Indian Journal of Animal Sciences **92** (4): 422–425, April 2022/Article
https://doi.org/10.56093/ijans.v92i4.124031

Lysyl oxidase *(LOX)* expression in normal and metabolic stressed cumulus oocyte complexes and effect of a *LOX*-inhibitor on oocyte maturational competence and viability

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Received: 21 October 2020; Accepted: 27 December 2021

ABSTRACT

In an earlier study, we have demonstrated that supplementation of lysyl oxidase (*LOX*) has the beneficial effect on the developmental competence of both normal and metabolic stressed cumulus oocyte complexes (COCs). The present study investigated the mRNA expression of *LOX* in normal COCs of different grades, both immature and matured ones and in metabolic stressed matured COCs by qPCR. The effects of different concentrations (0, 10, 20, 30, 40, 50, 60 µg/ml) of β-aminopropionitrile (β-APN), a *LOX*-Inhibitor on *in vitro* maturational competence and viability rates of ovine COCs were also examined. The mRNA expression of *LOX* in metabolic stressed COCs supplemented with a *LOX* inhibitor was also examined. mRNA expression level of *LOX* was higher in good quality oocytes followed by fair and poor quality ones. The mRNA expression of *LOX* reduced when COCs were exposed to metabolic stressors. Supplementation of 50 µg/ml β-APN during IVM significantly decreased both maturational competence and viability of COCs. Lower mRNA expression of *LOX* in 250 µM ammonia was followed by 6 mM urea, 210 µM NEFA and 1.0 µM β-OHB. In conclusion, lysyl oxidase (*LOX*) can be considered as a candidate biomarker of oocyte quality and may be useful for the selection of good quality oocytes for assisted reproduction.

Keywords: β-aminopropionitrile, COCs, Embryo development, *LOX, LOX*-inhibitor, Metabolic stressors

Paracrine communications are essential for the normal follicular development between somatic and germ cells (Jiang *et al.* 2010). Metabolic stressor levels were found to be significantly higher in follicular fluid of metabolic stressed ovine (Farman *et al.* 2015a, 2018). The developmental competence of the cumulus oocyte complexes (COCs) is impaired under metabolic stressful conditions, which leads to low maturation rate and embryo growth (Nandi *et al.* 2017, 2018). During the follicular growth, lysyl oxidase (*LOX*) is characterized by the ability to shape and sustain the extracellular matrix (ECM) by inducing covalent cross-linking of collagen and elastin (Smith-Mungo and Kagan 1998, Fang *et al*. 2016). Previous findings have shown that *LOX* is expressed in cattle and mouse granulosa cells (Kendall *et al.* 2003, Jiang *et al.* 2010), so this enzyme may play a critical role in governing the differentiation of granulosa cell maturation of oocytes and ovulation (Slee e*t al.* 2001, Jiang *et al.* 2010, Fang *et al.* 2016).

LOX is involved in miscellaneous cellular processes in folliculogenesis at some point however, the involvement of this factor remains an open question in the oocyte development cycle (Fang *et al*. 2016.). We had earlier demonstrated that supplementation of *LOX* at 20 µg/ml in the maturation medium increased the COCs developmental competence (Tripathi *et al*. 2018). In the present study, we hypothesized that the mRNA expression of *LOX* in COCs may act as marker for oocyte developmental competence. The present study was carried out to examine the mRNA expression of *LOX* in normal COCs of different grades, in metabolic stressed COCs and in metabolic stressed COCs supplemented with a *LOX* inhibitor. The effects of a *LOX* inhibitor β-aminopropionitrile (β-APN) on *in vitro* maturational competence and viability of ovine COCs were also examined to assess the level at which the *LOX* inhibitor significantly decreased the COC maturation rate and viability.

MATERIALS AND METHODS

Materials: Unless stated otherwise, all chemicals were obtained from Sigma, Aldrich (St Louis, MO). Sheep ovaries were collected from a local slaughterhouse and transported to the laboratory within 2 h in a warm 0.9% normal saline.

Collection and grading of COCs: Oocyte collection and their grading were based on Nandi *et al.* 2016 and Talukder *et al.* 2011. Briefly, oocytes were graded as good quality COCs (oocytes completely surrounded by cumulus cells and with homogenous ooplasm); fair quality COCs (oocytes partially surrounded by cumulus cells and with homogenous ooplasm) and poor quality COCs (oocytes

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not surrounded by cumulus cells [denuded] and with dark ooplasm). All three grades of oocytes were aspirated from all surface follicles and were chosen for the study.

Culture of COCs: COCs culture was carried out as described earlier (Tripathi *et al.* 2018). COCs were washed three times in TCM-199 + 10% (v/v) fetal bovine serum. COCs were matured (8-10) 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) and 50 µg/ml gentamicin. 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 rate was evaluated as described earlier (Nandi *et al.* 2017) and the oocytes viability rate was evaluated by using the trypan blue staining technique (Gupta *et al.* 2002).

RNA isolation and reverse transcription quantitative polymerase chain reaction (RTqPCR) analysis: Total RNAs from matured COCs were extracted using RNeasy Mini kit according to manufacturer's instructions and DNA contamination was removed by DNase-I digestion (Promega, Southampton Science Park, Southampton, UK). All RNA specimens were quantified and the absorbance values at 260 and 280 nm were checked to assess the RNA concentration and purity for protein impurities in the samples.

Complementary DNA (cDNA) was prepared by using commercially available kit (iScriptcDNA synthesis kit, California, USA,) according to manufacturer description. RT-PCR was done with KAPA SYBR FAST qPCR Master Mix (2X) (Kapa Biosystems, Wilmington, Massachusetts, United States). The PCR conditions used to amplify *LOX* genes were: initial denaturation at 95°C for 3 min with 40 cycles of denaturation at 95°C for 3 sec followed by annealing and extension at 60°C for 30 sec. All reactions were performed in quadruplicate. The gene expression levels in the normal and metabolic stressors-treated COCs were analysed with the 2−ΔΔCT method. Details of the primers used in current study is shown in Table 1.

Experimental designs: In experiment 1, the different grades of COCs were cultured for 24 h in oocyte maturation medium. The mRNA expression of *LOX* in different grades of COCs both in immature and matured oocytes were studied by qPCR. The experiment was replicated for three times.

In experiment 2, good quality COCs were cultured at 38.5°C with 5% CO_2 in air in the presence of ammonia: 100 µM (basal level) and 250 µM (significant level causing oocytes maturation impairment), urea: 4 mM (basal level) and 6 mM (significant level causing oocytes maturation impairment)], non-esterified fatty acids (NEFA): basal level-70 µM and 210 µM (significant level causing oocytes maturation impairment) and β-hydroxybutyric acid (BHB): Basal level, 0.5 µM and 1.0 µM (significant level causing oocytes maturation impairment) in oocyte maturation medium for 24 h. The level of metabolic stressors was selected based on the result of an earlier study conducted in our laboratory (Nandi *et al.* 2017, 2018). The mRNA expression of LOX in metabolic stressed exposed with ammonia, urea, NEFA, and BHB COCs were analysed by qPCR.

Table 1. Primer sequence

Gene name	Primer sequence (5′–3′)	Reference / GenBank accession no.
FSHR	F-ATGGCCTTGTTCCTGGTGG R-CAAACCTCAGTTCGACCGC	Nandi *et al.* 2018
GAPDH	F-GGGTCATCATCTCTGCACCT R-GGTCATAAGTCCCTCCACGA	Nandi *et al.* 2018
LOX	F- AATCCCAGCTATTTGGTGCCT R- GGCTTGCTTTCTAATACGGTGA	XM_027969995.1

In experiment 3, the good quality COCs were cultured in TCM-199 medium supplemented with different concentrations (0, 10, 20, 30, 40, 50 and 60 µg/ml) of *LOX* inhibitor, β-aminopropionitrile (β-APN), and 10% Fetal Bovine Serum (FBS) and gentamicin (50 µg/ml). The maturation and viability rates were evaluated.

In experiment 4, good quality COCs were exposed with β-APN (the dose selected based on the result of experiment 3) and mRNA expression of *LOX* was studied by qPCR.

The experiments 1, 2 and 4 were replicated for four times, the experiment 3 was replicated for 8 times.All PCR related work were replicated for 4 times to minimize the cost. The experiment no 3 was replicated for 8 times to get samples for proper statistical analysis.

Statistical analysis: The data was analysed by ANOVA followed by Tukey's multiple comparison tests (percentage values were transformed to arcsine values before analysis). The percentage values were arcsine square root transformed before analysis. The statistical package of Graph Pad Prism (Version), San Diego, USA was used for analyzing the data. A value of $p<0.05$ was considered statistically significant

RESULTS AND DISCUSSION

The mRNA expression of *LOX* in different grade of COCs is shown in Fig. 1 (Expt 1). Good quality COCs showed significantly (($p<0.05$) higher expression of *LOX* followed by fair and bad quality COCs. No significant difference of mRNA expression of *LOX* was observed between immature and matured COCs.

The mRNA expression of *LOX* on metabolic stressed COCs is shown in Fig. 2 (Expt 2). The mRNA expression of *LOX* was significantly ($p<0.05$) decreased in metabolically stressed COCs [metabolic stressors exposed COCs (250 µM ammonia; 210 µM NEFA; 1.00 µM BHB and 6 mM urea)] compared with COCs exposed with basal levels of metabolic stressors.

The effect of different concentrations of *β-APN* on maturation and viability rates of COCs is shown in Fig. 3 (Expt 3). Supplementation of *β-APN* at 50 µg/ml in maturation media significantly decreased ($P<0.05$) the maturation and viability rates in COCs as compared to those observed in other lower levels and control. Increment in the *β-APN* level to 60 µg/ml did not cause any further

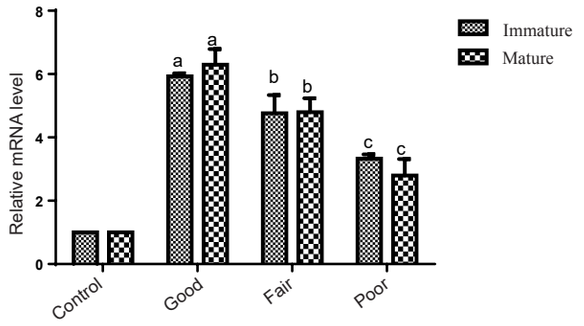

Fig. 1. Expression profile of *LOX* in different grade of COCs.

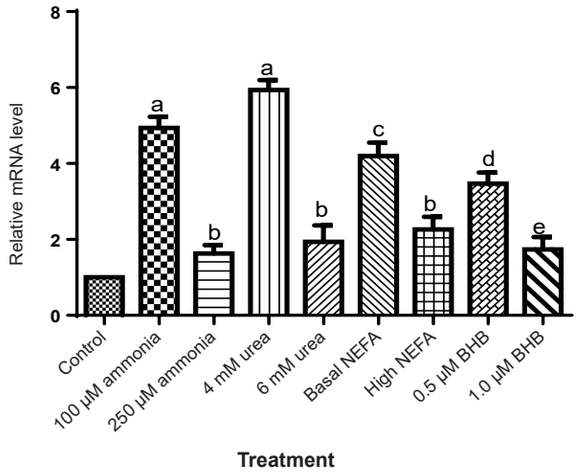

Fig. 2. *LOX* gene expression in good quality COCs exposed with different metabolic stressors.

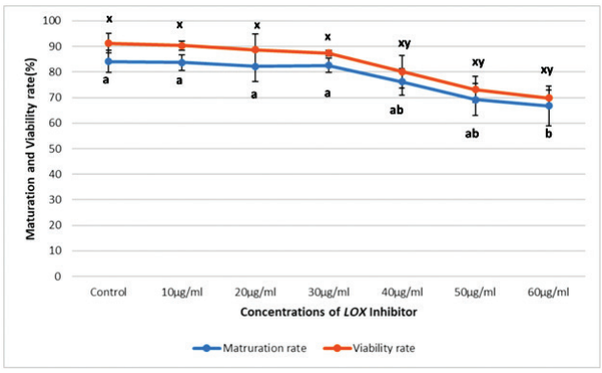

Fig. 3. Maturation and Viability rates of COCs exposed in different concentrations of a *LOX* inhibitor.

significant changes in maturation and viability rates of COCs.

Expression profile of *LOX* and *FSHR* mRNA in good quality COCs and COCs exposed with a *β-APN* (*LOX* inhibitor) is shown in Fig. 4 (Expt 4). Supplementation of *β-APN* at 50 µg/ml to good quality COCs significantly lowered the mRNA expression of *LOX* and *FSHR* compared

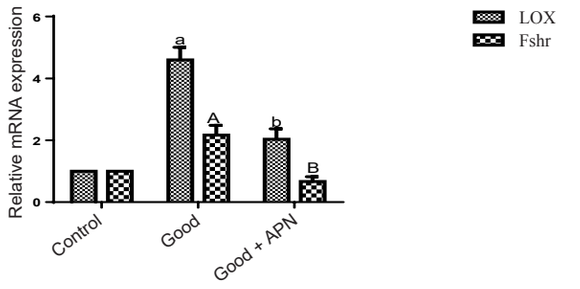

Fig. 4. Expression profile of LOX mRNA in good quality COCs exposed with β-APN (LOX inhibitor).

to good quality COCs without exposure with *β-APN.*

To the best of our knowledge, this is the first report of the mRNA expression of *LOX* of ovine normal and metabolic stressed COCs. Results indicated that the maturation and viability rates of ovine oocytes cultured in the presence of 50 µg/ml β-aminopropionitrile *(β-APN)* were significantly lowered in comparison to all the lower doses tested. The possible mechanism for reduced competence might be because of inhibition of *LOX.* Metabolic stress adversely affected female reproductive competence by disrupting the metabolic environment of follicular fluid that affected enclosed oocytes and cumulus cells, either directly or indirectly (Nandi *et al*. 2017). We recorded lower mRNA expression of *LOX* in poor and metabolically stressed oocytes in the present study which is in accordance with the findings of Jiang *et al.* (2010), wherein they reported higher mRNA expression of *LOX* in mural granulosa cells in follicles producing normal oocyte than poor oocyte developmental competence. It was reported that *LOX* oxidized residues of peptidyl lysine to peptidyl aldehyde in collagen and elastin and initiated the formation of covalent cross-connections which insolubilized these extracellular proteins (Li *et al*. 1997). Kendall *et al.* (2003) reported presence of mRNA expression of *LOX* in cultured bovine granulosa cells which were involved in the maintenance of cell differentiation.

The expression of *LOX* mRNA was dose dependent and regulated by FSH *in vitro*, and this was mirrored in reduced activity of the *LOX* enzyme matured COCs (Harlow *et al.* 2003). In the present study, we found decrease in the number of oocytes attaining maturational competence after addition of *LOX inhibitor* in the medium. In an earlier study we reported that *LOX* supplementation could improve the developmental competence of COCs (Tripathi *et al*. 2018). When cells were exposed to β-APN, a highly specific LOX inhibitor, it blocked the *Ras*-induced oocyte maturation (Saad *et al*. 2010) which is in accordance with our findings wherein we reported 50 µg/ml of β-APN significantly reduced maturation rate and viability of COCs. *LOX* was stated to be important for extracellular matrix (ECM) maintenance, as it was correlated with collagen and elastin cross-linking (Kendall *et al.* 2003). The ECM also served in cell migration, division, differentiation, death, and

anchorage (Kendall *et al.* 2003).

LOX, a key enzyme in extracellular matrix formation and stabilisation, is expressed in granulosa cells and plays a very important role in monitoring differentiation of granulosa cells, oocyte maturation and ovulation (Fang *et al.* 2016). *LOX* is reported to be the member of the FSH/androgen-regulated gene repertoire expressed in mammalian granulosa cells and plays physiologically significant roles in the regulation of ovarian follicular development (Slee *et al*. 2001). After hCG-induced ovulation, the activity of *LOX* enzyme increased in rabbit ovarian follicles and its mRNA expression was found to be upregulated at the time of ovulation in ovary (Langenau *et al.* 1999, Slee *et al.* 2001, Jiang *et al.* 2010). In addition, FSH inhibited dose-dependent *LOX* mRNA and enzyme activity in cultured granulosa cells of rats (Harlow *et al.* 2003, Jiang *et al.* 2010).

In conclusion, the mRNA expression profiles of *LOX* were significantly lower in poor and metabolic stressed oocytes which could be one of the possible mechanisms of action of reduced oocytes quality and its competence in metabolic stress. Our study also showed that the supplementation of β-APN (beta-aminopropionitrile), during *in vitro* maturation significantly reduced the maturation competence of COCs.

ACKNOWLEDGEMENT

We are grateful to the Director, ICAR-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.

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