Quantitative aspect of gene expression analysis in mammalian oocytes and embryos

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SUMMARY

In vitro produced embryos, due to their lower developmental potential when compared to the in vivo derived counterparts, have been currently subjected to an intensive scientific investigation. Qualitative as well as quantitative analyses of gene expression (reverse transcription – polymerase chain reaction; RT-PCR) belong to the powerful tools providing a wide spectrum of data on the quality of oocytes and embryos. The main research areas in this topic concern the following categories: oocyte quality, developmental competence of in vitro produced (IVP) embryos also with regard to their sex, embryo metabolism, gene expression levels, embryo manipulation and cloning. There are many methods that have been applied to study gene expression, but only some of them meet the requirement of analysing small cell samples which usually is the case with oocytes or early embryos. This paper deals with the description of the current methods commonly used for quantitative gene expression in mammalian oocytes and preimplantation embryos with a special attention paid to the real time PCR. Reproductive Biology 2 (3): 229-241

Key words: gene expression, oocyte, embryo, quantitation, real-time PCR

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INTRODUCTION

In vitro embryo production represents an alternative source of oocytes and embryos for both, experiments and routine embryo transfer (ET) procedures. There are, however, distinct differences between in vivo and in vitro derived embryos of domestic animals. Following the transfer to recipient females, embryos from both sources show similar implantation rates whereas embryonic and foetal losses appeared to be significantly higher for in vitro produced (IVP) embryos [20]. Moreover, about 30% of new-born ruminants show increased body size accompanied by several abnormalities described as large offspring syndrome [33]. Therefore, it has been postulated that conditions provided for in vitro culture are rather suboptimal and much effort has currently been concentrated on improving the quality of IVP embryos. The present paper reviews some aspects on the quality evaluation of IVP embryos with special attention paid to the molecular methods that can be used for quantitative gene expression study.

The viability of IVP embryos in cattle – brief summary of the current status

Despite a significant improvement in bovine in vitro embryo production procedures in recent decades, the quality of IVP embryos is still lower than their in vivo derived counterparts. Many reviews provide a careful analysis of a wide spectrum of data concerning differences between in vivo and in vitro derived embryos [5, 11, 17, 21, 26]. It is calculated, that 30-40% of in vitro matured oocytes develop into blastocyst. Moreover, the IVP embryos show a reduced survival rate (40%) when compared to the in vivo derived embryos (50-60%; [11, 26]). Several differences have been pointed out which distinguish between these two embryo groups in terms of their morphology and gene expression pattern. The main problems concerning in vitro embryo production are listed below.

1) Quality of oocytes and zygotes. Oocytes collected for in vitro maturation (IVM) do not undergo capacitation and final follicular maturation in the presence of a dominant follicle. In addition, elevated levels of chromosomal imbalance, polyspermy and asynchronous pronuclei development as well as an asynchrony between molecular, cytoplasmic
and cumulus maturation were noticed among oocytes and zygotes in vivo [11, 21, 26].

2) Developmental competence of embryos. Only 35% of in vitro matured and fertilised (IVM/IVF) oocytes reach the blastocyst stage in contrast to over 70% of in vivo matured and fertilised cells. The IVP embryos are characterised by a higher incidence of mixoploidy (72% versus 26%), early cavitation without a proper compaction, retarded forth cell cycle resulting in slower development and lower cell number in inner cell mass (ICM). The blastomeres are usually darker and swollen. The development rate is influenced by culture conditions, especially media composition; a phenomenon of male embryos developing faster is often observed [5, 11, 13, 26].

3) Embryo metabolism. IVP and in vivo embryos have similar rates of ATP production, glucose metabolism, pyruvate uptake and utilisation. However, considering slightly lower cell number in IVP embryos, significant differences are noticed on a cellular level [11, 26].

4) Gene expression level. Qualitative and quantitative differences have been observed in gene expression of IVP and in vivo derived embryos. For example in IVP embryos, there was no observable expression of the connexin (Cx43) gene; the transcript level for the heat shock protein (Hsp70) gene increased 15 fold; high expression of leukaemia inhibitory factor (LIF) gene contrasted to no detectable transcript in in vivo embryos; as well as an impaired expression of the glucose transporter isoform-1 gene (Glut-1) was reported in IVP embryos [17].

5) A significantly lower amount of IVP embryos survived being frozen and than thawed. The pregnancy rates of frozen-thawed IVP embryos after being transferred are significantly lower (10-40%) than those observed for in vivo embryos (40-70%). The IVP embryos show a higher sensitivity to cooling and freezing due to elevated lipid content; culture conditions can also affect their freezability [11, 26].

Application of gene expression analysis to in vitro production of mammalian embryos

Strategies for gene expression analysis have been successfully introduced to study qualitative and quantitative aspects of gene transcription which occurs during oocyte growth, maturation and embryonic development. Those
analyses were proved to provide data that help to explain some phenomena previously observed on a morphological level, e.g.:

1) the impaired compaction resulting in early cavitation and low cell number within IVP blastocyst was related to the absence of transcript for Cx43 gene responsible for gap junction formation [28];

2) the expression level of several genes was shown to be affected by the culture media composition. The transcript levels for Glut-1 and poly(A) polymerase were significantly increased in a medium supplemented with polyvinyl alcohol (PVA) but not with serum, whereas the expression level of the Hsp70 gene was enhanced in medium containing serum [29];

3) embryo manipulation and cloning were shown to cause changes in gene expression patterns. Blondin et al.[3] reported altered levels of insulin-like growth factor II (IGF-II) gene transcript in fetuses derived from IVP embryos. IGF-II is known to exert a distinct role in fetal development. As shown by Wrenzycki et al. [30], expression of some genes was affected by the protocols used for nuclear transfer. Moreover, the dosage compensation of few X-linked genes was impaired by nuclear transfer and in vitro culture conditions. For example the G6PD transcript level was 2-fold elevated in female blastocysts whereas, in in vivo derived embryos a similar expression pattern was observed in both sexes [31];

4) the morphology of oocytes and embryos has been attributed to the expression pattern of two genes belonging to the Bcl-2 family (apoptosis markers). Oocytes and embryos of good quality showed a high expression level for the Bcl-2 gene (anti-apoptotic) and low for the Bax gene (pro-apoptotic). The situation was reversed in low quality material [32]. Moreover, the Bcl-2 to Bax ratio was considered a survival marker;

5) expression level of some genes linked to the X-chromosome was related to the embryonic sex during preimplantation development [13]. Gutierrez-Adan et al. [8] demonstrated a sex related expression of two genes located on the X chromosome: glucose-6-phosphate dehydrogenase (G6PD) and hypoxanthine phosphoribosyl transferase (HPRT). Significantly higher levels of transcripts corresponding to those two genes were found in female embryos. This finding confirmed the previously reported phenomenon of sex differences in the rate of embryonic development prior to gonadal differentiation.
Methods used for quantitation of transcription

There are four methods known to be used for RNA quantitation: a. northern blotting (provides information on mRNA size, its integrity and alternative splicing); b. in situ hybridisation (allows localisation of transcript within an analysed tissue); c. RNAse protection assay (allows to distinguish from mRNA of similar size) and d. reverse transcription polymerase chain reaction (RT-PCR) [for review see: 4]. The main disadvantage of the first three approaches is their low sensitivity and relatively high complementary DNA (cDNA) copy number necessary for successful analysis. This limitation makes those techniques hardly applicable in case of analysing very rare transcripts or when a limited cell number is available for quantitation. In contrast, the RT-PCR enables analysis of various samples theoretically from as little as one cell. Since RNA cannot serve as a template for PCR, the amplification is preceded by reverse transcription of RNA into cDNA. It is important to remember however, that many variables can influence the efficiency of the RT process (RNA secondary structure, enzyme efficiency, primers used etc.) and PCR amplification (concentration of template, oligonucleotides - dNTP, MgCl$_2$, primers and polymerase [4, 16]). Even a small shift in amplification efficiency may lead to large differences in the amount of the final PCR product, even if the initial template concentration in all samples was the same [16]. Gilliland et al. [7] have shown, that the analysis of several replicates derived from the same sample and amplified in one PCR reaction may yield results that differ up to six fold among tubes. Therefore, in terms of quantitation one cannot rely on the direct measuring of the intensity of bands stained with ethidium bromide on agarose gel.

To overcome this limitation, strategies based on the concept of competitive RT-PCR have been developed [1]. In this approach a known amount of exogenous synthetic RNA is added to the RT mix. The exogenous sequence (competitor or internal standard) is co-amplified in the same tube with a target competing for the reagents. The target and competitor must be amplified by the same pair of primers. The ratio between the target and the competitor should remain constant during the amplification step. This method allows the initial target concentration to be determined. The PCR products derived from the target and competitor should be distinguishable by the presence of a small deletion, an addition of target unrelated motive or a modification of restriction site in the internal standard sequence [16].
Those authors have shown, that the competitor did not affect the quantitation process which indicates a similar amplification of both sequences. Moreover, the variation in reproducibility of this approach was found to be between 5% and 7%.

Another approach, known as non-competitive, semiquantitative RT-PCR involves co-amplification of the target mRNA and an endogenous (e.g. β-actin) or exogenous (e.g. rabbit β-globin RNA) control sequences contained within the sample whose levels are not affected by the treatment [22]. The target and control are amplified by the use of two different pairs of primers. For each pair of primers the range of cycle numbers over which the amplification is exponential (linear range) is established and the number of PCR cycles is kept within this range (a mid point in the linear range). PCR products are visualised on a gel usually stained with ethidium bromide and the relative band intensity is normalised to that of the control by using a computer aided system. The quantity of the RT-PCR product is determined by comparing the product obtained from the standards with the product of the gene of interest [8].

**Real-time PCR.** A big advantage of this approach is quantitation of the initial amounts of target sequence in contrast to the other methods which detect the final amount of the amplified product. In addition, the post PCR gel analysis is eliminated. The real time assay can detect as little as 1000 copies of mRNA or even 400 copies [6]. The unique part of this system is the use of fluorescent reporter (SYBR Green, TaqMan probes, Molecular Beacons) which emits signals increasing in a direct proportion to the PCR product during each cycle. In order for the method to be precise, the quantitative data is collected in the exponential phase of amplification when a reaction proceeds at a constant rate and the first significant increase in the amount of PCR product is observed. The program determines a threshold cycle ($C_T$) which is when a fluorescence signal is first recorded as statistically significant at the level higher than background. Heid et al. [10] demonstrated that the $C_T$ value is correlated with the number of initial copies in each sample. Bustin [4] also recommends the optimal amplicon length for the real time PCR which should be less than 100pb. However, longer products of 400bp have also been successfully amplified.

The simplest and most economical system utilises the DNA specific dye SYBR Green which binds to double stranded DNA and upon excitation emits light [15]. The dye exhibits little fluorescence when unbound but
during the elongation step an increasing amount of dye binds to DNA (fig. 1). After binding, its fluorescence increases over 100-fold. Fluorescence is measured at the end of the elongation step of every PCR cycle and thus product accumulation is monitored. This method has however one disadvantage; the dye binds to double stranded DNA of any origin, including primer-dimer and non-specific products. Therefore a precise optimisation of PCR conditions is of major importance. Moreover, the SYBR green system can be used to analyse only one transcript at a time.

TaqMan probes and Molecular Beacons are the most often used alternatives as to SYBR Green dye [4]. In both cases, fluorescence-labelled probes which

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**Denaturation of double stranded DNA**

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**Primer annealing**

*to complementary DNA strand*

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**Primer extension; dye molecules bind**

*to the double-stranded PCR product; start of fluorescence emission*

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**Polymerisation step; an increasing number of dye molecules bind to double-stranded DNA**

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**Final extension; fluorescence detection**

*Fig. 1. A diagram demonstrating one PCR cycle of the SYBR Green dye incorporation method.*
hybridise to accumulating, specific PCR product are utilised. TaqMan probes are oligonucleotides longer than primers, with a fluorescent dye attached to the 5′ end (reporter) and a second dye (quencher) at their 3′ end [10]. When the probe is intact, the reporter and quencher stay close to each other which prevents the emission of any fluorescence (fig. 2). For quantitation, a pair of amplicon-specific primers and the hybridisation probe, which is non-extendible, have to be used. The probe is designed to hybridise to the internal region of an amplicon. After hybridisation has occurred, the 5′ endonuclease activity of the DNA polymerase cleaves and displaces a probe which separates reporter and quencher dyes and fluorescence is detected.

**Fig. 2.** A diagram demonstrating one PCR cycle of the TaqMan real time assay.
Molecular Beacons are DNA hybridisation probes equipped with fluorescent and quenching dyes [27]. They are complementary to a sequence in the middle of the expected amplicon and are designed to adopt a hairpin structure while free in solution. The hairpin structure consists of a stem built of two complementary arms and a loop that is complementary to the target sequence. This configuration helps fluorescent dye and the quencher to stay very close to each other and therefore no fluorescence can be detected. During the annealing step, beacons hybridise to the target sequence which changes their conformation and separates reporter and quencher dyes resulting in fluorescence being emitted (fig. 3). Beacons stay intact during the PCR reaction and must rebind to the target in every cycle for signal detection. If the template is not complementary to the Molecular Beacons, they will not hybridise and no fluorescence will be emitted since the hairpin configuration is very stable [4]. Both, Molecular Beacons and Taq-Man probes allow a simultaneous detection of many DNA sequences by using different reporter dyes.

The quantitative gene expression studies are carried out under various experimental conditions like stress, growth or cell differentiation. Those

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**Fig. 3.** A diagram demonstrating one PCR cycle of the Molecular Beacon real time assay.
Gene expression in oocytes and embryos

Methods used for mRNA quantitation in mammalian oocytes and embryos

The semiquantitative RT-PCR is the most frequently used approach for analysing the relative transcript abundance (RA) in bovine oocytes and embryos [8, 12, 14, 18, 29, 30, 31]. It involves adding a known amount of rabbit β-globin RNA prior to RNA extraction or utilizing endogenous transcript for housekeeping genes as a standard. Oocytes or embryos are usually pooled into groups for analysis, but single blastocysts were also successfully investigated using this method [30, 31]. There are only a few reports on applying the Northern blot analysis [2]. This method requires significantly higher transcript concentrations than the RT-PCR and therefore more oocytes and embryos have to be pooled. The real time RT-PCR has been successfully introduced to study gene expression in single oocytes and embryos. Steuerwald et al. [23, 24] demonstrated the utility of the fluorescence monitored RT-PCR (light cycler) for quantitative analysis of two housekeeping genes (HPRT, β-actin) in single human oocytes and embryos. It has to be, however, kept in mind that both investigated genes are continuously transcribed at considerably high levels during mammalian treatments are usually accompanied by a wide variation in transcript levels. Therefore, the so called internal, exogenous standard (an mRNA that is not affected by the treatment) has to be included in the analysis [25]. An exogenous standard should be expressed at a constant level within various tissues and at a similar level as the analysed transcript. In the majority of studies, three different RNAs of housekeeping genes are usually utilised as an internal standard: glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), β-actin and ribosomal RNAs. None of the controls appeared to be ideal. It was shown that the GAPDH gene expression level was affected by glucose, insulin, heat shock and cellular proliferation. However, fewer examples are reported for the β-actin gene to be modified under experimental treatments [for review see: 25]. The ribosomal RNA production is less likely to vary under conditions affecting mRNA transcription, however it is not always a good representative of total mRNA population in a cell since rRNA is expressed at a much higher level than mRNA [4]. In conclusion, although β-actin seems to be a better choice, an internal control has to be selected for each experiment and all limitations need to be considered.
embryogenesis. Therefore, it seems necessary to show the utility of this approach in case of less abundant transcripts which usually need much more attention in terms of optimisation the PCR conditions. Recently, Hartshorn et al. [9] successfully applied the SYBR green approach to analyse the Xist gene expression in a single murine embryo.

In humans, real-time PCR appeared as a powerful tool in preimplantation genetic diagnosis (PGD), especially in the detection of chromosomal abnormalities and single-gene diseases in individual blastomeres biopsied from an embryo. Pierce et al. [19] have shown that the diagnostic efficiency reached 99.7% for single lymphocytes and 84% for individual blastomeres. The lower accuracy for embryonic cells may be partially due to the poor quality of analysed embryos. Recently, Zimmermann et al. [34] reported a novel test for trisomy 21 detection by real-time quantitative PCR.

In summary, the real time approach seems to be the method of choice for studying the quantitative aspect of gene expression in oocytes and embryos especially after considering a possibility of carrying out a multiplex amplification within a single reaction. This will make it possible to analyse several genes in a very limited cDNA sample derived from a single oocyte or embryo.

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