Expression pattern of transcription factors during zygotic genome activation in buffalo (Bubalus bubalis) embryos produced in vitro

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Following fertilization, the early embryo is transcriptionally silent and early development is directed by the complement of maternally-inherited mRNAs and proteins. At some point, however, a maternal to embryonic transition occurs, in which further development is directed by the zygotic transcripts. So to determine this transition stage, the quantitative expression pattern of HMGN-2 and CREB genes involved in transcription activation, and two other genes, EHMT-1 and EHMT-2, involved in epigenetic modification were evaluated in different embryonic stages with and without different concentration of α-amanitin. The results of the present study showed that the mRNA transcripts of EHMT-1, EHMT-2, HMGN-2 and CREB were present in immature, in vitro-matured oocytes, and in embryos at 2-, 4-, 8- to 16-cell, morula and blastocyst stages. However, all these four genes showed very low levels around the 8- to 16-cell stage, and their levels increased by at least 80% at the blastocyst stage compared to those initially found in immature GV or in vitro matured oocytes, which indicates that the zygotic genome activation occurs at 8-16 cells stage in in vitro produced buffalo embryo.

Keywords: Developmental block, in vitro fertilization, transcription factors, zygotic genome activation

Introduction

The ultimate goal of in vitro embryo culture systems is to perfectly mimic the in vivo condition of oocyte maturation, fertilization and embryo development. The in vivo development is more complex than the standard in vitro cell culture systems because of the various environments through which the gametes and embryos pass during in vivo development. Improvements in media and other culture conditions have led to increase in percentage of the fertilized oocytes to the blastocyst stage but the great majority of buffalo zygotes stop developing within a few cell cycles after initiating cleavage. Generally, embryos cultured in vitro fail to develop past the 8-16 cell stage, whereas embryos those survive in culture up to 16-cell stage or later stages frequently develop to compact morula and blastocysts1-2. This suggests the existence of a “block” during in vitro development at different stages in different species.

After fertilization, the early embryo development is carried by the maternally-inherited mRNAs and proteins. At some point, however, a maternal to embryonic transition occurs in which further development is directed by the zygotic transcripts. Zygotic gene activation (ZGA) is absolutely essential for continued development. There is a possibility that changes in chromatin structure, rather than changes in the intrinsic activity of the transcription machinery underlie ZGA in the buffalo3. Several speculations are put forth concerning how changes in chromatin structure may actively regulate ZGA, since very little is known about the molecular basis for ZGA in the mammal.

Distinct epigenetic modification events regulate gene expression and chromatin structure during the period between the immature oocyte and the blastocyst. Throughout this developmental period, important methylation fluctuations occur on genomic DNA and histones. Finding single or combinations of factors, which are at work during this period is essential to understand the entire epigenetic process. Histone methylation acts as an epigenetic regulator of chromatin activity through the modification of arginine and lysine residues on histones H3 and H4. In the case of lysine, this includes the formation of mono-, di- or tri- methyl groups, each of which is presumed to represent a distinct functional state at the cellular level4. Another transcription factor HMG, a
non-histone chromosomal protein decompacts the higher order chromatin structure and promotes the binding of nuclear factor, so that transcription initiation complex can start transcription of several developmentally important genes. Due to the inability of activation of transcription of important developmental genes, the developmental block arises. This block suggests that embryonic transcription is particularly sensitive to culture conditions. Finding the correct expression level at the different stages of the embryo development will not only help to identify the stage at which the developmental block occurs, but it will also reveal the importance and state of the chromatin modification. Quantitative gene expression data with the a-amanitin, a transcription inhibitor, add further evidence that the corresponding mRNA content of any gene is not resulted from the new transcription, but it is transported from the mature oocyte to the successive stages of the embryonic development. Therefore, the quantitative expression pattern of high mobility group nucleosome binding protein-2 (HMGN-2) and cAMP response element binding protein (CREB) genes that are involved in transcription activation, and two other genes, euchromatic histone-lysine N-methyltransferase (EHMT)-1 and EHMT-2 that are involved in epigenetic modification, were evaluated in different embryonic stages with and without different concentration of a-amanitin.

Materials and Methods

All chemicals, culture media and antibiotics were purchased from Sigma Chemical Company (St. Louis, MO, USA). All plasticware used were of tissue culture grade and obtained from Nunc (Rockslide, Denmark) unless otherwise indicated.

In Vitro Maturation and Fertilization of Buffalo Oocytes

Buffalo ovaries were collected from Delhi slaughter house and transported to the laboratory within 4-5 h of slaughter in warm isotonic saline (32-37°C) containing 400 IU/mL penicillin+50 µg/mL gentamycin sulphate. Follicular (2-8 mm diam) oocytes were aspirated with a 18-gauge needle in aspiration medium containing TCM-199+0.6% bovine serum albumin (BSA)+50 µg/mL gentamycin sulfate. Aspirated oocytes were washed 5-6 times in the washing medium containing TCM-199+10% FBS+0.81 mM sodium pyruvate+50 µg/mL gentamycin sulfate. Only those cumulus-oocytes complexes (COCs) had a compact and unexpanded cumulus mass with ≥2 layers of cumulus cells and with homogenous evenly granular ooplasm were used for IVM. The oocytes were washed 2-3 times in IVM medium (TCM-199 supplemented with 10% FBS, 5 µg/mL pFSH, 0.81 mM sodium pyruvate and 5% buffalo follicular fluid) and groups of 15-20 COCs were placed in 100 µL droplets of the IVM medium, overlaid with sterile mineral oil in 35 mm Petri dishes, and cultured for 24 h in a humidified CO2.

The spermatozoa used for IVF throughout the study were from the same donor and had been tested for IVF earlier. The spermatozoa were prepared for fertilization as described earlier. Briefly, two straws of frozen-thawed ejaculated buffalo semen were washed twice with washing Bracket and Oliphant (BO) medium (BO medium containing 10 µg/mL heparin, 137.0 µg/mL sodium pyruvate and 1.942 mg/mL caffeine sodium benzoate). The pellet was resuspended in around 0.5 mL of the washing BO medium. The in vitro matured oocytes were washed twice with the washing BO medium and were transferred to 50 µL droplets (15-20 oocytes/droplet) of the capacitation and fertilization BO medium (washing BO medium containing 10 mg/mL fatty acid-free BSA). The spermatozoa in 50 µL of the capacitation and fertilization BO medium (2-4 million spermatozoa/mL) were then added to the droplets containing the oocytes, covered with sterile mineral oil and placed in a CO2 incubator (5% CO2 in air) at 38.5°C for 18 h for IVF.

After the end of sperm-oocyte incubation, the cumulus cells were washed off the oocytes by gentle pipetting. The oocytes were then washed several times with modified Charles Rosenkrans medium with amino acids (mCR2aa) containing 0.6% BSA and cultured in this medium for 48 h post insemination. After which, the embryos were cultured in IVC (in vitro culture) medium (mCR2aa+0.6% BSA+10% FBS) and then cultured in 100 µL droplets of this medium on original beds of granulosa cells (co-culture) for up to 9 d post-insemination in a humidified CO2 incubator (5% CO2 in air) at 38.5°C. The medium was replaced with 50% of fresh IVC medium every 48 h.

Effects of a-Amanitin on Embryo Development

To see the effect of a-amanitin on embryo development, two different concentrations, viz., 15 and 25 µg/mL, of a-amanitin were tested on in vitro produced buffalo embryos.
RNA Isolation and Reverse Transcription (RT)

To detect the expression of developmentally regulated genes like HMGN-2, CREB, EHMT-1 and EHMT-2, qRT-PCT was performed in different developmental stages of embryo. For this total RNA was isolated from different developmental stages (immature, mature oocytes, 2-cell, 4-cell, 8-16-cell, morula and blastocyst) and α-amanitin treated buffalo embryos using RNeasy kit (QIAGEN, Hilden, Germany) as per manufacturer’s instructions. The protocol was standardized for 20 oocytes and 10 embryos of each stages and RNA was dissolved in 10 µL DEPC (diethyl pyrocarbonate) water. The concentration of isolated RNA was measured using spectrophotometer (ND-1000, NanoDrop) and diluted to 50 ng/µL. The RNA was reverse transcribed to cDNA using Sensiscript kit (QIAGEN, Hilden, Germany) as per manufacturer’s instructions.

Quantification of RT-PCR Products

The quantification of RT-PCR products was done by using Roche Light cycler apparatus 480 (Roche Applied Science, Mannheim, Germany). For real time PCR, cDNA from mature oocytes was used for standard curve preparation. For that 1:10 serial dilution of the template was used. Real time PCR was performed using SYBR green (Roche Applied Science, Mannheim, Germany) as described by the kit protocol. The real time PCR product specificity was confirmed by the analysis of the melting curve given by the Light cycler software (Roche). Primers and their amplifying condition are mentioned in Table 1.

Standard Curve

For relative quantification by real time PCR, standard curves with high efficiency were prepared for all genes examined. These were prepared by plotting log concentration versus Ct (the point at which the fluorescence crosses the threshold). The Ct value was calculated by the software for each target gene from the amplification curves. For a standard reaction, efficiency = 2.00 is required, which means that there is a doubling of PCR product in every successive cycle. For calculating the relative mRNA abundance of the target gene and reference gene, the software compares the Ct values of the target gene and reference gene with external standard curves.

Statistical Analysis

Data was analyzed using SYSTAT 7.0 (SPSS Inc., USA) after arcsine transformation. Differences between mean percentages were analyzed by one way ANOVA, followed by Fisher’s LSD test for pair-wise comparison of means.

Results

In Vitro Embryos Production

A total of 876 good quality COCs (Fig. 1A) were cultured (in vitro maturation medium) for in vitro maturation (Fig. 1B). The per cent cleavage (Figs 1C & ID) and blastocyst (Fig. 1E) production rate obtained were 57.2±1.61 and 13±0.70, respectively in control group without α-amanitin (Table 2). On the other hand, 861 COCs were cultured in IVC medium containing α-amanitin in two different concentrations

Table 1—PCR primers used for buffalo real time quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene product*</th>
<th>Primer 5′−3′</th>
<th>Reaction condition</th>
<th>Product size (bp)</th>
<th>Acc. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGN-2</td>
<td>f-CCAAGAGAAAGGCTGAAGG</td>
<td>40 Cycle at 51°C</td>
<td>267</td>
<td>AF022987</td>
</tr>
<tr>
<td></td>
<td>r-ACCTGGCATCTCCAGGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CREB</td>
<td>f-CCAAGAGAGGAGCAATACAC</td>
<td>40 Cycle at 54°C</td>
<td>344</td>
<td>DQ487022</td>
</tr>
<tr>
<td></td>
<td>r-GACACTCTCGTGCTGCCTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHMT-1</td>
<td>f-CCAAGAGAAAGGCTGAAGG</td>
<td>40 Cycle at 60°C</td>
<td>189</td>
<td>NM174121</td>
</tr>
<tr>
<td></td>
<td>r-ACCTGGCATCTCCAGGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHMT-2</td>
<td>f-CCAAGAGAAAGGCTGAAGG</td>
<td>40 Cycle at 60°C</td>
<td>175</td>
<td>K00622</td>
</tr>
<tr>
<td></td>
<td>r-ACCTGGCATCTCCAGGC</td>
<td></td>
<td></td>
<td>BC016624</td>
</tr>
<tr>
<td>18S-RNA</td>
<td>f-GAGAAACGGGCTAAGGCTCA</td>
<td>40 Cycle at 60°C</td>
<td>337</td>
<td>AF176811</td>
</tr>
<tr>
<td></td>
<td>r-GGACACTCTCCAGGCATCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>f-ATCCACCATCTCCAGGAGC</td>
<td>40 Cycle at 60°C</td>
<td>495</td>
<td>AF077815</td>
</tr>
<tr>
<td></td>
<td>r-TAGGAACACGGGAAGGCATG</td>
<td></td>
<td></td>
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</tbody>
</table>

*HMGN-2, High mobility group protein N-2; CREB, c-AMP response element binding protein; EHMT-1 & 2, Euchromatic histone-lysine N-methyltransferase 1 and 2; 18S-RNA, 18S ribosomal RNA; GAPDH, Glyceraldehydes-3-phosphate dehydrogenase.
15 and 25µg/mL. The per cent cleavage and blastocyst production rate obtained with 15 µg/mL of α-amanitin was 53.0±2.02 and 7.6±1.2, respectively. At the higher concentration of 25 µg/mL, none of the α-amanitin-treated presumed zygotes reached the blastocyst stage. (Table 2)

<table>
<thead>
<tr>
<th>α-Amanitin conc. (µg/mL)</th>
<th>No. of oocytes taken (n)</th>
<th>No. of cleaved embryos n (%)</th>
<th>Blastocyst n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>876</td>
<td>572</td>
<td>130</td>
</tr>
<tr>
<td>15</td>
<td>422</td>
<td>(57.2±1.61)</td>
<td>(13±0.70)</td>
</tr>
<tr>
<td>25</td>
<td>439</td>
<td>(53.0±2.02)</td>
<td>(7.6±1.2)</td>
</tr>
</tbody>
</table>

Data from 5 trials; values are mean±SEM. Values within the same column with different superscripts differ significantly (P< 0.05).

### HMGN-2 and CREB mRNA Transcript Level in Oocytes and Embryos

The relative mRNA abundance of HMGN-2 and CREB genes is presented in Figs 2A and B, respectively in immature (IMO) and in vitro matured (MO) oocytes and in embryos at 2-cell (C2), 4-cell (C4), 8- to 16-cell (C8-16), morula (MR) and blastocyst stage (BT). For both genes, the level of mRNA was highest in the oocytes that tended to decrease during the first few cell divisions until the 8-cell stage, where it reached significantly lower level. In the subsequent cell divisions, the level remained stable until the blastocyst stage, where the level was significantly higher than those at the 8-cell stage. The HMGN-2 mRNA transcripts were nearly
eliminated in the 8-cell stage embryos, whereas a significant increase was observed between the 8-cell and the blastocyst stages.

**EHMT-1 and EHMT-2 mRNA Transcript Levels in Oocytes and Embryos**

The relative mRNA abundance of *EHMT-1* and *EHMT-2* genes are presented in Figs 3A and B, respectively in immature (IMO) and in vitro matured (MO) oocytes, and in embryos at 2-cell (C2), 4-cell (C4), 8- to 16-cell (C8-16), morula (MR), and blastocyst stages (BT). In the present study, both genes showed a high expression in immature and in vitro matured oocytes and very low level in the 8-16 cell stage.

**Comparison of mRNA Transcript Levels in α-Amanitin Treated Embryos**

Since the embryonic development did not proceed beyond the 8-16 cells stage following the treatment with α-amanitin at 25 µg/mL concentration, the mRNA transcript level of the all four genes were compared between the control and α-amanitin-treatment groups in embryos at the 2-, 4- and 8- to 16-cell stages. All the four genes did not show any significant difference between the two groups at all the embryonic stages examined (Figs 4A & B; 5A & B). These results suggested that the mRNA transcript of *EHMT-1*, *EHMT-2*, *HMGN-2* and *CREB* genes were present in the cytoplasm and these were not transcribed from the DNA in the early embryonic
developmental stages, which is consistent with the reports of in vitro fertilized embryos in cattle\textsuperscript{12}.

**Discussion**

The expression pattern of *HMGN-2* in cattle has been found similar to that observed in buffalo in the present study, except that no increase in mRNA level was observed at the blastocyst stage in cattle. A possible implication has been suggested for *HMGN-2* in early bovine embryonic development and genome activation\textsuperscript{13}. The mRNA levels of the four genes (*HMGN-2, CREB, EHMT-1 & EHMT-2*) were higher in the oocytes as compared to that in embryos at all stages and that did not increase at the blastocyst stage after the genome activation. This strongly suggests an important role for these factors in the early developmental stages of bovine embryos. Earlier expression studies with *HMGN-2* in mouse embryos showed that its mRNA was present in high quantities in the mouse GV oocytes. However, its level decreased till the 2-cell stage embryos (at the MZT) and then increased from the 4-cell to the blastocyst stage\textsuperscript{14}. In the case of *CREB* and *HMGN-2* gene, the first reduction in mRNA level was observed between the MII stage oocyte and the 2-cell stage embryo in bovine embryonic development\textsuperscript{15}. The expression patterns of *CREB* illustrated in Fig. 2 B was slightly different from the patterns observed in *HMGN-2*. This suggests possible production of proteins for pre-MZT transcription and the following steps of development. The other mRNA decrease happened between the 4- and 8-cell stage, which could be related to the translation of new proteins implicated in embryonic gene activation. Considering that *CREB* was present during early embryo development, one could propose that it played a significant role in transcription before or during the MZT.

The mRNA expression levels of *EHMT-1* and *EHMT-2* were similar in their general patterns, although levels of *EHMT-1* tended to remain constant between the oocyte and the 4-cell stage embryos before diminishing significantly at the 8-cell stage. In bovines, these genes have been reported to reach their highest relative peak levels in MII oocytes, whereas their lowest levels occurred around the MET stage, *i.e.*, at the 8- to 16-cell stage\textsuperscript{15}. The mRNA expression of *EHMT-2* exhibited a significant reduction after the MII and the 2-cell stage. These similar patterns are not surprising because *EHMT-1* and *EHMT-2* were involved in a heteromeric complex critical for the mono- and di-methylation of H3-K9 in euchromatin\textsuperscript{16}. The di-methylation state of H3-K9 in mouse oocytes and zygote reveals that only the maternal histone H3 shows clear staining, whereas the male pronucleus remains unstained. This asymmetrical methylation is maintained even in the 2-cell embryo and only becomes symmetrical in the 4-cell embryo\textsuperscript{17-18}. Methylation of H3-K9 is usually linked to the transcriptional repression of developmental genes and, in EHMT-2 deficient mice, lack of euchromatic H3-K9 methylation leads to early embryo mortality\textsuperscript{19}. Shi et al\textsuperscript{19} also proposed that a complex that included HDAC1/2 (histone deacetylases1/2) would remove the acetyl group from the histone tails of active chromatin, allowing EHMT-1 and EHMT-2 to methylate H3-K9. HDAC1 and HDAC2 transcripts were also detected in these stages in buffalo oocytes and embryos\textsuperscript{20}. The hypothesis of Shi et al\textsuperscript{19} could also apply to the oocytes, where HDAC1/2 could deacetyl ate the transcriptionally active chromatin of the GV oocytes during maturation, allowing EHMT-1 and EHMT-2 to methylate H3-K9, which would result in a repressive chromatin state.

We have demonstrated that the 4 genes (EHMT-1, EHMT-2, HMGN-2 & CREB) examined in the present study were present in buffalo immature and in vitro-matured oocytes and in embryos at 2-, 4, 8- to 16-cell, morula and blastocyst. All these four genes showed low levels around the 8- to 16-cell stage but their levels increased by at least 80% at the blastocyst stage compared to those initially found in immature GV or in vitro-matured oocytes. This confirms that the cytoplasm pool of all these four genes, which was inherited from the oocyte, degraded at this stage and the very high levels in the next stages, like in the blastocyst, apparently synthesized from the embryonic genome.

\textit{α}-Aminitin is a cyclic peptide of eight amino acids, which interacts with the bridge helix in RNA polymerase (pol) II and interferes with the translocation of RNA and DNA needed to empty the site for next round of RNA synthesis. As such, it is a potent RNA pol II inhibitor but works only mildly on RNA pol III and has no effect on RNA pol I. In the presence of the \textit{α}-amanitin, embryo progressed through initial stages like 2-cell, 4-cell and 8-16 cell, which certainly required proteins inevitable for the cell division. So, this concludes that the protein is important for the cell division and possibly other proteins present into the cytoplasm are inherited...
from the oocyte and they are not synthesized in these initial stages. Nonetheless, after the 8-16 cell stages α-amanitin hindered the further progress of the embryo development, which clearly shows that the newly synthesized protein replaces the cytoplasm pool and the zygotic genome in the buffalo embryos is activated at this stage.

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References