Indian Journal of Animal Sciences **88** (9): 1041–1043, September 2018/Short communication

Beneficial effect of lysyl oxidase on *in vitro* development of cultured ovine normal and metabolic stressed cumulus oocytes complexes

S K TRIPATHI[1], S NANDI[2], P S P GUPTA[3] and S MONDAL[4]

ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, Karnataka 560 030 India

Received: 20 February 2018; Accepted: 1 June 2018

Key words: COCs, Embryo development, Lysyl oxidase (*Lox*), Metabolic stressors

Oocyte maturation is one of the most crucial steps *in vitro* embryo production protocol. During development, the oocytes continue meiosis, meet spermatozoa for fertilization, cleave, and embryonic development takes place. We had documented that metabolic stressors like elevated levels of total NEFA, ammonia, urea and beta-hydroxy butyrate (BHB) were significantly higher in follicular fluid of metabolic stressed ewes (Farman *et al.* 2015a). In metabolic stressed conditions, the developmental competence of oocyte is compromised which leads to poor maturation rate and embryo development (Nandi *et al.* 2017a, b). Supplementation of oocytes maturation medium with various additives is one of the methods to improve developmental competence of oocyte. Positive effects of lysyl oxidase (*Lox*) mRNA expression in granulosa cell had previously been described (Kendall *et al.* 2003, Jiang *et al.* 2010). *Lox* involved in miscellaneous biological processes at some stage in folliculogenesis, but the function of this factor throughout the period of oocyte development remains unknown. Hence the present study was accomplished to examine the role of *Lox* in ovine normal and metabolic stressed cumulus oocytes complexes (COCs). We propose that providing *Lox* supplementation in maturation medium during IVM may possibly have beneficial effect for oocyte maturation and the subsequent embryo development in both normal and metabolic stressed COCs groups.

Ovine ovaries were collected at a local abattoir from adult sheep (2–4 years old). All materials were obtained from Sigma-Aldrich Co, USA unless stated otherwise.

The COCs were aspirated from ovarian follicles and washed 3 times in TCM-199+ 10% (v/v) fetal bovine serum. COCs (8–10) were matured in 50 µl drops of maturation medium. The control maturation medium consisted of TCM-199 supplemented with 10% (v/v) fetal bovine serum (FBS), 10 µg/ml ovine-follicle stimulating hormone (oFSH), 50 µg/ml gentamicin. Metabolic stressors and different *LOX* concentration (0, 5, 10, 20, 30 µg/ml) were added to the maturation medium. The drops were covered by mineral oil and incubated for 24 h in air containing 5% CO_2 with maximum humidity at 38.5°C. Maturation of oocytes was assessed on the basis of cumulus cell expansion and first polar body extrusion after 24 h of incubation as described earlier (Nandi *et al.* 2017a). For *in vitro* insemination, freshly collected ram sperm cells were added to the fertilization medium to a final concentration of (2–3) $\times 10^6$ sperm cells/ml. The embryo culture medium consisted of TCM-199 + FBS (10%) + gentamicin (50 µg/ml). After 40–42 h of inseminating the oocytes, the presumptive zygotes were evaluated under stereo zoom microscope at 110× magnification for evidence of cleavage. Results were recorded in terms of cleavage rate (percentage of oocytes inseminated and that were cleaved to 2 cell stage). The cleaved embryos were further cultured for 7 days for production of morulae/blastocytes. The determination of ROS in matured oocyte and granulosa cells was as described earlier (Waiz *et al.* 2016). Blastocysts obtained after 7 h days of culture were collected and subjected to a differential staining protocol for embryos (Nandi *et al.* 2017a) for counting of cells.

In experiment 1, the isolated COCs were cultured in TCM 199 medium supplemented with different concentrations (0, 5, 10, 20 and 30 µg/ml) of *Lox* and 10% FBS fetal and gentamicin (50 µg/ml). In experiment 2, the concentrations of ammonia, urea, free fatty acids, and β-OHB used were based on results of an earlier study (Nandi *et al.* 2017a) conducted in our laboratory. Based on the result of the first experiment, ovine COCs were cultured at 38.5°C with 5% CO_2 in air in the presence of 250 µM ammonia (significant level causing oocytes maturation impairment), 6 mM urea (significant level causing oocytes maturation impairment), 210 µM NEFA (significant level causing oocytes maturation impairment) and 1.0 µM β-OHB (significant level causing oocytes maturation impairment) in oocyte maturation medium for 24 h. The total NEFA (210 µM) consisted of stearic acid (30 µM) + palmitic acid (60 µM) + oleic acid (1200 µM). *Lox* was supplemented at the level of 20 µg/ml. The maturation, viability, reactive oxygen species production, cleavage, blastocysts production rate

Present address: [1]Senior Research Fellow (tshiv@ymail.com), [2,3,4]Principal Scientist (snandi71@gmail.com, pspgupta@hotmail.com, sukanta781@gmail.com).

Table 1. Effect of *Lox* on developmental competence of normal COCs

Treatments (*Lox*; µg/ml)	Maturation rate (%)	ROS (µM)	Viability rate (%)	Cleavage rate (%)	Blastocyst yield (%)	Total no. of cells in blastocysts (mean±SEM)
0-Control	83.2±0.51^{a}	12.1±0.22^{a}	96.2±1.41^{a}	55.068±1.2^{a}	25.4±0.44^{a}	93.4±4.9^{a}
5	83.4±0.63^{a}	11.21±0.58^{a}	94.35±3.41^{a}	56.2±1.5^{a}	24.8±0.94^{a}	95.14±3.42^{a}
10	83.7±1.28^{a}	10.64±0.46^{a}	94.68±1.66^{a}	56.5±1.4^{a}	25.1±0.86^{a}	98.43±2.94^{a}
20	92.1±0.94^{b}	9.52±0.12^{a}	93.49±1.39^{a}	66.1±2.5^{b}	34.8±0.23^{b}	112±1.21^{b}
30	93.2±0.35^{b}	9.69±0.32^{a}	93.4±2.361^{a}	66.3±3.2^{b}	34.7±0.75^{b}	114±3.53^{b}

Superscripts bearing different letters in the same column differ significantly (P>0.05). Values are mean±standard error of the mean based on 10 replicates/treatment with 8 to 10 oocytes/plot.

Table 2. Effect of *Lox* on developmental competence of metabolic stressed COCs

Treatments (*Lox*; µg/ml)	Maturation rate (%)	ROS (µM)	Viability rate (%)	Cleavage rate (%)	Blastocyst yield (%)	Total no. of cells in blastocysts (mean±SEM)
Ammonia (A), 250 µM	62.2±2.0^{a}	24.5±2.6^{a}	74.1±0.2	33.7±0.6	9.0±0.58	73.2±5.4
A + *Lox*	79.1±2.4^{b}	11.3±2.4^{b}	74.9±0.3	31.64±0.8	11.5±0.1	74.1±3.1
Urea (U), 6 mM	64.1±1.4^{a}	28.8±1.4^{a}	69.21±0.5	35.3±0.3	15.4±0.6	79.6±31
U + *Lox*	77.2±1.3^{b}	13.1±2.9^{b}	68.46±0.2	34.42±0.2	16.2±0.1	78.2±1.2
NEFA (N), 210 µM	63.4±1.6^{a}	25.9±2.7^{a}	70.16±0.5	36.3±0.6	12.5±0.2	73.1±3.6
N + *Lox*	76.2±1.8^{b}	12.3±2.2^{b}	69.8±1.3	38.5±1.5	12.2±0.3	72.89±0.6
b-OHB (B), 1.0 µM	61.8±1.3^{a}	19.5±2.9^{a}	67.8±2.7	38.6±0.9	14.2±0.7	72.4±2.4
B + *Lox*	74.21±1.3	12.3±1.5^{b}	67.2±1.1	36.8±0.4	15.8±0.2	73.54±3.1

Superscripts bearing different letters in the same column differ significantly (P> 0.05). Values are mean±standard error of the mean based on 10 replicates/treatment with 8 to 10 oocytes/plot. *Lox* supplemented @ 20 µg/ml.

and total cell number in blastocysts were evaluated in both the experiments.

The maturation rates, fertilization rates and embryos yield were analysed by ANOVA followed by Tukey's multiple comparison tests (percentage values were transformed to arcsine values before analysis). The statistical package of Graph Pad Prism, San Diego, USA was used for analyzing the data. A value of P<0.05 was considered statistically significant.

The effect of different concentrations of *Lox* on IVM, IVF and embryo development of normal ovine oocytes is shown in Table 1. Supplementation of *Lox* at 20 µg/ml in maturation media significantly increased (P<0.05) the maturation, cleavage, blastocysts production and total number of blastomere production from COCs as compared to that observed in control and 10 µg/ml *Lox* supplemented COCs. Incrementing the *Lox* level to 30 µg/ml did not cause any further improvement of developmental rates of COCs. We did not observe any significant improvement in ROS production and viability rates in any of the treatment. The effect of *Lox* on developmental competence of metabolic stressed COCs is shown in Table 2. Supplementation of 20 µg/ml of *Lox* in metabolic stressors exposed COCs (250 µM ammonia, 210 µM NEFA, 1.00 µM BHB and 6 mM urea) significantly increased maturation rate and significantly decreased ROS production. However we did not find any significant changes in viability, cleavage rate, blastocyst formation yield and total cell number in blastocyst when metabolic stressors exposed COCs were supplemented with 20 µg/ml of *Lox* in culture media.

To the best of our knowledge, this is the first study to report the impact of *Lox* on *in-vitro* development of ovine normal and metabolic stressed COCs. Results indicated that the maturation and cleavage rates of ovine oocytes cultured in the presence of 20 µg/ml *Lox* was significantly higher in comparison to all the lower doses tested. The possible mechanism for improved maturation might be because of *Lox* protects metabolic stressed oocyte against oxidative stress through decline of free oxygen radicals. Metabolic stress negatively affected female reproductive competence either directly or indirectly by disturbing metabolic environment of follicular fluid which affected enclosed oocytes and cumulus cells (Nandi *et al.* 2017b). This disturbance in microenvironments of follicular fluids leads to elevation of NEFA, total cholesterol, BHB, ammonia, urea and low concentrations of glucose. Recruitment of body reserves in metabolic stress was linked with changes in blood metabolites and other body fluid metabolite profiles that negatively impact fertility (Farman *et al.* 2015, 2016, Tripathi *et al.* 2016, Leroy *et al.* 2017).

Lox was involved in the regulation of granulosa cell differentiation as *Lox* mRNA expression was higher in granulosa cells in follicles producing normal oocyte than poor oocyte developmental competence (Jiang *et al.* 2010). *Lox* oxidized peptidyl lysine to peptidyl aldehyde residues which helps in formation of the covalent cross-linkages that

in-solubilize these extracellular proteins (Li *et al.* 1997). Kendall *et al.* (2003) stated that there was mRNA expression of *Lox* in cultured bovine granulosa cells which involved in the maintenance of cell differentiation. We found an increase in the number of oocytes attaining two-cell stage after addition of *Lox* to the medium. When combination of *Lox* and metabolic stressors was used, there were significant improvements of IVM compared to the control group. Therefore we propose that both cases (normal and metabolic stressed oocytes) *Lox* could improve IVM quality.

Several growth factors played a crucial role in development, cell growth, tissue repair, granulosa cell differentiation and transformation (Mandal *et al.* 2015, Rios *et al.* 2015, Ascari *et al.* 2017). Similarly, *Lox* was reported to be necessary for the maintenance of extracellular matrix (ECM) as it was associated with the cross-linking of collagen and elastin (Kendall *et al.* 2003). The ECM also act as an active supervisor of cell migration, division, differentiation, death and anchorage (Kendall *et al.* 2003).

In conclusion, the results of this study showed that the supplementation of *Lox* during *in vitro* maturation was beneficial for enhancing the maturation and cleavage rates of ovine oocytes. The expression profiles and mechanisms of action of *Lox* on oocytes physiology is under progress.

SUMMARY

The present study was undertaken to investigate the effect of lysyl oxidase (*Lox*) on *in vitro* development of normal and metabolic stressed ovine cumulus oocyte complexes (COCs). The isolated COCs (normal and metabolic stressed) were cultured in TCM-199 medium supplemented with different concentrations (0, 5, 10, 20 and 30 μg/ml) of *Lox* and 10% fetal bovine serum (FBS) and gentamicin (50 μg/ml). The metabolic stressors used in the maturation medium were ammonia (250 μM), urea (6 mM), total non-esterified fatty acid (NEFA, 210 μM) and b-hydroxyburyric acid (BHB, 1 mM). The matured oocytes were *in vitro* inseminated with fresh semen. The maturation, viability, reactive oxygen species production, cleavage, blastocysts production rate and total cell number in blastocysts were evaluated. Supplementation of 20 μg/ml *Lox* during IVM significantly improved both developmental competence and quality of the produced embryos in normal oocytes. Addition of 30 μg/ml *Lox* improved the maturation rate and caused less ROS production, however it did not make any significantly improvement in the developmental rates of embryos. Results indicated that addition of *Lox* in IVM culture media is associated with improved developmental competence of COCs.

ACKNOWLEDGEMENT

We are grateful to the Director, NIANP, Bengaluru for providing the necessary facilities. We thank Mr. Sanjit Rabidas for technical assistance. Financial help from the Department of Biotechnology, Government of India (Grant number BT/PR7131/AAQ/1/526/2012) is gratefully acknowledged.

REFERENCES

Ascari I J, Alves N G, Jasmin J, Lima R R, Quintão C C R, Oberlender G, Moraes E A and Camargo L S A. 2017. Addition of insulin-like growth factor I to the maturation medium of bovine oocytes subjected to heat shock: effects on the production of reactive oxygen species, mitochondrial activity and oocyte competence. *Domestic Animal Endocrinology* **60**: 50–60.

Farman M, Tripathi S K, Nandi and Girish K V. 2015a. Follicular fluid concentrations of metabolic stressors in normal, obese, metabolic stressed and emaciated ewes. *Asian Journal of Animal Science* **9**(6): 466–70.

Farman M, Nandi S, Girish K V, Tripathi S K and Gupta P S P. 2015b. Effect of stearic acid on *in vitro* formation of sheep oocytes. *Indian Journal of Animal Sciences* **85**: 584–87.

Farman M, Nandi S, Girish K V, Tripathi S K and Gupta P S P. 2016. Effect of metabolic stress on ovarian activity and reproductive performance of dairy cattle—review. *Iranian Journal of Applied Animal Science* **6**: 1–7.

Jiang J Y, Xiong H, Cao M, Xia X, Sirard M A and Tsang B K. 2010. Mural granulosa cell gene expression associated with oocyte developmental competence. *Journal of Ovarian Research* **3**: 6.

Kendall N R, Marsters P, Scaramuzzi R J and Campbell B K. 2003. Expression of lysyl oxidase and effect of copper chloride and ammonium tetrathiomolybdate on bovine ovarian follicle granulosa cells cultured in serum-free media. *Reproduction* **125**: 657–65.

Leroy J L M R, Bie De J, Jordaens L, Desmet K, Smits A, Marei W F A, Bols P J E and Hoeck V V. 2017. Negative energy balance and metabolic stress in relation to oocyte and embryo quality: an update on possible pathways reducing fertility in dairy cows. *Animal Reproduction* **14**(3): 497–506.

Li W, Nellaiappan K, Strassmaier T, Graham L, Thomas K M and Kagan H M. 1997. Localization and activity of lysyl oxidase within nuclei of fibrogenic cells. *Proceeding of National Academy of Science USA* **94**: 12817–822.

Mondal S, Mor A, Reddy I J, Nandi S and Gupta P S P. 2015. Effect of fibroblast growth factor 2 (FGF2) and insulin transferrin selenium (ITS) on *in vitro* maturation, fertilization and embryo development in sheep. *Brazilian Archives of Biology and Technology* **58**: 521–25.

Nandi S, Tripathi S K, Gupta P S P and Mondal S. 2017a. Nutritional and metabolic stressors on ovine oocyte development and granulosa cell functions *in vitro*. *Cell Stress and Chaperones* **23**(3): 357–71.

Nandi S, Tripathi S K, Gupta P S and Mondal S. 2017b. Effect of metabolic stressors on survival and growth of *in vitro* cultured ovine preantral follicles and enclosed oocytes. *Theriogenology* **104**: 80–86.

Ríos G L, Buschiazzo J, Mucci N C, Kaiser G G, Cesari A and Alberio R H. 2015. Combined epidermal growth factor and hyaluronic acid supplementation of *in vitro* maturation medium and its impact on bovine oocyte proteome and competence. *Theriogenology* **83**(5): 874–80.

Tripathi S K, Farman M, Nandi S, Girish Kumar V and Gupta P S P. 2016. Oviductal and uterine fluid analytes as biomarkers of metabolic stress in ewes (Ovis aries). *Small Ruminant Research* **144**: 225–28.

Waiz S A, Raies-ul-Haq M, Dhanda S, Kumar A, Goud S T, Chauhan M S and Upadhyay R C. 2016. Heat stress and antioxidant enzyme activity in bubaline (*Bubalus bubalis*) oocytes during *in vitro* maturation. *International Journal of Biometerology* **60**(9): 1357–66.