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Effects of a Sonic Hedgehog agonist on ovine oocyte maturation, epigenetic changes and development of parthenogenetic embryos

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Purmorphamine is a Sonic Hedgehog agonist that plays an important role in activity regulation of receptors and transcription factors. This study was carried out to assess the effects of Purmorphamine on nuclear and cytoplasmic maturation, epigenetic changes of *in vitro* matured oocytes and development of parthenogenetic ovine embryos. Ovine ovaries were randomly collected from industrial slaughterhouse, and cumulus-oocyte complexes were cultured in media containing 250 or 500 ng/ml Purmorphamine or without Purmorphamine as a control group. Then, after *in vitro* maturation (IVM), Hoechst stain, cell tracker blue and histone H4K12 antibody were used, respectively to assess the nuclear and cytoplasmic maturation, and histone acetylation rate of matured oocytes. Matured oocytes were activated and cultured to the blastocysts stage in order to determine the parthenogenetic embryo cleavage rate. Expression of *Histone Deacetylase 1, 2 and 3 (Hdac1*, *Hdac2* and *Hdac3*) genes were evaluated in matured oocytes by quantitative Real Time PCR. Results showed that although the concentration of 500 ng/ml Purmorphamine had no significant influence on the oocyte nuclear maturation, it led to an increase in oocyte cytoplasmic maturation. Also, no significant difference in the rates of cleavage and blastocysts per cleavage was observed ($P > 0.05$) in the groups treated with Purmorphamine compared to the control. Quantitative PCR analysis indicated that in 500 ng/ml Purmorphamine treatment, *Hdac* 2 and 3 transcripts decreased and the H4K12 acetylation increased significantly ($P < 0.05$). Consequently, this study showed that 500 ng/ml Purmorphamine can induce the cytoplasmic maturation of oocytes, and may possibly be a good addative for improvement of ovine oocyte cytoplasmic maturation.

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1. Introduction

Oocyte maturation can be divided into nuclear and cytoplasmic steps. Nuclear maturation causes the formation of the first polar body and encompasses the process driving the progression of meiosis to metaphase II. Cytoplasmic maturation determines the quantity and quality of blastocysts (Pavlok et al., 1992; Sirard, 2001). Cytoplasmic glutathione (L-γ- glutamyl-L-cysteinyl-glycine; GSH) is the major non-protein sulphydryl compound in mammalian gametes (Pastore et al., 2003), which has been introduced as an important indicator of cytoplasmic maturation (Kim et al., 2007).

There have been wide-spread studies focusing on *in vitro* culture (IVC) systems of the oocytes and embryos which have improved *in vitro* applications of oocytes (Van Langendonckt et al. 1997; Lonergan et al. 2003; Rizos et al. 2003; Wrenzycki et al., 2004). Altering the culture conditions of oocyte maturation and embryo development will help manipulate gene expression patterns in order to simulate *in vivo* conditions and enhance embryo quality (Nguyen et al., 2011). Maturation of oocytes is influenced by different factors such as steroids and growth factors (Driancourt and Thuel, 1998). Therefore, researchers have been trying to enhance the environmental conditions by introducing these factors, which may improve the process of oocyte maturation and subsequent development of *in vitro* embryos (Ikeda et al., 2000). Sonic Hedge-

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hog (SHH) is a factor that seems to have an effect on oocyte maturation, proliferation and embryonic development in human and animal species (Nguyen et al., 2011). Sonic Hedgehog signaling pathway is mediated through a cell surface receptor system consisting of two proteins: the receptor Patched (Ptc) and its co-receptor Smoothened (Smo) (Hooper and Scott, 2005). In the absence of SHH, SMO is inhibited by PTC receptors resulting in inactivation of Glioma-Associated Oncogene (GLI) transcription factor. However, in the presence of SHH, SMO is released causing translocation of GLI protein to the nucleus, which could increase transcription of *Histone Deacetylase* (*Hdac*) genes in the nucleus (Ruiz i Altaba et al., 2002; Gueripel et al., 2006; Canettieri et al., 2010). Histone Deacetylase 1, 2 and 3 enzymes belong to the Hdac family and are encoded by *Hdac 1*, *2* and *3* genes in human, respectively. These proteins have notable roles in epigenetic programming, regulation of gene expression and transcription, cell cycle progression and development of embryos (Mehnert and Kelly, 2007). Several porcine studies have demonstrated that inclusion of exogenous SHH in the IVM or IVC media enhances oocyte maturation and development of parthenogenetic embryos (Nguyen et al., 2009b, 2010).

Hedgehog (Hh) molecular signaling pathway has been shown to be affected by many growth factors and small molecules such as Purmorphamine. Purmorphamine is a SHH signaling activating agonist that has been developed by Wu and his colleagues (Wu et al., 2002). This small molecule plays a crucial role in regulating the activity of SMO and PTCH receptors and GLI transcription factors (Nguyen et al., 2011). According to the positive effect of SHH on porcine oocyte maturation and embryo development (Nguyen et al., 2011), this idea has emerged that Purmorphamine may also affect ovine oocyte maturation process in the same way. Therefore, the aims of this study were to investigate the effects of this factor on ovine oocyte nuclear and cytoplasmic maturation, histone H4K12 acetylation, expression of *Hdac1*, *2* and *3* genes in ovine *in vitro* matured oocytes and also on development of parthenogenetic embryos. The findings of this study may be helpful in giving an insight for possible application of Purmorphamine in IVM media in order to enhance the quality of resulting ovine embryos.

2. Material and methods

All chemicals and media were obtained from Sigma-Aldrich Company (St Louis, MO, USA), unless otherwise specified. Experimental procedures for collecting the samples of the animals were approved by the review committee of the Stem Cell Technology Research Center (Tehran, Iran), and animal experimentations including ovine slaughter and ovarian sample collections were in agreement with the ethical commission.

2.1. Oocytes collection and in vitro *maturation (IVM)*

Ovaries from adult sheep (an Iranian breed called Shal) were randomly collected from an industrial abattoir and maintained at 35 °C in physiological saline, and transported to the laboratory within 2 h. Cumulus oocyte-complexes (COCs) were aspirated from medium-sized follicles (3–7 mm in diameter) with an 18 gauge needle. Then COCs were washed in Hepes-TCM 199 supplemented with 10% FBS (Gibco, Grand Island, NY, USA). Only COCs with at least two layers of cumulus cells and homogeneous ooplasm were collected under a microscope and washed three times in maturation medium. Ten to 12 COCs were randomly allocated to each 50 μl droplet of TCM 199 maturation medium supplemented with 10% FBS, 5 μg/ml LH, 5 μg/ml FSH, 1 μg/ml estradiol and different concentrations of Purmorphamine (250 ng/ml and 500 ng/ml Purmorphamine; (Santa Cruz Inc., California, USA). For control group, concentration of Purmorphamine was zero. Then COCs were cultured at 39 °C, in a 5% CO_2 incubator for 22 h (Thompson et al., 1995). The selected Purmorphamine concentrations were according to a previous study (Nguyen et al., 2009b, 2010).

2.2. Staining of oocytes nuclei

Immediately after IVM, oocytes were denuded using Hyaluronidase enzyme. In order to stain the nuclei and determine nuclear maturation rate, the oocytes were washed with 100 ml phosphate buffer saline, containing 1 mg PVA and then fixed in 4% paraformaldehyde for 30 min. Then, oocytes were stained in 10 μg/ml Hoechst solution for 5 min. Oocytes were inserted in 10 μl glycerol droplets, squashed on a glass slide and then observed under an epifluorescence microscope (Nikon, Tokyo, Japan) (Mohammadi-Sangcheshmeh et al., 2011). Based on maturation stages, oocytes were classified as being in germinal vesicle (GV), germinal vesicle break- down (GVBD), metaphase-I (MI), and metaphase-II (MII) according to Mohammadi-Sangcheshmeh et al. (2011).

2.3. Glutathione assessment in matured oocytes

Glutathione content was measured according to a previously method described by You and Park, (2010). Briefly, immediately after IVM, oocytes were denuded and incubated in a medium containing 100 ml phosphate buffer saline (PBS), 1 mg PVA (Polyvinyl Alcohol) and 10 μg/ml Cell Tracker Blue (Invitrogen, USA) for 30 min. The oocytes were subsequently washed in 100 ml PBS medium, containing 1 mg PVA and were placed into 10 μl glycerol droplets, observed under an epifluorescence microscope (Nikon, Tokyo, Japan) with UV filters and then all fluorescent images were saved as graphic files. The assessment of blue pixels was analyzed by ImageJ software (Version 1.45 s, National Institutes of Health, USA), and compared with control oocytes.

2.4. RNA extraction and cDNA synthesis

In order to analyze the effect of Purmorphamine on the expression of *Hdac1*, *2* and *3* genes in *in vitro*-matured oocytes, three biological replicates for each sample, each containing 10 denuded matured oocytes, were used for RNA extraction. The oocytes were washed in PBS droplets and transferred into Eppendorf tubes containing 1.5 ml cellular lysis buffer (Zuccotti et al., 2002). The RNA from oocytes was extracted using Qiazol reagent (Qiagen, Hilden, Germany). Complementary DNA was synthesized using First Strand cDNA Synthesis Kit (Fermentas, Germany) according to the manufacture's instruction. Briefly, 3 μg/ml random hexamer and 5 μl nuclease-free water were added to RNA, and tubes were placed in a thermal cycler (Bio-Rad, Hercules, CA, USA) for 5 min at 75 °C. Then tubes were placed on ice and 5 μl RT buffer 5X, 1 μl RT enzyme (200 u/μl), 3 μl dNTP (10 mM) and 0.25 μl RNA inhibitor (20 u/μl) were added in a 10 μl-total reaction volume. The reverse transcription program was as follows: 25 °C for 10 min, 37 °C for 15 min, 42 °C for 45 min and 72 °C for 10 min.

2.5. Real time PCR

Quantitative Real-time PCR was performed to assess the expression of *Hdac1*, *2* and *3* genes in three biological replicates (each cDNA in duplicates) for each sample using Rotor Gene Q instrument (Qiagen, Hilden, Germany). Reactions were in a total volume of 13 μl including 6.5 μl SYBR Green PCR master mix (Takara, Japan), 4.5 μl distilled water, 1 μl of forward and reverse primers (10 pmol/μl) and 1 μl cDNA. Tubes were transferred to the Rotor-Gene cycler. The amplification program was as follows: 3 min at 95° C for enzyme activation and 40 cycles of 5 s denaturation at

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