

—Original Article—

Laminarin enhances the quality of aged pig oocytes by reducing oxidative stress

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Abstract. Laminarin (LAM) is a β -glucan oligomer known to possess biological activities such as anticancer and antioxidant effects. This study explored the influence of LAM supplementation on *in vitro* aged porcine oocytes and the underlying mechanisms behind this influence. We found that LAM delayed the aging process and improved the quality of aged oocytes. LAM supplementation enhanced the subsequent developmental competence of aged oocytes during the *in vitro* aging process. The blastocyst formation rate was significantly increased in aged oocytes treated with 20 μ g/ml LAM compared to non-treated aged oocytes (45.3% vs. 28.7%, $P < 0.01$). The mRNA levels of apoptosis-related genes, B cell lymphoma-2-associated X protein (*Bax*) and *Caspase-3*, were significantly lower in blastocysts derived from the LAM-treated aged oocytes during the *in vitro* aging process. Furthermore, the level of intracellular reactive oxygen species was significantly decreased and that of glutathione was significantly increased in aged oocytes following LAM treatment. Mitochondrial membrane potential was increased, and the activities of caspase-3 and cathepsin B were significantly reduced in the LAM-treated aged oocytes compared with the non-treated aged oocytes. Taken together, these results suggest that LAM is beneficial for delaying the aging process in porcine oocytes.

Key words: Embryo development, Laminarin, Oocyte aging, Porcine

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Failure in the fertilization or activation of oocytes at the optimum time after ovulation may result in the deterioration of oocyte quality in a time-dependent manner. This process is termed as postovulatory aging [1]. As the aging time of the oocyte increases, the accumulation of reactive oxygen species (ROS) in the oocytes increases, which may impair the dynamic equilibrium of calcium and result in mitochondrial dysfunction [2, 3]. Although oocytes exhibit some intracellular glutathione (GSH) mediated defense against oxidative damage, this defense system gradually decreases with aging in postovulatory oocytes [4]. The drastic increase in ROS production, as well as the loss of antioxidant protection, may induce oxidative stress in oocytes that have aged after ovulation. Oxidative stress may directly affect the initiation of apoptosis in aged oocytes after ovulation [5]. Antioxidant therapy may relieve the oxidative stress and delay the process of apoptosis during oocyte aging [1, 6, 7]. Therefore, it is important to reduce oxidative stress

in aged oocytes to delay oocyte aging and increase the time for oocyte-assisted reproduction [8].

Laminarin (LAM) is the main storage polysaccharide in algae and is composed of β -(1,6) branched β -(1,3)-D-glucan [9]. Unlike classic glucose metabolism, many studies have demonstrated that LAM can be delivered as a therapeutic cream, drug, or gene-transfer vector, based on its different molecular structures [10, 11]. LAM may serve as a functional food supplement or nutraceutical to inhibit lipid peroxidation and enhance immune function by altering the gene expression level of proteins involved in immune responses [12–20]. According to current studies, LAM has the potential to reduce both sepsis-induced oxidative stress and lipid peroxidation, as well as prevent cancer cell migration [15, 21, 22]. Although LAM displays beneficial biological and physiological functions for immune activation, information regarding LAM's influence on oocyte and embryo quality is limited.

Mammalian oocytes, enclosed by cumulus cells and denuded oocytes, are able to mature both *in vivo* and *in vitro*. Maturation of oocytes coincides with the aging process, which negatively affects oocyte quality both with and without cumulus cells [8, 23–25]. In this study, we eliminated the potential impact of cumulus cells on oocyte aging. We then investigated the ability of LAM to maintain oocyte quality and promote the subsequent embryonic development of porcine oocytes. We also evaluated the intracellular GSH and ROS levels as well as the mitochondrial membrane potential in aged oocytes exposed to LAM to gain insights into how LAM affects oxidative stress in aged oocytes.

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Materials and Methods

All reagents and chemicals were purchased from Sigma (St. Louis, MO, USA) unless expressly stated otherwise.

Collection and in vitro maturation (IVM) of porcine oocytes

Porcine ovaries from slaughtered pigs were obtained at a local slaughterhouse and transported to the laboratory within 2 h in sterile saline solution (0.9% sodium chloride, 75 µg/ml penicillin G, and 50 µg/ml streptomycin sulfate) at 35–37°C in a vacuum flask. Cumulus-oocyte-complexes (COCs) were aspirated from the follicles (3–8 mm in diameter) of porcine ovaries. COCs were washed three times in Tyrode's lactate-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (TL-HEPES) supplemented with 0.1% polyvinyl alcohol (PVA, w/v) and 0.05 mg/ml gentamycin. COCs were matured in IVM medium (medium 199 (Gibco, NY, USA) supplemented with 10% (v/v) porcine follicular fluid, 1 µg/ml insulin, 75 µg/ml kanamycin, 0.91 mM sodium pyruvate, 0.57 mM L-cysteine, 10 ng/ml epidermal growth factor, 0.5 µg/ml porcine follicle-stimulating hormone, and 0.5 µg/ml sheep luteinizing hormone) for 44 h at 38.5°C and 5% CO₂.

In vitro aging of porcine oocytes

Cumulus cells were removed from the COCs by pipetting in TL-HEPES supplemented with 1 mg/ml hyaluronidase for 3 min after porcine oocyte maturation. Only oocytes with first polar bodies were used for the subsequent experiments. For *in vitro* oocyte aging, denuded oocytes were cultured in mineral oil covered fresh IVM medium with (treatment group) or without (control group) LAM for an additional 24 h at 38.5°C, 5% CO₂, in humidified air. The optimal concentration of LAM was predetermined by a dose-response study, wherein the dose of LAM with the highest blastocyst formation rate was noted. Based on these previously obtained results, a final concentration of 20 µg/ml LAM was used in the experiments [26].

Parthenogenetic activation and in vitro culture of embryos

Denuded fresh oocytes (cultured in IVM for only 44 h without aging) and aged oocytes were gradually equilibrated in the parthenogenetic activation solution (297 mM mannitol containing 0.1 mM calcium chloride, 0.05 mM magnesium sulfate, 0.01% PVA, and 0.5 mM HEPES) and subjected to a 1.2 kV/cm electric pulse for 60 µsec. The activated oocytes were then incubated in bicarbonate-buffered porcine zygote medium (PZM-5) [27] containing 4 mg/ml bovine serum albumin (BSA) and 7.5 µg/ml cytochalasin B for 3 h to suppress the extrusion of the pseudo-second polar body. Next, fresh or aged post-activation oocytes were cultured in PZM-5 for 7 days and the resultant embryo cultures were maintained at 38.5°C and 5% CO₂.

Immunofluorescence staining and fluorescence intensity analysis

The oocytes were fixed with 3.7% paraformaldehyde (PFA) dissolved in phosphate-buffered saline (PBS) for 30 min and permeabilized in 0.5% Triton X-100 for 30 min. After 1 h incubation in a blocking buffer (1% BSA/PBS), the oocytes were incubated overnight at 4°C with a primary rabbit anti-caspase-3 antibody, followed by incubation with an Alexa Fluor 488-conjugated secondary antibody (1:200) for 1–2 h at 25°C. Hoechst 33342 (10 µg/ml in PBS) was

used for DNA counterstaining. Then the oocytes were mounted on glass slides and analyzed. Approximately 30 oocytes were examined in each group. For immunofluorescence intensity measurements, samples were imaged using an inverted fluorescence microscope, and the data was analyzed using Image-J software.

Total cell count of blastocysts

To quantify the total number of blastocysts at 7 day, the blastocysts were collected and washed in 1% PVA/PBS. Then they were stained with 10 µg/ml Hoechst 33342 for 20 min and washed three times with 1% PVA/PBS. After the final wash with PVA/PBS, the embryos were fixed with 3.7% PFA/PBS for 30 min at 25°C, and then were mounted onto glass slides. More than 90 embryos were examined in each group at 400 × magnification using a fluorescence microscope (Nikon Corp.).

Quantification of ROS and GSH in metaphase II (MII) oocytes

To measure ROS levels, oocytes were incubated with 10 µM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Thermo Fisher Scientific, Waltham, USA) for 30 min, followed by spectroscopic analysis (green fluorescence, UV filters, 490 nm). To measure GSH levels, oocytes were incubated with 10 µM Cell Tracker Blue dye 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CMF2HC) (Thermo Fisher Scientific) for 30 min, followed by spectroscopic analysis (blue fluorescence, UV filters, 370 nm). The fluorescence intensity of the oocytes was analyzed using ImageJ software.

Quantification of cathepsin B activity and mitochondrial membrane potential in MII oocytes

The activity of cathepsin B in oocytes was quantified using a commercial Magic Red Cathepsin B Assay Kit (ImmunoChemistry Technologies LLC, Bloomington, MN, USA). In brief, oocytes were incubated in 50 µl PVA/PBS with 2 µl reaction mix in a humidified atmosphere of 5% CO₂ at 39°C for 30 min. After incubation, the oocytes were washed three times in PVA/PBS. were examined in each group at magnification using a fluorescence microscope (Nikon Corp.).

To measure mitochondrial membrane potential, oocytes were incubated with 2 µM 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylimidacarbocyanine iodide (JC-1 dye; Invitrogen), and the mitochondrial membrane potential was calculated as the ratio of red fluorescence (activated mitochondria/J-aggregates) to green fluorescence (less-activated mitochondria/J-monomers).

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Blastocysts at 7 day were collected and mRNA was extracted using the Dynabeads mRNA DIRECT Kit (Invitrogen, Oslo, Norway). cDNA was obtained by reverse transcription of mRNA using the Oligo (dT) 12–18 primer and SuperScript III Reverse Transcriptase (Invitrogen). cDNA amplification was conducted in a thermocycler using the following protocol: 95°C for 3 min, 40 cycles at 95°C for 15 sec, 60°C for 30 sec, 72°C for 20 sec, and a final extension at 72°C for 5 min. The target genes were *Caspase-3*, B-cell lymphoma 2 (*Bcl-2*), and Bcl-2-associated X protein (*Bax*). The gene encoding glyceraldehyde-3-phosphate (*Gapdh*) was used as a reference. The primers used to amplify each gene are shown in Table 1. Quantification

Table 1. Sequences of primers used for RT-PCR

Genes	Primer sequence (5'-3')	Product size (bp)
<i>Bcl2</i>	F: GCCGAAATGTTTGTCTGAC	154
	R: GCCGATCTCGAAGGAAGT	
<i>Bax</i>	F: GATCGAGCAGGCGAATG	277
	R: GGGCCTTGAGCACCAAGTTTA	
<i>Gapdh</i>	F: TTCCACGGCACAGTCAAG	117
	R: ATACTCAGCACCAAGCATCG	
<i>Casp3</i>	F: GACGGACAGTGGGACTGAAGA	101
	R: GCCAGGAATAGTAACCAGGTGC	

Annealing temperature for all reactions is 60°C. F: forward primer; R, reverse primer.

of the mRNA data was performed using the $2^{-\Delta\Delta Ct}$ method [28]. Three separate experiments were carried out.

Statistical analysis

Results are presented as mean \pm standard deviation (SD). Statistical analysis was performed using the SPSS software version 19.0 (IBM, IL, USA). Total numbers (n) of oocytes/embryos used in each group and replicates (R) of each experiment are shown in the data column and the notes of each figure, respectively. Data obtained from only two groups were compared using the Student's t-test. Data with three or more means were analyzed using a one-way ANOVA (Tukey post hoc multiple comparisons).

Results

LAM supplementation improves the blastocyst formation rate, and the quality of aged oocytes, after parthenogenetic activation

We induced parthenogenetic activation to determine whether LAM improves the blastocyst formation rate in aged oocytes and the subsequent blastocyst quality. The optimal concentration of LAM was predetermined by a dose-response study, wherein the dose of LAM with the highest blastocyst formation rate was noted. A final concentration of 20 $\mu\text{g}/\text{ml}$ LAM was used in the experiments. After parthenogenetic activation, the blastocyst formation rate of fresh oocytes (cultured in IVM without the additional 24 h of culturing) in the absence of LAM was 56.73%. However, the blastocyst formation rate of oocytes aged for the additional 24 h was 28.68%. Yet aged oocytes treated with 20 $\mu\text{g}/\text{ml}$ LAM, after the 24 h of aging, displayed an increased blastocyst formation rate of 45.33% compared to untreated aged oocytes (Fig. 1A).

To determine the effects of LAM on the development of aged oocytes, we counted the total number of cells in blastocysts formed from fresh and aged oocytes (Fig. 1B and 1C). The total number of cells was significantly lower in aged blastocysts than in fresh blastocysts. However, LAM treatment slightly increased the total number of cells in the aged blastocysts. To determine the effect of LAM on the expression of apoptosis-related genes, we measured the mRNA levels of *Caspase-3*, *Bax*, and *Bcl-2* in blastocysts from each group (Fig. 1D). The expression levels of *Caspase-3* and *Bax* were significantly higher in the blastocysts derived from the aged oocytes than in those derived from the fresh oocytes. However, LAM

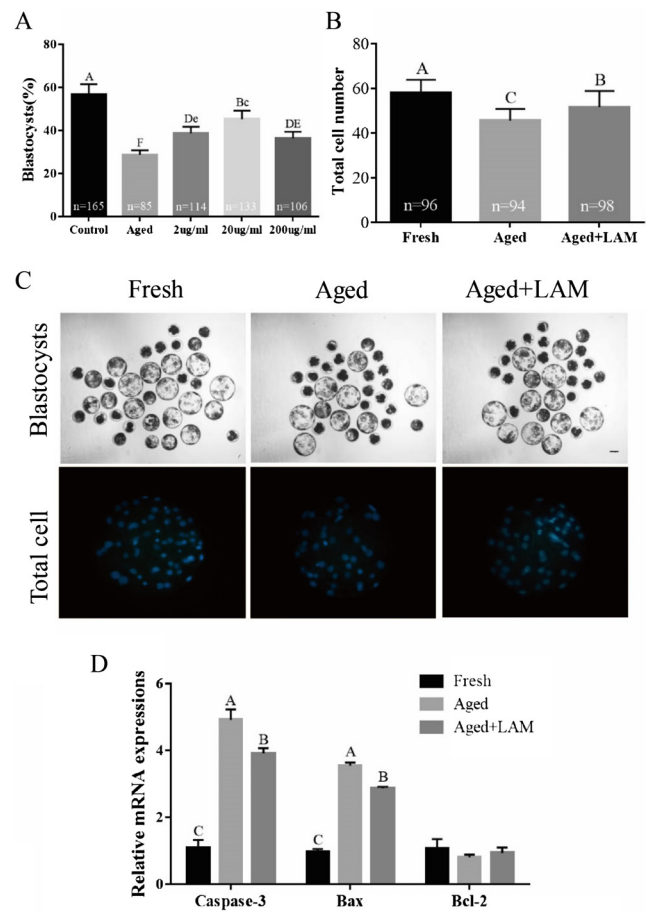


Fig. 1. LAM improves embryo development after parthenogenetic activation. (A) Blastocyst formation rate of fresh oocytes and of those aged for 24 h in the presence of different concentrations (0, 2, 20, and 200 $\mu\text{g}/\text{ml}$) of LAM. R=7. (B) Total cell number of embryos. R = 6. (C) Representative images of blastocysts and total number of cells. Scale bar: 100 μm . (D) The relative mRNA levels of apoptosis-related genes encoding *Caspase-3*, *Bax*, and *Bcl-2*, as analyzed by RT-PCR. R = 3. Significant differences between two groups are represented with different single capital letters ($P < 0.01$), and without lower-case letters at the same time ($P < 0.05$). The same letter denotes no significant difference between groups ($P > 0.05$).

treatment decreased the expression of *Caspase-3* and *Bax* genes in aged oocytes. No significant difference in the *Bcl-2* transcript level was observed between the LAM-treated oocytes and the control groups.

LAM supplementation ameliorates oxidative stress in aged oocytes

To study the antioxidative effect of LAM, we analyzed the intracellular ROS and GSH levels in MII oocytes treated with or without LAM after 24 h of aging as well as in fresh oocytes (Fig. 2A–D). ROS levels were significantly higher in aged oocytes than in LAM-treated aged oocytes ($P < 0.01$). In addition, aged oocytes had higher ROS levels than the fresh oocytes ($P < 0.01$). The intracellular GSH level significantly decreased in aged oocytes compared to fresh oocytes

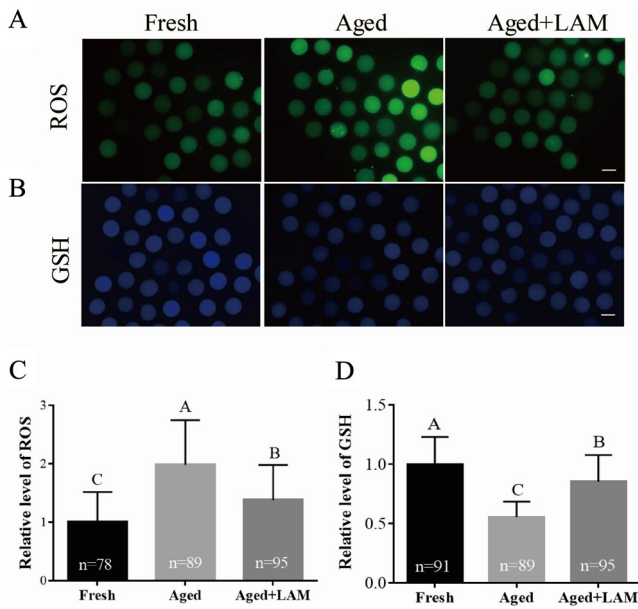


Fig. 2. The effects of LAM on ROS and GSH levels in aged oocytes. (A) Oocytes were stained with H2DCFDA to detect the intracellular level of ROS. Scale bar: 100 μ m. (B) Oocytes were stained with Cell Tracker Blue CMF2HC dye to detect the intracellular level of GSH. Scale bar: 100 μ m. (C) and (D) The relative level of intracellular ROS and GSH in *in vitro*-matured porcine oocytes from the three groups (fresh, aged, and aged + LAM). Significant differences are represented with different capital letters ($P < 0.01$). R = 3.

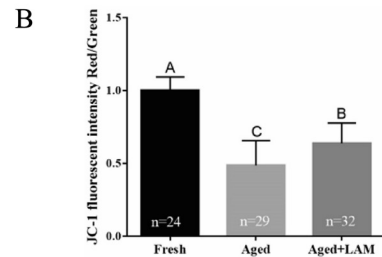
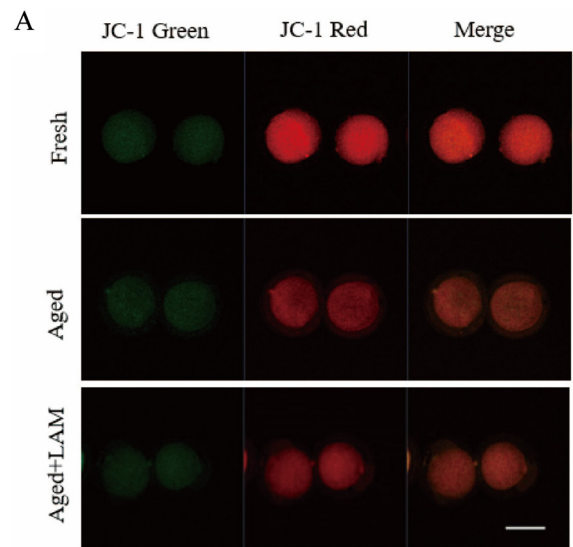


Fig. 3. Evaluation of the effect of LAM on mitochondrial membrane potential. (A) 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylimidacarbocyanineiodide (JC-1) staining of oocytes. Scale bar: 100 μ m. (B) Fluorescence intensity for JC-1 in oocytes. Significant differences are represented with different capital letters ($P < 0.01$). R = 3.

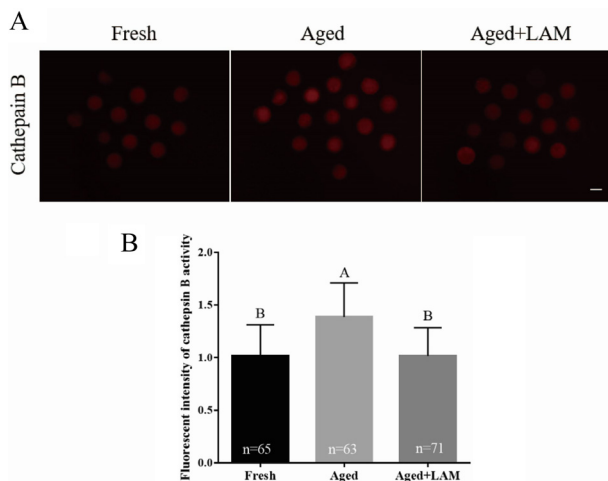


Fig. 4. The effect of LAM on cathepsin B activity of aged oocytes. (A) Representative images showing cathepsin B activity in fresh, aged, and LAM-treated MII oocytes. Scale bar: 100 μ m. (B) Quantified fluorescence intensity for cathepsin B in oocytes. Significant differences are represented with different capital letters ($P < 0.01$). R = 3.

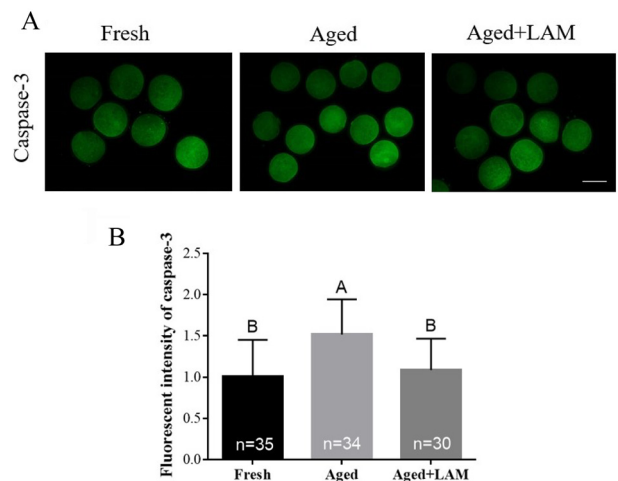


Fig. 5. The effect of LAM on the caspase-3 activity of aged oocytes. (A) Representative images showing caspase-3 activity in fresh, aged, and LAM-treated MII oocytes. Scale bar: 100 μ m. (B) Quantified fluorescence intensity for caspase-3 in oocytes. Significant differences are represented with different capital letters ($P < 0.01$). R = 3.

($P < 0.01$) but increased in LAM-treated aged oocytes compared to untreated aged oocytes ($P < 0.01$). These data suggested that LAM can reduce the oxidative stress in oocytes and improve the quality of oocytes during aging.

LAM supplementation enhances mitochondrial membrane potential in aged oocytes

Mitochondria dysfunction is one of the major factors that induces an increase in ROS levels and compromises embryo development [29]; therefore, we evaluated the mitochondrial membrane potential status during oocyte aging by examining the ratio of red/green fluorescence using the JC-1 dye. At aged group this dye emits green fluorescence and at fresh group it aggregates and displays red fluorescence. The fresh oocytes showed the maximum value for mitochondrial membrane potential, whereas the ratio of the fluorescence signal was much lower in aged oocytes than in LAM-treated aged oocytes ($P < 0.01$) (Fig. 3A and 3B). This observation indicates that LAM has a remarkable efficacy in maintaining the mitochondrial membrane potential of aging oocytes.

LAM supplementation decreases cathepsin B and caspase-3 activity in porcine oocytes

To analyze the effect of LAM on porcine oocyte quality after aging for 24 h, we measured the activity of cathepsin B in oocytes treated with or without LAM aged for 24 h as well as in fresh oocytes (Fig. 4A and 4B). Aged oocytes treated with LAM showed a significant decrease in cathepsin B activity compared with the untreated aged oocytes ($P < 0.01$). We also evaluated the activity of caspase-3 in oocytes after 24 h of aging (Fig. 5A and 5B). Aged oocytes treated with LAM showed a significant reduction in caspase-3 activity compared with the untreated aged oocytes ($P < 0.01$). However, the level of caspase-3 in fresh oocytes was significantly lower than that in aged oocytes ($P < 0.01$).

Discussion

Oocyte aging is a complex and irreversible biological process that may lead to several structural changes and a decrease in the functional status of mammalian oocytes through DNA damage, fertilization rate reduction, parthenogenetic activation, abnormal mitochondria structure, oxidative damage, and early oocyte apoptosis [29–31]. Here, we have demonstrated that LAM treatment may effectively delay the aging of oocytes and enhance subsequent embryonic development. Furthermore, LAM may enhance the mitochondrial membrane potential and reduce the apoptotic activity of aged oocytes. These results suggest that LAM supplementation may eliminate free radicals produced during oocyte aging, maintain the quality of oocytes, and promote subsequent embryonic development of porcine oocytes.

Oocyte quality is usually affected by oxidative stress or damage [32, 33]. As oocytes age, there is an increase in ROS accumulation [8, 34]. Oocytes appear to be particularly sensitive to elevated ROS levels. GSH, present in oocytes, resists oxidative stress and protects the oocytes from oxidative damage; however, the level of GSH gradually decreases with increasing oocyte age [35, 36]. GSH level is a critical factor known to influence oocyte quality. It has been reported that ROS accumulation accelerates the aging process in

oocytes and influences their subsequent fertilization ability [37, 38]. Analysis of ROS and GSH levels revealed that aged oocytes in the LAM-treated group had significantly lower levels of ROS and higher levels of GSH, which resulted in the reduction of oxidative stress and an improvement in oocyte quality compared with the control group. This observation is similar to those of other reports where LAM significantly increased GSH levels and inhibited ROS production, thereby reducing oxidative damage and improving oocyte quality [26, 39].

Mitochondrial function is compromised as oocytes age after ovulation and may be one of the factors that severely affects oocyte quality [40]. Proton pumps on each side of the mitochondrial membrane determine the mitochondrial membrane potential through ATP formation, ensuring the accuracy of the electron transport chain and oxidative phosphorylation reaction. In addition, mitochondrial membrane potential is directly associated with oocyte maturation, fertilization, cell signal transduction, maintenance of calcium homeostasis, and cell apoptosis [41–43]. Therefore, mitochondrial membrane potential is one of the major factors that affect oocyte and embryo development. In this study, we found that LAM supplementation increased the mitochondrial membrane potential of aging oocytes. This observation may explain the increase in the developmental competence of aging oocytes following LAM supplementation.

Given that the end point of oocyte aging is cell death via apoptosis [44, 45], we evaluated the effect of LAM on the activity of apoptosis-associated proteins. Caspase-3 is a member of the cysteine-aspartic acid protease (caspase) family [46]. The sequential activation of caspases plays a central role in cell apoptosis. Cathepsin B is a lysosomal cysteine protease involved in the process of apoptosis, and it has been shown that oocytes with lower developmental abilities have higher cathepsin B expression [47, 48]. Additionally, recent studies have reported that cathepsin B impairs oocyte developmental competence possibly through the stimulation of apoptotic pathways [49]. The results of this study showed that LAM treatment reduced cathepsin B expression in oocytes subjected to aging for 24 h. We evaluated caspase-3 activity in fresh oocytes and untreated or LAM-treated oocytes after 24 h of aging. We found that treatment with LAM resulted in a significant reduction in cathepsin B and caspase-3 activities in oocytes subjected to aging for 24 h and reduced the damage caused by apoptosis in the aged oocytes. These results support the hypothesis that LAM can exert beneficial effects during the process of aging in oocytes and can promote the subsequent embryonic development of porcine oocytes.

In conclusion, our research reveals that LAM supplementation can effectively relieve oxidative stress, decrease the levels of early apoptosis mediators, maintain mitochondrial membrane potential, and remarkably improve the blastocyst formation rate in aged oocytes.

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