

Growth hormone gene expression in oocytes and zygotes produced by cows heterozygous (Leu127Val) at GH locus

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Received: 10 July 2004; accepted: 19 October 2004

SUMMARY

The present work describes analysis of the growth hormone (GH) gene expression in immature and *in vitro* mature bovine oocytes as well as zygotes after *in vitro* fertilization. The Leu/Val polymorphism described in the 5th exon of the bovine GH gene was applied to investigate the expression of genetic variants in the analyzed material. Experiments were performed on oocytes collected from heterozygous (LV) cows. Since developmental stages analyzed in this study are not transcriptionally active with regard to the GH gene, the analyzed transcripts were exclusively of maternal origin. According to our findings, GH transcript was observed in each analyzed sample. The expected heterozygous pattern of GH gene expression was found in immature oocytes. However, an unexpected variation in allelic distribution was noticed in mature oocytes and zygotes. A tendency was observed of a gradual disappearance of the heterozygous pattern (variant LV) ranging from 100% in immature oocytes, 70% in mature cells and 20% in zygotes. In some of analyzed samples,

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the expected heterozygous pattern was replaced by the VV variant in mature oocytes and either by VV or LL variants in zygotes. Possible mechanisms underlying the described phenomenon (*in vitro* culture conditions, transcript polyadenylation) are discussed. These results indicate a possible influence of sub-optimal *in vitro* culture conditions on the distribution of genetic variants of GH gene transcripts in bovine oocytes and zygotes. *Reproductive Biology* 2004 4 (3): 259-269.

Key words: Leu127Val polymorphism, GH gene expression, oocyte, zygote

INTRODUCTION

In vitro embryo culture (IVC) is an alternative method for embryo production in cattle. It is well known however, that embryos produced *in vitro* (IVP) display lower quality and developmental potential than their *in vivo* derived counterparts [19, 26, 27]. Several differences between *in vivo* and *in vitro* produced embryos were identified [e.g. cell number and morphology, timing of development, chilling sensitivity; 10]. Also the frequency of chromosomal abnormalities was found to be higher in IVP embryos [17, 31]. As a consequence, only 30% - 40% of the mature oocytes reach the blastocyst stage after *in vitro* fertilization (IVF; [30]). The conditions for *in vitro* maturation (IVM) and *in vitro* culture (IVC) were shown to affect the transcriptional activity of several genes in embryos [23], what may lead to the *large offspring syndrome* (LOS) noticed in about 30% of calves resulting from IVP embryos [34].

The pre-implantation period of embryonic development is characterized by dynamic quantitative and qualitative changes of mRNA and protein contents [25, 29]. A lack of *de novo* transcription in early embryos coincides with a control of maternally derived mRNA synthesized during oogenesis. The onset of the major embryonic genome activation is a species-specific phenomenon occurring in cattle during the fourth cell cycle [8].

The growth hormone (GH) gene was shown to play an important role in mammalian reproduction [11]. The presence of transcripts for the GH gene in bovine oocytes [12] and embryos [14], as well as for the growth hormone

receptor (GHR) gene in all stages of pre-attachment development has been previously reported [13]. Several point mutations in the bovine GH gene have been described. The Leu127Val polymorphism described by Seavey et al. [28] has been extensively investigated with regard to production traits in cattle. This polymorphism was successfully applied in the experiments investigating GH gene expression onset in bovine embryos [14].

The aim of the present study was to investigate the distribution of polymorphic mRNA forms of GH gene in bovine immature and *in vitro* matured oocytes as well as in zygotes derived from cows heterozygous at the GH locus.

MATERIALS AND METHODS

Oocyte collection and *in vitro* maturation

The IVM/IVF/IVC protocol described by Makarevich and Markkula [21] was applied. Cumulus oocyte complexes (COCs) were collected by slicing individual ovaries. The majority of oocyte donors represented the Polish Friesian cattle, since in our area individuals of meat breeds are only occasionally slaughtered. Single ovaries were randomly picked up from a pool of gonads; consequently one ovary represented a donor. After slicing, COCs were washed 2-3 times in Hepes-Talp and washing medium. Only oocytes showing an evenly granulated ooplasm and several layers of non-expanded cumulus cells were submitted for IVM. *In vitro* maturation was carried out for 24 hours in TCM 199 (Gibco, Invitrogen Co, Scotland, UK) supplemented with 10% FCS (Gibco, Invitrogen Co, Scotland, UK) and hormones (Sigma, Steinheim, Germany: 2 µg/ml FSH, 10 µg/ml LH, 1 µg/ml 17β – estradiol) in humidified 5% CO₂ atmosphere at 39°C. Oocytes were mature in groups reflecting their origin from individual ovaries.

DNA isolation from follicular cells and donor's genotyping at GH locus

At the time of oocyte collection, follicular cells derived from each ovary were washed in 0.9% NaCl and stored at -20°C for further DNA analysis.

The genomic DNA was isolated by proteinase K (20 mg/ml) digestion followed by phenol/chloroform extraction and ethanol precipitation. The conditions for donor genotyping (PCR reaction and restriction analysis) were previously described [18]. A 492-bp amplified fragment was digested with *AluI* enzyme.

***In vitro* fertilization**

In vitro fertilization was carried out in IVF medium with addition of heparin (10 µg/ml) in humidified 5% CO₂ atmosphere at 39°C. Frozen-thawed sperm samples collected from an AI (artificial insemination) bull of known *in vitro* fertilization potential was used for oocyte insemination. It was washed twice in Sperm Talp medium, adjusted to the final concentration of 1.5×10⁶/ml and co-incubated with oocytes for 18 hours [21]. Afterwards, the presumptive zygotes were frozen in liquid nitrogen for further analysis, whereas a control group of zygotes from each IVF replicate was cultured for seven more days in order to monitor the cleavage rate (day 2) and the blastocyst rate (day 7).

RNA isolation

Immature and mature oocytes as well as zygotes were frozen in liquid nitrogen in groups reflecting their origin from single ovaries. Before freezing, cumulus cells attached to immature and mature oocytes were removed by a short incubation in 0.15% hyaluronidase and by pipetting. All samples were washed three times in PBS supplemented with 0.25% PVP. By the time of RNA isolation, the donors had already been genotyped at *GH locus*. Since the gene expression analysis in single oocytes is still difficult to perform, oocytes/zygotes originating from LV cows were pooled (60-80 cells/sample). RNA isolation was carried out using the GenElute™ Mammalian Total RNA kit (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's protocol. Afterwards each sample was treated with DNase (0.5 IU/sample; Promega, Biosciences Inc, Madison, Wi, USA).

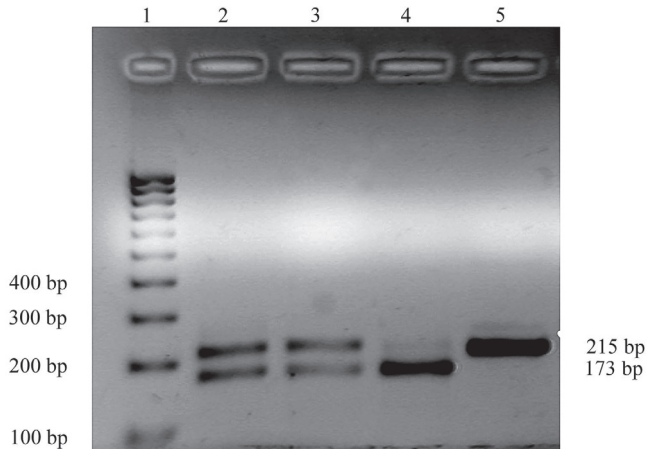


Fig. 1. Expression of polymorphic variants of GH gene in bovine oocytes and zygotes [lane 1 – DNA size marker (Gene Ruler™ 100 bp DNA Ladder), lane 2, 3 – variant LV, line 4 – variant LL, line 5 – variant VV]. Adapted from: Lechniak-Cieślak [18].

Reverse transcription and PCR reaction (RT-PCR)

The first step of reverse transcription (RT) was sample incubation for 10 min at 70°C with a primer mix (random hexamers and oligo-dT, 0.5 µg · µl each) followed by 1.5 – 2h incubation in a RT mix (1x reaction buffer, 0.5 mM dNTP, 20 U Rnasin, 5 mM MgCl₂, 1 µl Sensiscript, Qiagen, Hilden, Germany). The final volume of each cDNA sample was 20 µl. At the end of the procedure, the enzyme was inactivated (94°C for 5 min) and cDNA was stored at -20°C. The protocol for nested PCR reaction and restriction analysis previously described by Joudrey *et al* [14] was used. The amplified PCR product of 220 bp was digested with *AluI* enzyme (*fig. 1*). Each cDNA sample was amplified and subjected to restriction analysis in two independent replicates.

RESULTS

A total of 89 individual ovaries representing oocyte donors were included in the present study. The genotype frequencies at the GH locus among cows are presented in Table 1. The analyzed population consisted mainly of the

Table 1. The distribution of genotypes at the GH locus among cows analyzed in the present study

	Genotype			Total
	LV	LL	VV	
Number of donors	30	52	7	89
Genotype frequency	0.34	0.58	0.08	1.00

LL (0.58) and the LV individuals (0.34), whereas the VV females were very rare (0.08). Altogether, 226 immature oocytes, 307 mature oocytes and 219 zygotes collected from heterozygous LV cows were analyzed. On average, 26 oocytes (from 7 to 53) were derived from one donor represented by one ovary. The average cleavage rate evaluated on day 2 after insemination was 75% and the average blastocyst rate on day 7 equaled 20%.

The distribution of polymorphic variants of the GH transcript is shown in Table 2. The expected heterozygous pattern of GH gene expression was found in immature oocytes, whereas a variation in allelic distribution was noticed in mature oocytes and zygotes. The rate of the heterozygous pattern (variant LV) decreased from 100% in immature oocytes to 70% in mature cells and 20% in zygotes. In some of the analyzed samples, the heterozygous pattern of GH gene expression was replaced by the VV variant in mature oocytes and either by the VV or LL variants in zygotes. Thus, the LL variant was noticed only in zygotes, whereas the VV form appeared in mature oocytes and zygotes.

Oocytes derived from ovaries of homozygous LL and VV cows were analyzed as a control. The variant distribution appeared as expected: LL only in oocytes of LL cows and VV in oocytes of VV females (fig. 1).

DISCUSSION

Despite the previous opinion that GH does not influence early embryonic development, recent findings have demonstrated the presence of GH transcript in murine and bovine oocytes and embryos [12, 17, 24]. Moreover,

Table 2. Variants of growth hormone gene mRNA expressed in bovine oocytes and zygotes derived from cows heterozygous (LV) at the GH locus

Developmental stage	Replicates / genetic variants									
	1	2	3	4	5	6	7	8	9	10
immature oocytes	LV	LV	LV	LV	LV	LV	LV	LV	LV	LV
mature oocytes	LV	LV	LV	VV	LV	VV	LV	LV	VV	LV
zygotes	LL	VV	LV	LL	VV	VV	LV	LL	LL	VV

we have recently shown by use of the Leu127Val polymorphism, that the onset of the GH gene expression takes place in 8-16 cell stage bovine embryos which coincides with the major activation of embryonic genome in this species [14]. The aim of the present work was to investigate the expression pattern of the GH gene in bovine oocytes and zygotes derived from cows heterozygous at this locus. Since all developmental stages included into this study are not transcriptionally active with regard to the GH gene, the analyzed transcripts were exclusively of maternal origin. Therefore, we assumed that transcripts representing both alleles would be present in cytoplasm of all oocytes produced by heterozygous LV cows.

The previous experiment carried out by Joudrey¹ on bovine tissues derived from heterozygous cows revealed an unexpected variation in terms of expressed GH gene variants. In all examined tissues, the expected heterozygous pattern (variant LV) appeared with a various frequency ranging from 17% to 34%. The rate of the LV pattern varied among investigated animals but also among different tissues derived from the same individual. In a given tissue, all possible patterns of GH gene expression could be observed (variants LL, VV and LV). The LL variant was found to be the most prevalent (45%) compared to VV (32%) and LV (23%). Concentrating on those findings we aimed to investigate the pattern of GH gene expression in bovine oocytes and zygotes

¹Joudrey EM 2002 GH gene expression in bovine oocytes, embryos and tissues. MSc thesis, University of Guelph, Ontario, Canada.

produced by the heterozygous LV cows. Although the immature and mature bovine oocytes were subjected to gene expression analysis in the study by Joudrey et al. [14], the authors did not find any disturbances in the GH gene expression pattern in oocytes after maturation *in vitro*.

In the present study the heterozygous pattern of GH expression was constantly observed only in immature oocytes whereas a variation was noticed for mature oocytes and zygotes (tab. 2). In some of the examined cDNA samples we have also found a similar variation in distribution of the GH transcripts to that described in bovine tissues. This observation could not be attributed neither to sample origin nor to the time of sample storage.

The mechanism underlying the described phenomenon is not known, but a few assumptions can be provided based on the recently published data: i) the conditions of *in vitro* oocyte maturation and culture affect the gene expression in cattle, ii) the process of polyadenylation of maternal mRNA in oocytes can be influenced by the sub-optimal *in vitro* conditions. *In vitro* culture conditions have been shown to affect transcriptional activity in embryos at various stages of development in a number of species, including mice [22], sheep [35] and cattle [2, 3, 27, 33]. Moreover, the IVM process alone may change the pattern of gene expression in cattle as well as affect further embryonic development [7, 15, 20, 32]. Goto et al. [9] analysed gene expression pattern in human oocytes by the use of differential display method (DD-RT-PCR). Several populations of immature oocytes at germinal vesicle stage were similar to each other with regard to the gene expression profiles. The situation changed dramatically among oocytes at metaphase two stage when a big variation in detected transcripts was observed. Moreover, Dalbies-Tran and Mermillod [6] described a profile of nearly 300 transcripts present in bovine oocytes during *in vitro* maturation with some of them (about 70) showing significant changes in their relative abundance.

Transcripts of maternal origin are characterised by a unique stability, since some of them may be detected even in the blastocyst stage in mice [1]. Oocyte maturation is a very dynamic and complex process in terms of transcript metabolism and sub-optimal culture conditions may

significantly modify its regulation. Changes in poly(A) tail length affect the translational activity of transcripts and may be also related to the developmental competence of oocytes and embryos in cattle. Generally, oocytes displaying a lower polyadenylation level showed poor developmental competence. Similarly, poorly developing embryos were characterized by altered amounts of maternally derived transcripts and abnormal polyadenylation level [4, 5].

In summary, an unexpected variation in allelic expression was noticed after *in vitro* maturation and fertilization of bovine oocytes produced by heterozygous cows. It is suggested that this phenomenon may be caused by sub-optimal *in vitro* conditions, which were already demonstrated to affect gene expression. However further investigations, including *in vivo* studies are necessary to confirm our findings that we consider as preliminary.

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