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

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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 TC-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 TC-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% CO₂ 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) × 10⁶ 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/blastocysts. 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% CO₂ 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

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

<table>
<thead>
<tr>
<th>Treatments (Lox; µg/ml)</th>
<th>Maturation rate (%)</th>
<th>ROS (µM)</th>
<th>Viability rate (%)</th>
<th>Cleavage rate (%)</th>
<th>Blastocyst yield (%)</th>
<th>Total no. of cells in blastocysts (mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-Control</td>
<td>83.2±0.51a</td>
<td>12.1±0.22a</td>
<td>96.2±1.41a</td>
<td>55.068±1.2a</td>
<td>25.4±0.44a</td>
<td>93.4±4.9a</td>
</tr>
<tr>
<td>5</td>
<td>83.4±0.63a</td>
<td>11.21±0.58b</td>
<td>94.35±3.41a</td>
<td>56.2±1.58a</td>
<td>24.8±0.94a</td>
<td>95.14±3.42a</td>
</tr>
<tr>
<td>10</td>
<td>83.7±1.28a</td>
<td>10.64±0.46a</td>
<td>94.68±1.66a</td>
<td>56.5±1.48a</td>
<td>25.1±0.86a</td>
<td>98.43±2.94a</td>
</tr>
<tr>
<td>20</td>
<td>92.1±0.94b</td>
<td>9.52±0.12a</td>
<td>93.49±1.39a</td>
<td>66.1±2.58b</td>
<td>34.8±0.23b</td>
<td>112±1.21b</td>
</tr>
<tr>
<td>30</td>
<td>93.2±0.35b</td>
<td>9.69±0.32a</td>
<td>93.4±2.361a</td>
<td>66.3±3.2b</td>
<td>34.7±0.75b</td>
<td>114±3.53b</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Treatments (Lox; µg/ml)</th>
<th>Maturation rate (%)</th>
<th>ROS (µM)</th>
<th>Viability rate (%)</th>
<th>Cleavage rate (%)</th>
<th>Blastocyst yield (%)</th>
<th>Total no. of cells in blastocysts (mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia (A), 250 µM</td>
<td>62.2±2.0a</td>
<td>24.5±2.6a</td>
<td>74.1±0.2</td>
<td>33.7±0.6</td>
<td>9.0±0.58</td>
<td>73.2±5.4</td>
</tr>
<tr>
<td>A + Lox</td>
<td>79.1±2.4b</td>
<td>11.3±2.4b</td>
<td>74.9±0.3</td>
<td>31.6±0.8</td>
<td>11.5±0.1</td>
<td>74.1±3.1</td>
</tr>
<tr>
<td>Urea (U), 6 mM</td>
<td>64.1±1.4a</td>
<td>28.8±1.4a</td>
<td>69.21±0.5</td>
<td>35.3±0.3</td>
<td>15.4±0.6</td>
<td>79.6±3.1</td>
</tr>
<tr>
<td>U + Lox</td>
<td>77.2±1.3b</td>
<td>13.1±2.9b</td>
<td>68.46±0.2</td>
<td>34.2±0.2</td>
<td>16.2±0.1</td>
<td>78.2±1.2</td>
</tr>
<tr>
<td>NEFA (N), 210 µM</td>
<td>63.4±1.6a</td>
<td>25.9±2.7a</td>
<td>70.1±0.5</td>
<td>36.3±0.6</td>
<td>12.5±0.2</td>
<td>73.1±3.6</td>
</tr>
<tr>
<td>N + Lox</td>
<td>76.2±1.8b</td>
<td>12.3±2.9b</td>
<td>69.8±1.3</td>
<td>38.5±1.5</td>
<td>12.2±0.3</td>
<td>72.89±0.6</td>
</tr>
<tr>
<td>B-OH (B), 1.0 µM</td>
<td>61.8±1.3a</td>
<td>19.5±2.9a</td>
<td>67.8±2.7</td>
<td>38.6±0.9</td>
<td>14.2±0.7</td>
<td>72.4±2.4</td>
</tr>
<tr>
<td>B + Lox</td>
<td>74.2±1.3</td>
<td>12.3±1.5b</td>
<td>67.2±1.1</td>
<td>36.8±0.4</td>
<td>15.8±0.2</td>
<td>73.54±3.1</td>
</tr>
</tbody>
</table>

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, Ascarí 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 acted 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-hydroxybutyric 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.

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REFERENCES


