated that supplementation of maturation medium with ALA at a concentration of lower than 100 µm had no adverse effect on cumulus cell expansion in cattle. The deleterious effect of ALA at a concentration of 150 and 300 µm in the present study is consistent with the previous studies (Coyral-Castel *et al*., 2010; Ghaffarilaleh *et al*., 2014; Veshkini *et al*., 2015) that reported decreased viability of granulosa cells with higher concentration of ALA after 24 h of maturation. In the present study, high levels of ALA in maturation medium might have been outside the tolerance threshold of the cells, exerted toxic effect leading to a reduction in cell viability (Andrade *et al*., 2005).

Cumulus cells are important for keeping the oocytes under meiotic arrest, inducing meiotic resumption and supporting cytoplasmic maturation. These functions have been attributed to their gap junctions and their specific metabolizing capabilities (Tanghe *et al*., 2002). It is anticipated that cumulus cells mediate signals and regulate the synthesis of important cytoplasmic factors which support nuclear maturation (Albertini *et al*., 2001). However, it is not fully understood that expansion of cumulus cells is prerequisite for nuclear maturation or both processes are not inter-related. It has been reported in the ovine that cumulus cell expansion is prerequisite for nuclear maturation (Amini *et al*., 2016), at the same time no such connection has also been reported in the bovine (Marei *et al*., 2010; Khalil *et al*., 2013).

Meiotic competence of oocytes determines the developmental capacity for subsequent embryo development. In the present study, supplementation of maturation medium with 100 µm ALA increased the MII rate of buffalo oocytes. High doses of ALA in IVM medium inhibited maturation progression, manifested by high percentage of oocytes arrested at GVBD or MI stage and a concomitant decrease in percentage of oocytes that completed second meiotic division metaphase. Our results are in agreement with the results of Veshkini *et al*. (2015) who reported improved nuclear maturation in sheep oocytes at 100 µm ALA in maturation medium. The positive effect of ALA on oocyte's meiotic competence may be mediated directly by improvement of cytoplasmic maturation via the mitogen-activated protein kinase pathway and indirectly through PGE2 synthesis (Marei *et al*., 2009). The PGE2 is a key paracrine and/or autocrine regulator of cumulus cell functions and has been proposed to play a major role in oocyte nuclear maturation (Elvin *et al*., 2000). An elevated concentration of PGE2 positively stimulated the extent of cumulus expansion and nuclear maturation by enhancing the phosphorylation of MAPK1 and MAPK2 in both oocytes and cumulus cells through the elevation of cAMP levels (Marei *et al*., 2009).

In addition to the positive effects observed on cumulus expansion and oocyte nuclear maturation, improvement in all developmental stages of buffalo embryos was observed when oocytes were matured in the presence of 50-100 µm ALA in the maturation media. Similar to the findings of our study, an increased rate of embryonic development has been observed with supplementation of ALA in maturation media in the bovine (Marei *et al*., 2009), sheep (Ghaffarilaleh *et al*., 2014) and goat oocytes (Veshkini *et al*., 2015). Provision of ALA in maturation media might have improved the cellular processes during different phases of maturation, which in turn influenced the developmental competence of subsequent embryos (Krisher and Bavister, 1998). In another study on the bovine, addition of ALA to the culture medium did not improve the cleavage and blastocyst rates; however, the viability of the embryos was influenced in a positive manner by the presence of ALA in culture medium (Al Darwich *et al*., 2010). It is relevant to mention that the improvement in embryo development rate in present study can only be attributed to supplementation of ALA during oocyte maturation as maturation medium was serum free and there was no supplementation during *in vitro* embryo culture. Thesefinding s suggest that ALA might have been more important to the oocytes if added to the maturation medium rather than to the culture medium after fertilization.

In the present study, ALA at higher concentrations (300 µm ALA) resulted in reduced number of cleaved embryos and no morula stage embryos. Since PUFAs are more susceptible to peroxidation than monounsaturated and saturated fatty acids, supplementation at higher levels might have increased the oxidative stress (Song *et al*., 2000; Gladine *et al*., 2007). Higher levels of ALA might have disturbed the balance of unsaturated and saturated fatty acids, so it might be the ratio of saturated vs unsaturated FAs that is more critical in the oocyte's micro-environment rather than a certain concentration of the unsaturated FAs per se. Therefore, one may speculate that the higher concentrations of ALA in our study might have generated oxidative stress after oxidative phosphorylation of ALA (Wakefield *et al*., 2008) which might have compromised the developmental competence of these oocytes and be responsible for the observed reduction of embryonic growth. Further studies may be suggested to investigate whether antioxidant supplementation of ALA-supplemented IVM medium could abrogate negative effects as observed for higher concentrations of ALA. In conclusion, the results of the present study have shown that supplementation of *in vitro* maturation media with

ALA at 100 μm improves the *in vitro* maturation of buffalo oocytes that in turn improves early embryonic development.

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