Gonadotropin-Mediated Dynamic Alterations During Bovine Oocyte Maturation In Vitro

Cui-Ling Lu, Tian-Ren Wang, Li-Ying Yan, Xi Xia, Xiao-Hui Zhu, Rong Li, Hong-Cui Zhao, Jie Yan, Tai-Lang Yin, Hong-Yan Jin, Yan Zhang, Wen-Xin Zhang, Huai-Liang Feng, and Jie Qiao

ABSTRACT

Gonadotropins have been widely used in human-assisted reproduction and animal science for the past four decades. However, the effects of gonadotropins on oocyte maturation at the molecular and biochemical levels are poorly understood. To determine the effects of gonadotropins (recombinant follicle stimulating hormone and urinary human menopausal gonadotropin) on oocyte maturation, we used the bovine oocyte in vitro maturation model. First, we studied the effects of increasing gonadotropin concentrations on nuclear maturation and mitochondrial function in oocytes. Gonadotropins at concentrations of 0.075 and 0.75 IU/ml improved nuclear maturation and increased inner mitochondrial membrane potential and ATP levels; however, there were no beneficial effects at concentrations of 7.5 and 75 IU/ml. Second, we studied the effects of increasing gonadotropin concentrations on the status of methylation in matured (MII) oocytes. Aberrant methylation and demethylation of H19, SNRPN, and PEG3 genes were observed in MII oocytes at all concentrations except 0.075 IU/ml. The expression of genes that function in spindle formation, cell cycle control, and methylation was also downregulated by high gonadotropin concentrations. In conclusion, we established the optimal gonadotropin concentration (i.e., 0.075 IU/ml) to be used for bovine oocyte in vitro maturation studies. These results may provide a guide for clinical stimulation protocols and help to reduce the risks associated with gonadotropin administration during in vitro fertilization treatment.

gonadotropin, methylation, mitochondria, oocyte

INTRODUCTION

Gonadotropins have been used for nearly 40 years in assisted reproductive technology (ART). Because they are critical for infertility treatment, it is important that any side effects from gonadotropin treatment be identified. Gonal-F (recombinant follicle stimulating hormone [rFSH]) and urinary human menopausal gonadotropin (HMG: FSH:luteinizing hormone [FSH:LH] = 1:1) are commonly used in vivo to stimulate human oocyte maturation and ovulation. A previous in vitro study has demonstrated a positive association between mammalian oocyte maturation and gonadotropin concentration [1], which was confirmed in other studies that showed gonadotropins could enhance porcine oocyte maturation in vitro in a dose-dependent manner [2], and only high concentrations (>75 IU/ml) of gonadotropins can elicit negative effects [3–7]. Although these studies provided support that high concentrations of gonadotropins have positive effects on mammalian oocyte maturation in vitro, the culture systems employed in human in vitro maturation (IVM) are still afflicted by no direct evidence. No proof is available regarding the fact that the concentration normally adopted in IVM media, approximately 0.1 IU/ml, is appropriate. Recent experiments in the cow suggest that even a 0.1 IU/ml concentration of FSH is too high to ensure the maintenance of cumulus-oocyte gap junction communication [8]. Therefore, the optimal gonadotropin concentration for oocyte maturation and developmental competence studies has not yet been determined, and neither has whether gonadotropins supplied in the culture system could have any harmful effects on oocytes at the cellular and molecular levels. To determine these factors, we used the bovine oocyte IVM model, which has been applied to human IVF research.

The method used to define oocyte quality during IVM is critical because oocyte development involves careful synchronization of nuclear and cytoplasmic maturation. Cytoplasmic maturation, which follows meiotic maturation, is much more difficult to assess microscopically, and abnormalities in maturation may go undetected. For example, abnormalities in organelle (e.g., mitochondria) function, maternal mRNA storage, protein synthesis, or imprinting can decrease oocyte developmental competence and affect embryo quality and development [9]; thus, oocyte competence can be understood if the signaling cascades in IVM oocytes are identified.

During oocyte maturation, an elevated ATP level is needed for the breakdown of the nuclear membrane. Increasing the dynamic behavior of the cytoplasm also requires an elevated ATP level [10]. The ability of mitochondria to balance ATP
supply and demand is one of the most critical factors for oocyte fertilization and embryo developmental competence [11]. The ability of mitochondria to maintain ΔΨm (the potential across the inner mitochondrial membrane) is consistent with normal levels of ATP generation [12, 13]. ΔΨm has been defined as a developmentally relevant factor in oocyte and embryo functions [11].

Assisted reproductive technology-conceived children with Angelman syndrome or Beckwith-Wiedemann syndrome have a more widespread disruption of genomic imprinting than spontaneously conceived children with these syndromes [14, 15]. Because imprint acquisition has been shown to occur relatively late in oogenesis [16–18], these imprints may be more susceptible to exogenous hormones. DNA methylation is an important epigenetic mechanism for differentially marking the parental alleles of imprinted genes [19]. It occurs at cytosine residues, mainly within CpG dinucleotides, and is catalyzed by a family of DNA methyltransferases [20]. Therefore, it is important to determine whether different concentrations of gonadotropins influence genomic imprinting of matured (MI) oocytes in vitro.

In the present study, we investigated the effects of increasing concentrations of gonadotropins (rFSH + HMG) on mitochondrial function, methylation, and other molecular events in oocytes. These results provide a guide for clinical stimulation protocols and help reduce the risks associated with gonadotropin administration during in vitro fertilization (IVF) treatment.

MATERIALS AND METHODS

Oocyte Collection and IVM

Approximately 30 bovine ovaries were collected from an abattoir in 20 separate visits and were transported to the laboratory in 0.9% (w/v) NaCl containing penicillin G (75 µg/ml) and streptomycin sulfate (50 µg/ml). The NaCl solution was maintained at 35°C–37°C during transport. Cumulus-oocyte complexes (COCs) were aspirated from follicles using a 20-gauge needle that was fixed to a 20-ml disposable syringe. The COCs with intact morphology were scanned at the equatorial plane with a Zeiss model laser scanning confocal microscope (Zeiss). Fluorescence intensity parameters were kept constant for all experiments. This experiment was repeated at least three independent times.

Peak MII Nuclear Maturation

To measure the cytoplasmic texture (no vacuoles) were scanned at the equatorial plane with a Zeiss model laser scanning confocal microscope (Zeiss). To measure the cytoplasmic texture (no vacuoles) were scanned at the equatorial plane with a Zeiss model laser scanning confocal microscope (Zeiss). To measure the cytoplasmic texture (no vacuoles) were scanned at the equatorial plane with a Zeiss model laser scanning confocal microscope (Zeiss). To measure the cytoplasmic texture (no vacuoles) were scanned at the equatorial plane with a Zeiss model laser scanning confocal microscope (Zeiss). To measure the cytoplasmic texture (no vacuoles) were scanned at the equatorial plane with a Zeiss model laser scanning confocal microscope (Zeiss).

Quantification of Mitochondrial Membrane Potential

Mitochondrial depolarization was measured by confocal laser scanning microscopy (Zeiss) using 5,5′,6,6′-tetrachloro-1,1′-stilbene-2,2′-disulfonic acid (TMRM) (Sigma). TMRM staining and fluorescence microscopy were carried out as described previously [21].

Determination of the Mitochondrial Membrane Potential by Confocal Laser Scanning Microscopy

For staining of mitochondria or quantification of the inner mitochondrial membrane potential, denuded oocytes were cultured in maturation medium containing 2 µM JC-1 dye (Molecular Probes, Life Technology) for 30 min in a humidified atmosphere of 5% CO₂ at 38.5°C. After washing two to three times, oocytes were fixed and stained with 5 µg/ml Hoechst 33258 (Sigma). To quantify mitochondrial activity, oocytes having a normal ooplasmic size and texture (no vacuoles) were scanned at the equatorial plane with a Zeiss model laser scanning confocal microscope (Zeiss). To measure the cytoplasmic texture (no vacuoles), a circle was drawn with the aid of Zen wide field image analysis software (Zeiss). Fluorescence intensity parameters were kept constant for all measurements. This experiment was repeated at least three independent times.

RNA Extraction and Quantitative Real-Time PCR

Total cDNA from a single oocyte was prepared as described previously [25]. In brief, a pipette was used to select a single cell and transfer it into lysis buffer. The reverse transcription reaction was performed directly on the whole-cell lysate sample. A poly(A) tail was added to the 3′ end of the first-strand cDNA by terminal deoxynucleotidyl transferase, and PCR (20 cycles) was performed to amplify the single-cell cDNA. Quantitative real-time PCR (Q-PCR) was performed using an ABI model 7700 real-time PCR system (ABI). Three different samples were analyzed for each gene, and RPS24 was used as a reference gene.

Statistical Analysis

Statistical analyses were performed using SPSS 19.0 for Windows (Analytical Software). Data were presented as means ± SD, and the significance between groups was compared by one-way ANOVA. The melting rate was analyzed by chi-square test. A probability level of P < 0.05 or P < 0.01 was considered to be statistically significant, and groups without a common letter were significantly different from each other.

RESULTS

Cumulus Expansion and Nuclear Maturation of Oocytes Treated with Gonadotropins

To determine the effects of different concentrations of gonadotropins on the maturation of bovine COCs, cumulus expansion (n = 100 ± 5 per group) and nuclear maturation were observed under a microscope after 26–28 h in vitro. In groups treated with 0 and 7.5 IU/ml gonadotropins, cumulus expansions were lowest (CEIs, 2.31 ± 0.16 and 2.40 ± 0.14, respectively), whereas in the 0.075 and 0.75 IU/ml groups, cumulus expansions were highest (CEIs, 3.56 ± 0.09 and 3.48 ± 0.06, respectively). There was almost no cumulus expansion in bovine oocytes treated with 75 IU/ml gonadotropins (CEI, 0.4 ± 0.08; Fig. 1A). In agreement with these results, the nuclear maturation rate was highest in bovine MII oocytes.
GONADOTROPIN-MEDIATED OOCYTE MATURATION IN VITRO

TABLE 1. Oligonucleotides used for nested PCR and Q-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nested PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H19</td>
<td>GATTTTTTAGTTTATGGTTTTT</td>
<td>ATAAAAACCTCCCTAAATCC</td>
</tr>
<tr>
<td>In</td>
<td>GAGGTGTTGGGTGAGGATGAA</td>
<td>TCCCTGACCCACTTCAGCA</td>
</tr>
<tr>
<td>SNRPN</td>
<td>AAAAAAATATCACCACACAG</td>
<td>GGGTTGGTGTGATATTATT</td>
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<tr>
<td>In</td>
<td>GTTTTTTTGGTGGAGGAGG</td>
<td>AAAGAAATTTATTTCCCA</td>
</tr>
<tr>
<td>PEG3</td>
<td>GTAATGGTTATTTATGGTTTGG</td>
<td>ACCCTAATCCCCCAATCCA</td>
</tr>
<tr>
<td>In</td>
<td>GTGGGATGATTAGTGGTGT</td>
<td>ACCCTAATCCCCCAATCCA</td>
</tr>
<tr>
<td>Q-PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPS24</td>
<td>AAGACCACACAGATGCTATCAC</td>
<td>TGCCAGGGCACTGCTTTG</td>
</tr>
<tr>
<td>CCNB1</td>
<td>ACCGCCGATGGTAAAGGAAAG</td>
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<td>CDDH1</td>
<td>GTGTCGACTGGATGGGATGTCG</td>
<td>CTTCCGACAGTGCAGCAGG</td>
</tr>
<tr>
<td>MAD2L1</td>
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</tr>
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<td>NDC80</td>
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<td>BUB3</td>
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<tr>
<td>DNM3B</td>
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<td>TGGATCTGGCCCTGTCCTTA</td>
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A High Dose of Gonadotropins Affects the Inner Mitochondrial Membrane Potential in Oocytes

The JC-1 dye remains as a monomer and fluoresces green when the ΔΨm of low-polarized mitochondria is <100 mV. However, the JC-1 dye forms J-aggregates and fluoresces red when the ΔΨm of highly polarized mitochondria is >140 mV [26]. Oocyte ΔΨm can be determined by the relative level of red to green fluorescence emission. The relative ΔΨm increased in oocytes treated with 0.075 and 0.75 IU/ml gonadotropins compared with those cultured in the absence of gonadotropins (1.79 and 1.69 vs. 1.29; n = 73, 70, and 65, respectively). By contrast, the relative ΔΨm decreased in MII oocytes treated with 75 IU/ml gonadotropins (0.71; n = 71) compared with those cultured in the absence or presence of 0.075 or 0.75 IU/ml gonadotropins. There was no significant difference in the relative ΔΨm between groups treated with 0 and 7.5 IU/ml gonadotropins (1.29 vs. 1.14; n = 65 and 68, respectively; Fig. 2).

A High Dose of Gonadotropins Compromises Mitochondrial Function in Oocytes

The ATP level in oocytes cultured in the absence of gonadotropins was 1.26 ± 0.07 pmol (n = 95). It increased to 1.44 ± 0.04 pmol (n = 108) and 1.47 ± 0.09 pmol (n = 121) in oocytes treated with 0.075 and 0.75 IU/ml gonadotropins, respectively. However, the ATP level sharply decreased to the level close to the absent gonadotropin group when the concentration of gonadotropins further increased to 7.5 IU/ml (1.23 ± 0.12; n = 128). By contrast, the ATP level in oocytes treated with 75 IU/ml gonadotropins decreased to 0.74 ± 0.13 pmol (n = 129; Fig. 3).

Effects of Gonadotropins on DNA Methylation of Imprinted Genes in MII Oocytes

To determine whether gonadotropins at increasing concentrations affect imprinting establishment and maintenance, the methylation profiles of three differentially methylated regions (DMRs; H19/IGF2 intergenic imprinting control region [ICR], PEG3, and SNRPN promoter DMRs) were analyzed by bisulfate treatment and sequencing. Because of its close proximity to the H19 gene, the H19/IGF2 intergenic ICR was defined as H19. In oocytes, H19 is normally demethylated, whereas SNRPN and PEG3 DMRs are methylated. The methylation status in individual clones of H19 DMR1 is shown in Figure 4. In 0 and 0.075 IU/ml treatment groups, the methylation status of H19 was very low; almost all CpG sites (155 of 160 and 226 of 240, respectively) were unmethylated. However, the degree of methylation increased to 26.18% (83 of 317) in the 0.75 IU/ml group, 58% (116 of 190) in the 7.5 IU/ml group, and 57.36% (113 of 197) in the 75 IU/ml group. The methylation status in individual clones of SNRPN DMR is also presented in Figure 4. In 0 and 75 IU/ml treatment groups, the methylation rates were 61.54% (184 of 299) and 60.34% (216 of 358), respectively. However, the methylation rates in the 0.075 and 0.75 IU/ml treatment groups were 91.15% (484 of 531) and 89.98% (377 of 419), respectively, which was significantly higher than that of the 7.5 IU/ml group (85.87%; 231 of 269). All clones of PEG3 in 0, 0.075, and 0.75 IU/ml treatment groups were almost completely methylated at virtually all sites, with a few stochastic methylation errors (98.61%, 98.31%, and 97.92%, respectively), whereas the cytosine methylation rates in the 7.5 and 75 IU/ml treatment groups (92.06%, 116 of 126; and 85.83%, 159 of 179, respectively) were lower than those of the other groups. The decrease in methylation was statistically significant (Fig. 4).
Relative Poly(A)$^+$ mRNA Abundance in a Single Oocyte Cultured in the Absence or Presence of Gonadotropins

The relative abundance of several poly(A)$^+$ mRNA transcripts that are critical for meiosis and DNA methylation in MII oocytes was determined. The protein encoded by CCNB1 gene is involved in mitosis and forms the maturation-promoting factor. The proteins encoded by BUB1, CDH1, and MAD2L1 are regulators of spindle assembly during meiosis. We used single-cell cDNA amplification and Q-PCR to quantify these genes’ expression in oocytes cultured in the absence and presence of gonadotropins. In general, mRNA transcripts increased in oocytes treated with 0.075 and 0.75 IU/ml gonadotropins but decreased in those treated with 7.5 and 75 IU/ml gonadotropins. NDC80, a regulator of microtubule-kinetochore attachment, was upregulated in oocytes treated with 0.075 and 0.75 IU/ml gonadotropins. We also detected genes involved in the maintenance of methylation marks (DNMT1a/b) and de novo methylation (DNMT3a/b) expression. These genes were all significantly downregulated in the 7.5 and 75 IU/ml treatment groups compared with the other groups. The expression of DNMT1a was also strongly upregulated in the 0.075 and 0.75 IU/ml treatment groups (Fig. 5).

DISCUSSION

Most studies in the literature focus on the effects of gonadotropins on ovarian function, embryo development, and oocyte quality after superovulation [27-30]; however, there are few reports on the effects of gonadotropins on MII oocytes. Therefore, our study concentrated on the effects of increasing concentrations of gonadotropins (rFSH + HMG) on the intracellular events during oocyte maturation in vitro. First, we studied the effects of increasing concentrations of gonadotropins on nuclear maturation, mitochondrial function,
FIG. 2. Analysis of the inner mitochondrial membrane potential ($\Delta\Psi_{\text{mit}}$) in oocytes cultured in the absence or presence of increasing concentrations of gonadotropins. A) Images of oocytes from different treatment groups ($n = 70 \pm 5$) stained with the JC-1 dye. Green and red fluorescence correspond to JC-1 monomers and aggregates, respectively. Bar = 20 µm. B) Relative ratio of red to green fluorescence in the cytoplasm of individual oocytes. Results are expressed as arbitrary units (a.u.). Bars indicate SD. Groups without a common letter are significantly different from each other ($P < 0.05$).
and ATP production in MII oocytes. Second, we studied the effects of increasing concentrations of gonadotropins on the status of methylation and gene expression in MII oocytes. Our results provide a better index by which gonadotropin-stimulated oocyte competence can be assessed, and may help to reduce the risks associated with gonadotropin administration during IVF treatment in humans.

Different concentrations of gonadotropins have been used for IVM. For example, a previous study has shown that 1 IU/ml rFSH and 10 IU/ml rLH enhance the maturation of bovine oocytes to the MII stage and improve human embryo developmental competence [4]. Another group cultured bovine oocytes in vitro using rFSH with concentrations up to 0.5 μg/ml (about 7 IU/ml) [7]. Two previous reports suggested that a 7.5 IU/ml concentration of gonadotropins can improve the blastocyst rate. All of the concentrations (include over high dosage 75 IU/ml) seemed normal in mitochondrial contribution, cortical granule migration, and global DNA methylation status, except that the concentration of 75 IU/ml induces spindle and chromosomal configuration abnormalities [5, 6]. In addition, another study demonstrated gonadotropins at concentrations up to 40 IU/ml can enhance oocyte maturation in vitro, without any of the negative effects observed in the above index [2]. These results are all based on a morphological study that did not examine the molecular changes inside MII oocytes, as the current study does.

In this study, gonadotropins at concentrations of 0.075 and 0.75 IU/ml increased cumulus expansion, nuclear maturation, mitochondrial function (i.e., the ΔΨm), and ATP production, whereas the 7.5 IU/ml gonadotropin concentration had no more positive effects compared with the no gonadotropin group. By contrast, oocyte maturation was negatively affected in the group treated with a concentration of 75 IU/ml, which is indicative of cytotoxicity. A decrease in the inner mitochondrial membrane potential and ATP level can affect the energy of a cell, which in this case may have disrupted oocyte maturation and subsequently resulted in poor oocyte quality, a low fertilization rate, and abnormal embryonic development [31, 32]. Therefore, low concentrations of gonadotropins (0.075 and 0.75 IU/ml) can enhance nuclear maturation and mitochondrial function in oocytes, whereas a high concentration (7.5 IU/ml) has no additional positive effects.

Besides nuclear maturation in oocytes, there is also a variety of other processes that take place in the cytoplasm that are required for complete developmental competence. More studies are limited to the effects of gonadotropins on the imprinting genes in oocytes after superovulation. The DMR methylation occurs during imprint erasure (demethylation; i.e., the paternal H19 methylation imprint is not erased) or imprint establishment (de novo methylation) and maintenance during oogenesis (i.e., SNRPN or PEG3 remain unmethylated in oocytes). One study observed normal methylation patterns at SNRPN, PEG3, and H19 loci in pooled oocytes from superovulated female mice [32]. Another study found an aberrant gain of DNA methylation at the normally unmethylated H19 locus in pooled mouse oocytes [33]. In addition, human oocytes from women undergoing multiple hormone treatments exhibited aberrant imprinting at the H19 loci [34]. Superoovulation also affects the imprinting of SNRPN, PEG3, and H19 genes in blastocyst-stage embryos in a hormone dose-dependent manner. A greater frequency of aberrant methylation occurs at the high concentrations of hormones [33, 35]. These studies illustrated that superovulation can lead to production of oocytes with or without affecting primary imprinting, and thus there may need to be more investigation on gonadotropins’ effects during use in ARTs [36–38].

We observed a loss of SNRPN and PEG3 maternal methylation and a gain of H19 methylation in MII oocytes after IVM. This aberrant imprinting also occurred in a dose-dependent manner, with perturbations occurring more frequently at high gonadotropin concentrations (7.5 and 75 IU/ml) compared with a low concentration (0.75 IU/ml). Our results were obtained by eliminating all biases based on oocyte pools and undetected cumulus cell contamination. We conclude that oocytes are susceptible to gonadotropin stimulation, and 0.075 IU/ml is the optimal concentration for oocyte imprinting during IVM.

Because of the nature of oocytes, the majority of cytoplasmic mRNA is in storage for later development and fertilization. An increase in the rate of oocyte maturation in vitro by different gonadotropin concentrations may affect the synthesis and storage of sufficient amounts of RNA. Two laboratories respectively reported the total protein content reduction in bovine or human MII oocytes after IVM compared with oocytes matured in vivo [39]. Although a decrease in the total protein in IVM oocytes may be due to transcriptional or translational defects that are currently unknown, it is certain that the decrease in the RNA level in MII oocytes is not related to good oocyte quality.

The functions of cell cycle checkpoint gene CCNB1, which forms the maturation-promoting factor, as well as spindle assembly checkpoint genes BUB1, CDH1, MAD2L1, and NDC80, in cell meiosis expression were determined. The expression of these genes increased in oocytes treated with low gonadotropin concentrations (0.075 and 0.75 IU/ml), which is indicative of oocyte maturation. By contrast, the expression of these genes decreased in oocytes treated with high concentrations of gonadotropins. Therefore, the decrease in gene transcripts, especially those of the meiosis-related genes, in high-dosage groups should be closely related to poor oocyte competence. These results may also partially explain the findings from previous reports that demonstrated an increase in abnormal spindle formation and chromosome alignment in oocytes treated with high gonadotropin concentrations [2, 6, 40]. The lower abundance of the methylation marks (DNMT1a/b) and de novo methylation (DNMT3a/b) mRNA transcripts in high-dose groups may explain the aberrant methylation profiles observed in Figure 4. On the whole, the lower abundance of cell cycle, spindle assembly checkpoint, and methylation-related genes in oocytes treated with high gonadotropin

![FIG. 3. The effect of increasing concentrations of gonadotropins on the ATP level in individual oocytes. Data are presented as mean ± SEM. Different letters indicate statistically significant differences (P < 0.05 or P < 0.01).](image-url)
FIG. 4. Methylation patterns of representative DMRs in matured oocytes cultured in the absence or presence of increasing concentrations of gonadotropins. Each row represents the unique methylation profile of an individual allele (DNA molecule) after Biqanalyzer software analysis (Max-Planck-Institut). The frequency of the methylation is indicated below. Filled and open circles indicate methylated and unmethylated CpG sites, respectively. The analyzed H19 ICR contains 20 CpG sites. These are different methylated alleles with a paternal germ line methylation pattern. The analyzed SNRPN DMR contains 30 CpG sites. These are completely methylated alleles with a maternal germ line methylation pattern. The analyzed PEG3 DMR contains 19 CpG sites. These are completely methylated alleles with a maternal germ line methylation pattern. Different superscripts indicate statistically significant differences (P < 0.05 or P < 0.01).
concentrations (7.5 and 75 IU/ml) was indicative of their poor quality.

In conclusion, we established the optimal gonadotropin concentration (i.e., 0.075 IU/ml) to be used for bovine oocyte IVM studies. At a gonadotropin concentration of 7.5 IU/ml, oocyte quality was negatively affected. These results may provide a guide for clinical stimulation protocols and help to reduce the risks associated with gonadotropin administration during IVF treatment.

REFERENCES