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GHRELIN PROMOTION OF OOCYTE MATURATION VIA ERK1/2 PATHWAY IN OVIS ARIES

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Abstract

Ghrelin has recently garnered increasing attention in biomolecular studies. Ghrelin's growth hormone secretagogue receptor type 1a (GHS-R) is a pleiotropic modulator of diverse biological functions, including energy homeostasis and reproduction. This study sought to understand the ways in which ghrelin impacts ERK1/2 and p90^{rsk} during the ovis aries oocyte maturation process. We applied different concentrations of ghrelin and of ghrelin receptor inhibitor (D-Lys3-GHRP-6) to ovis aries oocytes and observed the effects on the ERK1/2 and p90^{rsk} pathway. The ERK1/2 and p90^{rsk} pathway plays an essential role in the *in vitro* maturation of ovis aries oocytes. This study discovered that ERK1/2 and p90 rsk pathway, during the ovis aries oocyte maturation, was associated with maturation of ovis aries oocyte in vitro.

Key words: Ghrelin, Oocyte maturation, ERK1/2.

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INTRODUCTION

Researchers began to pay increased attention to ghrelin in December of 1999, when the endogenous ligand for the growth hormone secretagogue receptor (GHS-R) type 1a was discovered (20, 27). Further studies reported the effective isolation of a 28-aminoacid peptide with a fatty acid chain modification (n-octanoic acid) in the serine 3 residue (14). This hydrophobic peptide (ghrelin) is the first known natural bioactive peptide modified by an acyl acid. Ghrelin has been shown to stimulate GH secretion, and it has orexigenic and adipogenic effects (15). It also influences glucose and lipid metabolism, and plays various roles in myriad tissues and organs, such as regulation of food intake, regulation of whole-body energy homeostasis, regulation of mucosa protection, regulation of gastrointestinal motility and gastric acid secretion et al. Ghrelin has been shown to positively effect immune system modulation, cardiovascular and gastroenteropancreatic physiology, and memory and learning (13). Ghrelin is mainly produced in the stomach, and to a lesser extent in other organs, where it is produced by a distinct group of endocrine cells that are located within the gastric oxyntic mucosa (12, 16). A certain amount of ghrelin has been observed along the gastrointestinal tract and in the pancreas. Minor expressions of ghrelin have been reported in the brain, testes, pituitary, kidney, thyroid, placenta, and various areas of the reproduction system (2). Recently reported data have conclusively demonstrated the expression of ghrelin (at the mRNA and peptide levels) in the adult rat ovary. To better understand the physiological regulation of ghrelin gene expression in the rat ovary, researchers have assessed relative mRNA signal levels throughout the different stages of the estrous cycle (3). Despite persistent expression of the signal throughout all stages of the estrous cycle, ghrelin mRNA levels varied significantly depending upon a given phase. The lowest expression levels were observed in the proestrus phase, and maximum values in the diestrous phase (Day 1).

Immature mammalian oocytes are known to express two isoforms of nonphosphorylated ERK, referred to as ERK1 (44 kDa) and ERK2 (42 kDa) (22). Around the period of germinal vesicle breakdown (GVBD), both isoforms become active through phosphorylation, and express a corresponding change in their electrophoretic mobilities. ERK1/2 belongs to the mitogen-activated protein kinase superfamily (MAPKs). MAPKs are serine-threonine protein kinases, which, among other things, mediate cellular responses to a variety of extracellular stimuli (11), and it is well known that ERK1/2 controls meiotic maturation in oocytes (28).

ERK1/2 phosphorylation allows for the regulation of cytoplasmic molecular and nuclear protein activity, which can control gene expression (28). ERK1/2 also phosphorylate p90 rsk (p90^{rsk}), MSK, and MNK, as well as the proteins involved in migration and cell attachment, including paxillin and calpain, and transcription factors, such as Elk-1, c-Fos, and c-Myc. Additionally p90^{rsk} whose MW is 90kDa, is the downstream effector in the MAPK pathway (8), and contains two distinct kinase catalytic domains in a single polypeptide chain. p90^{rsk} is a signal-transducing serine/threonine kinase, and constitutes an essential member of a growing subfamily of MAPKs-activated protein kinases (5). In mammalian cells, p90^{rsk} is activated in response to a broad range of cellular perturbations, such as an onset of or shift in oncogenic transformation, insulin, growth factors, phorbol esters, and growth hormones. p90^{rsk} can also be activated by diversification in cAMP levels, heat shock, ionizing radiation, and T cell receptor activation in mammalian cells (4). The p90^{rsk} signal transduction pathways are involved in cell growth, proliferation, differentiation, and apoptotic death (23), and research has also illustrated

that $p90^{rsk}$ is the predominant $p90^{rsk}$ isoform expressed in eggs and embryos. Both MAPK and $p90^{rsk}$ are similarly activated during the meiotic maturation of mouse oocytes, indicating the conservation of the whole pathway (19).

The ERK1/2 / $p90^{rsk}$ pathway and ghrelin play an essential *in vitro* role in the maturation process of the ovis aries oocyte. The present study investigated the relationship between ghrelin and the ERK1/2 / $p90^{rsk}$ pathway during the process of ovis aries oocyte maturation. The aim of the study was to understand whether or not ghrelin promoted oocyte maturation via the MAPK pathway in ovis aries.

MATERIALS AND METHODS

Unless otherwise specified, all chemicals and reagents in the study were purchased from the Sigma Chemical Company (St. Louis, MO, USA). Antibodies to IgG, GAPDH, ERK1/2, phospho- ERK1/2, $p90^{rsk}$, and phospho-$p90^{rsk1}$(Ser380) were purchased from the Millipore Corporation, MA, USA. Unless otherwise specified, the oocyte culture took place at 38.5℃ with 5 % CO_2 under humidified air.

Animals

The oocytes used in this study were from ovis aries animals that were free of parasitic and infectious diseases. The animals were purchased from the Tecon Group, located in the Urumqi (Xinjiang Autonomous Region, PR China). All animals were taken care by trained animal keepers and fed with hay and a commercial pelleted ration. Water and mineralized salt were available *ad libitum*. All experimental animals in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at the Inner Mongolia Agriculture University before the onset of the study.

Animal handling, euthanasia, and experimental procedures were conducted in compliance with Tecon Group regulations (Licence NO.: SYXK, Xin, 2010-0005), and with the approval of the Animal Ethics Committee of the Inner Mongolia Agriculture University of Inner Mongolia. Euthanasia was conducted by intravenous injection of a barbiturate overdose, and was followed by exsanguination and immediate removal of the oviducts.

Collection and in vitro maturation (IVM) of oocytes

The oocytes collected from the ovaries of ovis aries were subsequently cultured for maturation and analysis. The collected oocytes were cultured in droplets of TCM-199 (Gibco, New York, USA) supplemented with Hepes, 0.12 IU/ml LH, 0.001 μg/ml E_2, 0.02 IU/ml FSH, and 1ml OES (Sigma).

The oocytes were cultured in four-well multidishes with 500 μL cultural media. After 20 hr of culturing, the oocytes were freed of cumulus cells. The oocytes that had a first polar body (Pb1) and no spontaneous ooplasmic protru-

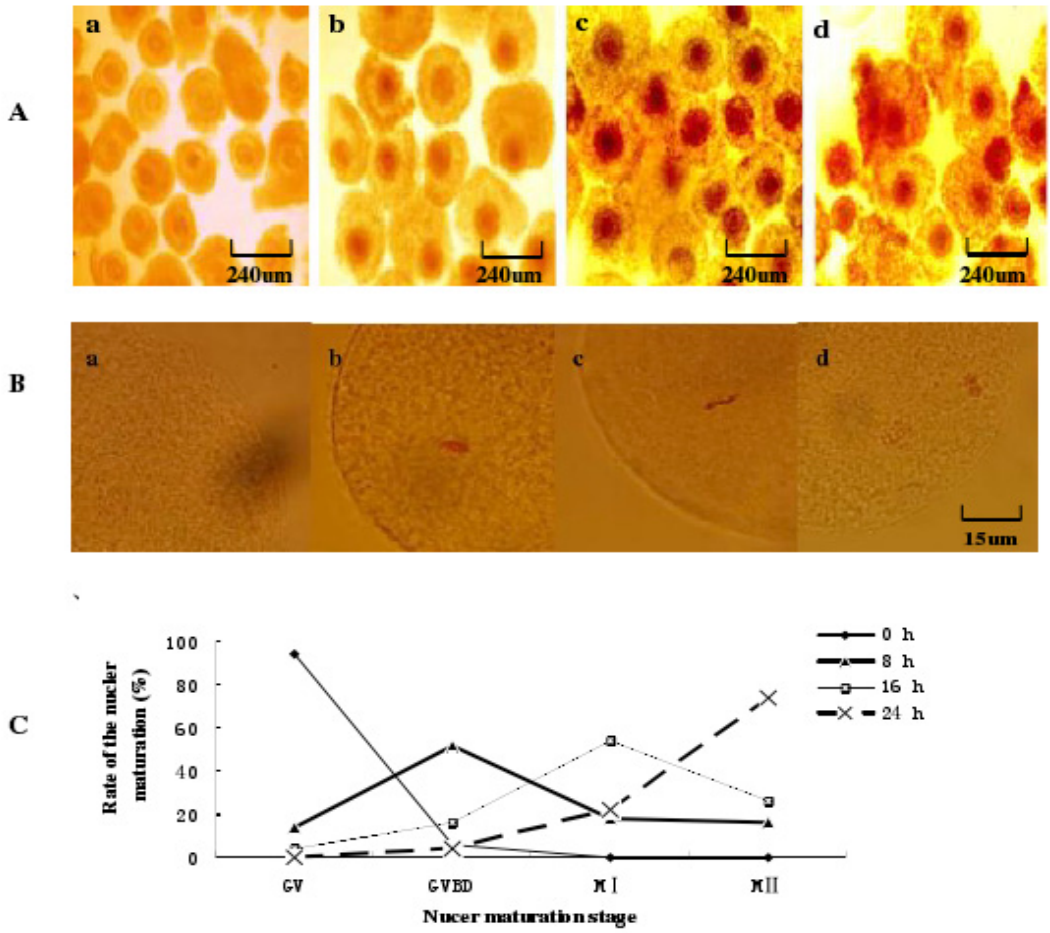

Figure 1. Ovine COCs matured *in vitro*. A. COCs morphological changes during *in vitro* maturation (40×): a. 0 hr; b. 8 hr; c. 16 hr; d. 24 hr. B. Different nuclear stages of oocyte meiosis (400×): a. GV; b. GVBD; c. MI; d. MII. C. Nuclear maturation competence of ovine COCs matured *in vitro*.

sions were selected for further treatments. The oocyte morphology was observed at 0 hr, 8 hr, 16 hr, and 24 hr after culturing (Fig. 1A).

Ghrelin and D-Lys3-GHRP-6 (ghrelin receptor inhibitor) assay

Ghrelin (Sigma) and D-Lys3-GHRP-6 (Sigma) were added to the oocyte media to final concentrations of 400, 500, 600, 700, 800 ng/ml, and 10^{-8}, 10^{-9}, 10^{-10}, and 10^{-11} mg/ml, respectively. The oocytes were cultured for 24 hr, and then the oocyte nuclear maturation was observed and analyzed. The optimum concentration of ghrelin, as determined by the prior experiment, in addition to D-Lys3-GHRP-6 at concentrations of 10^{-8}, 10^{-9}, 10^{-10}, and 10^{-11}mg/ml, was added together into the oocyte media, respectively. The oocytes were then cultured for 24 hr, and oocyte nuclear maturation was then observed and analyzed.

Determination of oocyte nuclear maturation

The stage of oocyte nuclear maturation was observed and analyzed following the above cultures. First, the oocytes were completely denuded of cumulus cells by vortexing in cultural medium for 2 min. The denuded oocytes were then washed twice, positioned on a grease-free slide, and overlaid with a coverslip supported by four droplets of a Vaseline: paraffin mixture (40:1). Subsequently, the oocytes were slightly compressed onto the slide and placed in an acetic acid : methanol fixative (1:3) for at least 48 hr. The oocytes were then stained with aceto-orcein (1 % orcein in 45 % acetic acid) and examined under phase-contrast microscope. The stage of nuclear maturation was determined according to the morphology of the nuclear material, using a system described by Hewitt et al. The oocyte nuclear status was classified as follows: 1) germinal vesicle (GV) stage, if a GV could be observed; 2) GVBD stage: Nucleolus and nuclear membrane disappeared. Chromatin trends to condensate lumpy. 3)MI stage, if no GV could be seen, and if chromatin was either compacted or in a typical chessboard fashion; and 4) MII stage, if the oocyte displayed a chromatin configuration indicative of anaphase I, telophase I, or metaphase II (Fig 1B&C) (25).

Western blot

The proteins that were homogenized from oocytes were separated by electrophoresis on 8 – 10 % SDS/polyacrylamide gels, and then transferred to immunoblot NC membrane. Membranes were blocked for 30 min at room temperature with PBS buffer containing 5 % fat-free milk and 0.1 % Tween20. Membranes were then incubated with primary anti-Rac1 antibody for at least 1 hr at room temperature, or overnight at 4 ºC. The membranes were subsequently washed three times with PBS containing 0.1 % Tween20, incubated with peroxidase-conjugated secondary antibodies, and developed using the ECL reagent (Pierce, Rockford, IL, USA).

Statistics

Data are presented as the mean6the standard error of the mean (s.e.m.). N indicates the number of separate experiments, while n indicates the total number of oocytes or eggs. Means of the data were compared using t-tests (two groups) or ANOVA (three or more groups). Where ANOVA was used, Tukey-Kramer's posthoc test was applied (Prism 5, GraphPad Software Inc., San Diego, CA). In all cases, $P<0.05$ was considered significant.

RESULTS

Effects of ghrelin and D-Lys3-GHRP-6 treatment on oocyte nuclear maturation

It was found that the oocyte nuclear maturation rate was promoted under the ghrelin treatments at 400, 500, 600, 700, and 800 ng/ml (24 hr culture) (Fig. 2A). The oocyte nuclear maturation rate under the 600 ng/ml ghrelin treatment was promoted more strongly than were those under the other ghrelin concentrations. It was also found that treatment with D-Lys3-GHRP-6 (10^{-8}, 10^{-9}, 10^{-10}, and 10^{-11} mg/ml) (24 hr culture) had no observable effects on the oocyte nuclear maturation rate (Fig. 2B). As a result, we developed a new treatment consisting of 600 ng/ml ghrelin (in cultural medium) for 2 hr. We added oocytes treated with D-Lys3-GHRP-6 at 10^{-8}, 10^{-9}, 10^{-10}, and 10^{-11} mg/ml to this medium, respectively. Following 24 hr culture, the previously observed promotion of the nuclear maturation rate was inhibited in the 10^{-8} mg/ml D-Lys3-GHRP-6 treatment group (Fig. 2C). Taken together, these results confirmed that the oocyte nuclear maturation rate had, in fact, been promoted under the 600 ng/ml ghrelin treatment. However, this observed increase in the oocyte nuclear maturation rate was reduced as concentrations of D-Lys3-GHRP-6 increased.

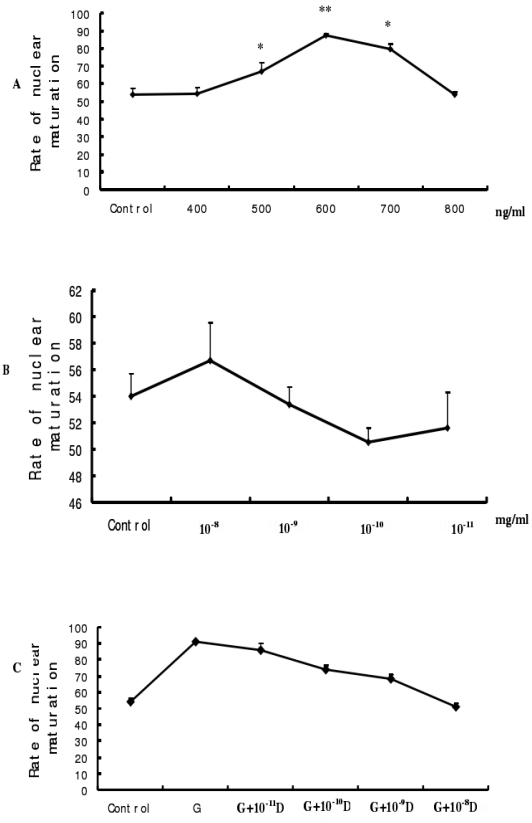

Figure 2. The rate of nuclear maturation in ovine oocytes. A. The rate of nuclear maturation in ovine oocytes with different concentrations of ghrelin. (*$p<0.05$, **$p<0.01$ vs. control; G: Ghrelin. B. The rate of nuclear maturation in ovine oocytes with different concentrations of D-Lys3-GHRP-6. D: D-Lys3-GHRP-6 C. Effect of ghrelin with D-Lys3-GHRP-6 on the rate of nuclear maturation in ovine oocytes. G: 600 ng/ml ghrelin; D: D-Lys3-GHRP-6.

Effects on ERK1/2 treatment with ghrelin and D-Lys3-GHRP-6 during in vitro oocyte maturation

It was found that ERK1/2 was expressed throughout the whole process of oocyte maturation, and the degree of expression remained consistent throughout the process (Fig. 3Aa, B). Following 8 hr treatment, ERK1/2 had not phosphorylated, and at 10 hr, the ERK1/2 had phosphorylated (Fig 3Ab, B). Under 600 ng/ml ghrelin, the amount of observed phosphorylated ERK1/2 was higher than that of the control (Fig. 3Ac,B). However, in the 600 ng/ml ghrelin + 10^{-6} mg/ml D-Lys3-GHRP-6 treatment, it could be seen that the effect of the ghrelin-promoted phosphorylated ERK1/2 had been disrupted (Fig. 3Ad, B).

Effects on p90^{rsk} treatment with ghrelin and D-Lys3-GHRP-6 during in vitro oocyte maturation

It was observed that p90^{rsk} was expressed at consistent rates throughout the whole process of oocyte maturation (Fig. 4Aa, B). Following 8 hr, the p90^{rsk} had not phosphorylated, but it had done so after 10 hr (Fig. 4Ab, B). Under the 600 ng/ml ghrelin treatment, levels of phosphorylated p90^{rsk} were higher than were those in the control (Fig. 4Ad, B). However, the effect of ghrelin-promoted phosphorylated p90^{rsk} was disrupted in the 600 ng/ml ghrelin + 10^{-6} mg/ml D-Lys3-GHRP-6 treatment (Fig. 4Ae, B).

DISCUSSION

Ghrelin is predominantly produced by the stomach, while substantially lower amounts of can be found in the bowel, pancreas, pituitary, kidney, and placenta (16). Ghrelin receptor, GHS-R, is a typical G-protein coupled-seven transmembrane receptor, (15). Two distinct ghrelin receptor cDNAs have thus far been isolated. The first receptor is GHS-R type 1a, and it encodes a 7-TM GPCR with binding and functional properties that are consistent with its role as a ghrelin receptor. Another GHS-R cDNA is GHS-R type 1b, which is produced by an alternative splicing mechanism (17). The ghrelin receptor is conserved across all examined vertebrate species, including a number of mammals, chicken, and pufferfish. Further, RT-PCR analysis showed evidence of ghrelin receptor mRNA expression in many peripheral organs, including the heart, lung, liver, kidney, pancreas, stomach, small and large intestines, adipose tissues, ovaries, and immune cells. Its maintain many species suggests that ghrelin and its receptor serve important physiological functions (18), while its widespread presence indicates that it serves myriad functions in various tissues (1, 7). Most notably for this study, ghrelin has been shown to impact the follicular development process, and more specifically, it has the capacity to promote oocyte maturation in ovis aries.

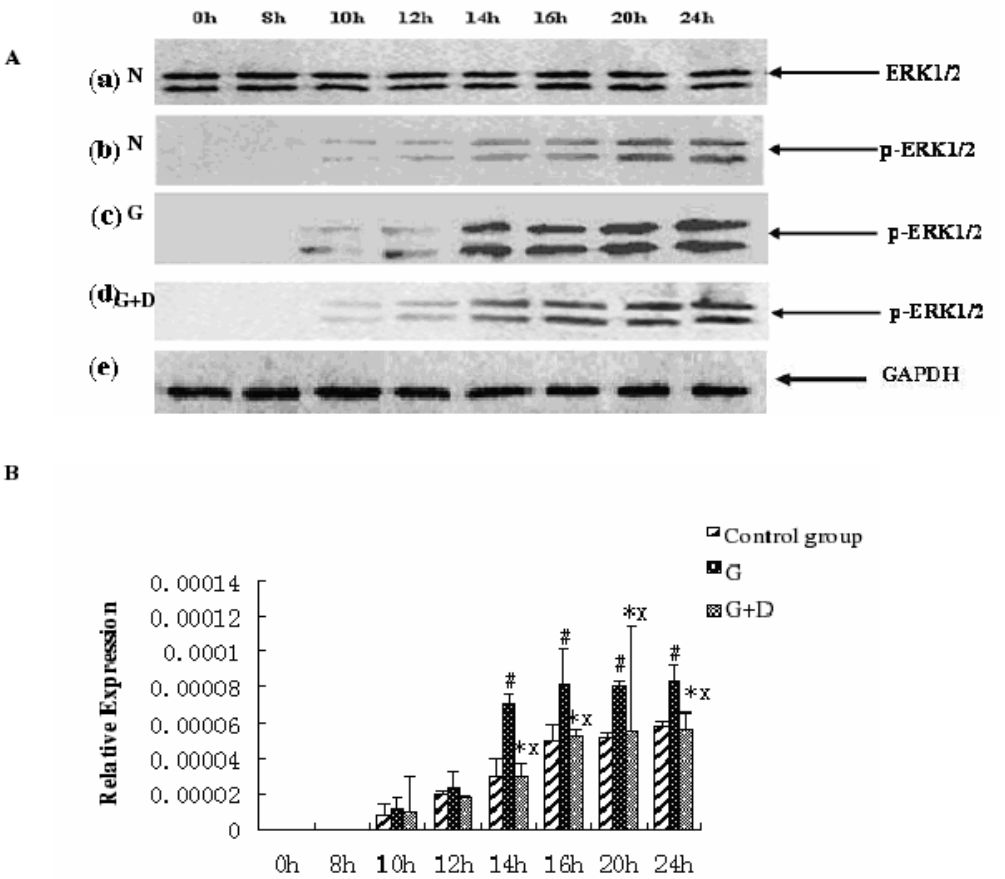

Figure 3. The expression of MAPK (ERK1/2) and phosphorylated MAPK (p-ERK1/2). A. Western blot result. (a) ERK1/2 was expressed in throughout the whole process of oocyte maturation (0, 8, 10, 12, 14, 16, 20 and 24h). And the expression level was consistent. (b) Cultural in vitro, ERK1/2 began phosphorylated at 10h. (c) Under 600 ng/ml ghrelin, the phosphorylated ERK1/2 was higher than (b) at the same time. (d) in the 600 ng/ml ghrelin + 10^{-6} mg/ml D-Lys3-GHRP-6 treatment, it could be seen that the effect of the ghrelin-promoted phosphorylated ERK1/2 had been disrupted. B. The expression level of p-ERK1/2. N: control group. G: 600 ng/ml Ghrelin added. G+D: 600 ng/ml ghrelin + 10^{-6} mg/ml D-Lys3-GHRP-6 added.

#: There is a significant different between the ghrelin (600 ng/ml) group and the control group ($p < 0.05$). *: There is a significant difference between the ghrelin (600 ng/ml) group and the ghrelin (600 ng/ml) + D-Lys3-GHRP-6 (10^{-6} mg/ml) group ($p < 0.05$). X: There is no significant difference between the control group and the ghrelin (600 ng/ml) + D-Lys3-GHRP-6 (10^{-6} mg/ml) group ($p > 0.05$).

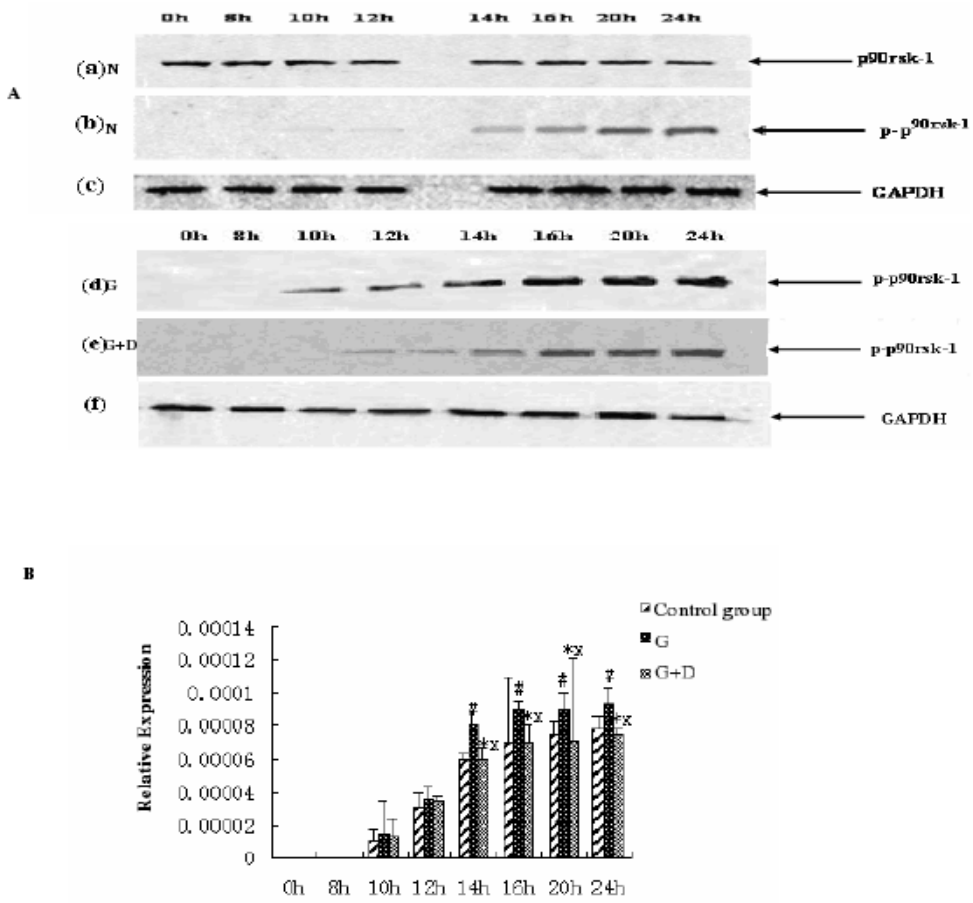

Figure 4. The expression of p90rsk-1 and phosphorylated p90rsk-1 (p-p90^{rsk-1}) A. Western blot result. (a) p90^{rsk} was expressed in throughout the whole process of oocyte maturation (0, 8, 10, 12, 14, 16, 20 and 24h). And the expression level was consistent. (b) Cultural in vitro, p90rsk began phosphorylated at 10h. (d) Under 600 ng/ml ghrelin, the phosphorylated p90^{rsk} was higher than (b) at the same time. (e) in the 600 ng/ml ghrelin + 10^{-6} mg/ml D-Lys3-GHRP-6 treatment, it could be seen that the effect of the ghrelin-promoted phosphorylated p90^{rsk} had been disrupted. B. The expression level of p- p90^{rsk}.

#: There is a significant difference between the ghrelin (600 ng/ml) group and the control group ($p < 0.05$). *: There is a significant difference between the ghrelin (600 ng/ml) group and the ghrelin (600 ng/ml) + D-Lys3-GHRP-6 (10^{-6} mg/ml) group ($p < 0.05$). X: There is no significant difference between the control group and the ghrelin (600 ng/ml) + D-Lys3-GHRP-6 (10^{-6} mg/ml) group ($p > 0.05$).

This study aimed to determine which ghrelin concentration had the greatest effect on oocyte nuclear maturation in ovis aries by treating oocytes with different concentrations of ghrelin (400, 500, 600, 700, and 800 ng/ml). Of the tested concentrations, the 600 ng/ml ghrelin treatment had the largest observable effects on oocyte nuclear maturation. This study additionally used ghrelin receptor inhibiter (D-Lys3-GHRP-6) to verify the above-observed effect of ghrelin on the oocyte nuclear maturation in ovis aries. The results illustrated that D-Lys3-GHRP-6 alone had no observable effect on oocyte nuclear maturation, so to verify ghrelin's effect on oocyte maturation, oocytes were treated with 600 ng/ml ghrelin and different concentrations of D-Lys3-GHRP-6 (10^{-8}, 10^{-9}, 10^{-10}, and 10^{-11} mg/ml) for 2 hr. The oocyte nuclear maturation rate was observed to be the highest in the treatment without D-Lys3-GHRP-6, and the oocyte nuclear maturation rate (in treatments with 600 ng/ml ghrelin) decreased as the D-Lys3-GHRP-6 concentration increased. Taken together, these results verified that ghrelin did, in fact, promote the rate of oocyte maturation.

Ghrelin helped to promote the oocyte maturation through several pathways. We observed the impact of ghrelin on the ERK1/2 pathway, and found that this pathway was critical to ghrelin's promotion of oocyte maturation. More specifically, we conducted experiments using ghrelin receptor inhibitor (D-Lys3-GHRP-6) and ghrelin and observed the impact on the ERK1/2 pathway. The results demonstrated that ghrelin promoted the overall level of phosphorylated ERK1/2. Phosphorylated ERK1/2 has been shown to be active in the oocytes, particularly in the GVBD, chromatin concentration, MII stage blocking, and cytoskeleton. Further, the total ERK1/2 expression level, including phosphorylated ERK1/2 and un-phosphorylated ERK1/2, has been shown to be constant in mammals (28). In the GVBD stage, ERK1/2 mainly was mainly found to be un-phosphorylated, and thus it did not show any activity. The phosphorylated ERK1/2 was found either in or after the GVBD stage, and the maximum amount of phosphorylated ERK1/2 was observed in the MII stage. In pig and cattle, ERK1/2 has been shown to be phosphorylated at the GVBD stage (6, 9); in the mouse, however, ERK1/2 was phosphorylated after the GVBD stage (26). In the present study, western blot results demonstrated that ERK1/2 was phosphorylated after the GVBD stage, and thus phosphorylated ERK1/2 was not a key element in GVBD stage formation and had no relationship with the meiosis. ERK1/2 potentially joined in the process of recovering the maturation promoting factor(s) at the end of the first meiotic division.

In the present study, it was observed that the process of

p90^{rsk} activation and ERK1/2 activation were the same. In the ovis aries oocytes, p90^{rsk} joined in the MII stage blocking process, while p90^{rsk} transmitted the ERK1/2 signal pathway (10). These roles and processes were similar to those which were displayed in the maturation of rat oocytes (24), but not to those observed in the pig (21). These results perhaps illustrated that the roles and processes differ from species to species. Further, ghrelin may influence gestation in a way that is likely to promote the breeding rate.

The major findings of this study were as follows. The critical nature of the ERK1/2/ p90^{rsk} pathway in the oocyte maturation process was clearly demonstrated. Further, it was shown that ghrelin and its receptor GHS-R were able to promote oocyte maturation in ovis aries oocytes, via the ERK1/2 and p90^{rsk} pathway. so, the ERK1/2 and p90^{rsk} pathway was one of the pathway to regulate the oocytes muration in ovis aries. ERK1/2 and p90 rsk pathway was associated with maturation of ovis aries oocyte in vitro.

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