



Cloning, Expression and Hormonal Regulation of Steroidogenic Acute Regulatory Protein Gene in Buffalo Ovary

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ABSTRACT : In mammalian ovary, steroidogenic acute regulatory (StAR) protein mediates the true rate-limiting step of transport of cholesterol from outer to inner mitochondrial membrane. Appropriate expression of StAR gene represents an indispensable component of steroidogenesis and its regulation has been found to be species specific. However, limited information is available regarding StAR gene expression during estrous cycle in buffalo ovary. In the present study, expression, localization and hormonal regulation of StAR mRNA were analyzed by semi-quantitative RT-PCR in buffalo ovary and partial cDNA was cloned. Total RNA was isolated from whole follicles of different sizes, granulosa cells from different size follicles and postovulatory structures like corpus luteum and Corpus albicans. Semi-quantitative RT-PCR analyses showed StAR mRNA expression in the postovulatory structure, corpus luteum. No StAR mRNA was detected in total RNA isolated from whole follicles of different size including the preovulatory follicle (>9 mm in diameter). However, granulosa cells isolated from preovulatory follicles showed the moderate expression of StAR mRNA. To assess the hormonal regulation of StAR mRNA, primary culture of buffalo granulosa cells were treated with FSH (100 ng/ml) alone or along with IGF-I (100 ng/ml) for 12 to 18 h. The abundance of StAR mRNA increased in cells treated with FSH alone or FSH with IGF-I. However, effect of FSH with IGF-I on mRNA expression was found highly significant ($p < 0.01$). In conclusion, differential expression of StAR messages was observed during estrous cycle in buffalo ovary. Also, there was a synergistic action of IGF-I on FSH stimulation of StAR gene. (**Key Words :** StAR Gene, StAR mRNA, Semi-quantitative RT-PCR, Granulosa Cells, Ovary, Buffalo)

INTRODUCTION

The production of steroid hormones in response to trophic hormone stimulation begins in the theca interna cells by conversion of cholesterol to pregnenolone by cytochrome P450_{scc} (cholesterol side chain cleavage) enzyme (Oonk et al., 1989). The true rate limiting step in steroidogenesis is the transport of substrate cholesterol from outer mitochondrial membrane to the inner mitochondrial membrane and the P450_{scc} enzyme. Diffusion of hydrophobic cholesterol from the aqueous intermembrane space is extremely slow and, therefore, it became clear that transfer occurs in an assisted manner. Recently, steroidogenic acute regulatory protein, originally isolated from mouse Leydig tumor cell line MA-10 (Clark et al., 1994), has been identified as an acute regulator of the rate limiting transfer of cholesterol to the inner mitochondrial

membrane (Stocco and Clark, 1996). Methodologies such as Western analysis, Northern analysis, *in situ* hybridization, immunocytochemistry, RNase protection assay, and RT-PCR all indicate that StAR expression is confined to steroidogenic tissues. It is expressed in the fetal and adult testes, ovaries and adrenals (Pilon et al., 1997). Expression of StAR mRNA is restricted to and induced in the ovarian steroidogenic cell types. Appropriate expression of StAR gene represents an indispensable component of steroidogenesis and its regulation has been found to be species specific (Stocco, 2001).

Spatio-temporal expression pattern of StAR in rat ovary revealed that its expression is confined to pre-ovulatory follicles, whereas non-ovulatory follicles were devoid of StAR in their granulosa cell layers during follicular development (Ronen-Fuhrmann et al., 1998). A comparison in bovine tissues revealed no StAR expression in granulosa cells, very weak expression in early Corpus luteum (CL) and maximal expression during midcycle, which is maintained throughout pregnancy (Hartung et al., 1995). Using a similar Northern blot study, two isoforms (~2.9 kb

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and ~1.8 kb) of StAR mRNA were detected in bovine CL, theca and granulosa cells (Pescador et al., 1996). Studies on human ovary including *in situ* hybridization, histochemistry defined the cellular localization of StAR mRNA. Its expression was limited to thecal cells of preovulatory follicles and luteinized granulosa and theca cells of CL (Kiriakidou et al., 1996). Pollack et al. (1997) employed immunostaining to reveal similar results with occasional staining of granulosa cells; whereas antral follicles stained intensely in the thecal layer. Corpora albicans was not stained at all. Granulosa cells also revealed StAR mRNA transcripts of about 3.6 and 1.6 kb in rat ovary (Minegishi et al., 2000). Consistent study by Luigi Devoto et al. (2001) confirmed an increased expression of StAR in early and mid luteal phase CL through real time quantitative RT-PCR. In early studies, it was demonstrated that hormone-stimulated steroid synthesis was accompanied by rapid increase in StAR mRNA levels (Clark et al., 1995), since trophic hormone stimulation usually results in a rapid increase in intracellular cAMP. The role of cAMP in the regulation of StAR was investigated and found to have a positive and rapid effect (Sugawara et al., 1996, 1997; Caron et al., 1997; LaVoie et al., 1999). In recent years, the factors belonging to families of insulin and insulin-like growth factor, i.e., IGF-I, have been found to play a central role in intraovarian regulation of follicular development in a species specific manner (Adashi et al., 1985). Ovarian follicles synthesize IGF-I and IGF-II (Hammond et al., 1991; Spicer et al., 1995). Studies with rat, sheep and cattle showed that FSH alone or in synergy with other factors inhibit apoptosis (Palumbo et al., 1994; Jolly et al., 1994, 1997) and stimulated steroidogenesis in cultured granulosa cells (Chun et al., 1994; Silva et al., 2000).

In a study on luteinized porcine granulosa cells (Pescador et al., 1997), it was seen that FSH elevated both StAR and P450scc messages in a dose-dependent manner over 6 h and continually stimulated over 24 h. A partial porcine StAR cDNA generation by RT-PCR of 280 bp reflected the synergistic steroidogenic stimulation by two intrafollicular regulators (Balasubramanian et al., 1997). IGF-I stimulated FSH induced StAR expression up to 6-fold though treatment with FSH or IGF-I alone had a small but consistent stimulatory effect in porcine granulosa cells up to 1.6 and 2.7 fold, respectively. In a similar study by LaVoie (1999), FSH alone significantly stimulated StAR promoter activity in a dose-dependent manner. IGF-I had no effect by itself, yet significantly augmented FSH-stimulated StAR promoter activity. A preliminary study comparing the responses of P450scc and StAR gene in undifferentiated rat ovarian granulosa cells treated for 44 h with FSH and IGF-I revealed that IGF-I only modestly improved the effect of FSH on StAR mRNA (Eimerl and Orly, 2002). IGF-I alone had no effect. Effect of FSH on follicular development,

granulosa cell apoptosis and steroidogenesis was mediated by IGF-I in the goat ovary (Yuansong et al., 2003). Our recent study demonstrated that formation of high molecular weight complexes of GATA-4 and C/EBP beta within porcine StAR gene promoter was increased in granulosa cell nuclear extracts by treatment with FSH alone and was enhanced with the combination of FSH and IGF-I at 2 to 3 h of treatment (LaVoie et al., 2004). However, least information is available regarding StAR gene in buffalo ovary. Therefore, the present study was carried out to understand the expression, localization and hormonal regulation of StAR gene during estrous cycle in buffalo ovary by determining the expression of mRNA encoding StAR protein during estrous cycle and studying the effect of FSH alone or along with IGF-I on StAR gene expression in cultured granulosa cells.

MATERIALS AND METHODS

Collection of ovaries, isolation, classification of whole follicles and tissues from ovaries and total RNA isolation

Buffalo ovaries at random stages of estrous cycle were obtained from slaughter house and processed for isolation and characterization of follicles and tissues as per our previous report (Vinze et al., 2004). Total RNA was isolated using TRI reagent (Molecular Research Centre, Inc. #TR-118) as per the manufacturer's instructions. Identical procedures were followed for the extraction of the total RNA from whole tissues, isolated cells and cell culture monolayer, except that the whole tissues were homogenized and not the cellular lysates. The final RNA pellet was air dried and re-suspended in sterile water (sH₂O). RNA purity was determined by evaluating A₂₆₀/A₂₈₀ ratio. Total RNA with the ratio values greater than 1.7 or 1.8 were considered as deemed acceptable for subsequent procedure and was stored at -70°C. Integrity of RNA preparation was evaluated using agarose gel electrophoresis by denaturation of RNA with DMSO and glyoxal (Sambrook et al., 1989).

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed using M-MuLV RT-PCR Kit (Bangalore Genei Pvt. Ltd., Bangalore, India: #KT74) as per the manufacturer's instructions. Briefly, to sterile DNA/RNA/pyrogen free microfuge tube (Labware Scientific Inc., #PCR-02N), 1 µg of RNA sample was taken and diluted to 9 µl with sH₂O. Then added 1 µl of random hexamer and a brief spin was given. Solution was incubated at 65°C for 10 min in a thermocycler (Biometra) and thereafter a brief spin was given and following reagents were added in the order: 1 µl RNase inhibitor, 1 µl of 0.1 M DTT, 4 µl of 5× RT buffer, 2 µl of 30 mM dNTP mix, 0.5 µl of M-MuLV RT enzyme and sH₂O to bring the reaction volume to 20 µl. This

Table 1. Details of primers used

Primer name	Primer sequence	Length (bp)	GC (%)	T _m (°C)	Product size (bp)
StAR upstream	5' TCTTCTTGGTTCTCAGCTGGAAGAC 3'	25	48.0	57.2	307
StAR downstream	5' CTTCTGCAGGATCTTGATCCTTCTTG 3'	25	44.0	55.6	
β-Actin forward	5' CGTGGGCCGCCCTAGGCACCA 3'	21	76.0	64.6	243
β-Actin reverse	5' TTGGCCTTAGGGTTCAGGGGGG 3'	22	63.6	60.7	

solution was mixed, spun and placed in thermocycler and the reaction was carried out at 37°C for 1 h. followed by 95°C for 5 min and reaction was stopped at 4°C. After incubation, tubes were given a brief spin and were quickly placed on ice. For amplification of cDNA, the following PCR components were added to a DNA/RNA/pyrogen free PCR tube: 2 µl of cDNA product, 5 µl 10× assay buffer for Taq polymerase, 1 µl of 30 mM dNTP mix, 1 µl of 10 µM upstream primer, 1 µl of 10 µM downstream primer, 1 µl of Taq polymerase enzyme (1 U/µl) and sH₂O to bring the reaction volume to 50 µl. PCR reactions were performed in the thermocycler under following cycle conditions: 95°C for 2 min, followed by 35 cycles of 95°C for 1 min, annealing temperature (Table 1) for 30 sec, and 72°C for 1 min. A 7 min final extension at 72°C was performed following last cycle. In present case, the annealing temperature was averaged at 58°C. The primers used are mentioned in Table 1.

Gel electrophoresis of PCR products

RT-PCR product samples were visualized by 1-2% agarose gel electrophoresis. Gel photographs were taken with a digital camera using an orange filter (Olympus, C3030 zoom).

Cloning and sequencing of PCR-Product

The RT-PCR products were sub-cloned into the pCR II vector using TA cloning kit (Cat# 2060; Invitrogen, San Diego, CA, USA) and transformed into competent INVαF' strain of *Escherichia coli* as per the manufacturer's instructions. The nucleotide sequence of the product was confirmed by an automated sequencer (DNA sequencing service, Bangalore Genei, Bangalore, India). Two separate tissue preparations pooled from three or more animals were analyzed and independently sequenced to confirm reproducibility.

Standardization of PCR condition for semi-quantitative PCR

Optimization of primer concentration : In order to determine optimum concentration of primer for appropriate expression of desired and control genes, varying concentration of primer was used in standard PCR amplification as described above. Each primer was used in the concentration range of 0.05 to 0.25 µM. Lower

concentration may be limiting in which case the amplification could not be quantitated, whereas higher concentration may leave large amount of unused primer, which could give rise to non-specific amplification product.

Determination of MgCl₂ concentration : Determination of optimal concentration for each primer set was performed using different MgCl₂ concentrations in the PCR reaction conditions (as described above) and by adjusting water volume. Consequently, 1.0, 1.5, 2.0 and 3.0 mM MgCl₂ concentrations were tested. Products were run on an agarose gel to choose the condition that give highest yield and specificity.

Determination of cycling parameter : Standard program comprised a cycle of 2 min at 95°C, followed by 95°C at 1 min, 58°C for 30 sec, 1 min at 72°C for varying cycles, i.e. 20, 25, 30 and 35. Optimization of StAR expression was done by varying above cycle numbers in order to choose the condition giving the highest yield.

Control for competition : To determine whether the selected conditions are suitable for semi-quantitative PCR (StAR with β-actin and StAR with glyceraldehyde 3-phosphate dehydrogenase) at the same time, a competition control was performed by amplifying same sample and at same time, through internal control as β-actin and both set together. Samples were run on the same agarose gel for quantitation and competition was detected. Different reaction conditions were tested as described in results and discussion.

Granulosa cell culture

The granulosa cells were isolated from medium and large ovarian follicles as described earlier. Cells were washed at least three times with bicarbonate buffered MEM with antibiotics (plain media, pH 7.2) before culture. After washing granulosa cell pellet was suspended in 1.0 ml plain media. The number of viable cells among purified granulosa cells was studied by trypan blue dye exclusion test. Approximately 8×10⁶ viable granulosa cells were plated in a six-well culture plate (Costar Corporation, Cambridge, USA) using plain media, pH 7.2 and 3% fetal bovine serum (FBS) (Sigma #F7524) to permit cell anchorage. Cells were allowed to attach to culture dish for 24 h at 37°C at 5% CO₂. Monolayer was maintained in about 2.0 ml per well media containing serum in a water jacketed CO₂ incubator (Nuair, USA) for about one day. After 24 h of cell culturing in FBS, cells were observed under inverted microscope (Olympus CK2) for cell

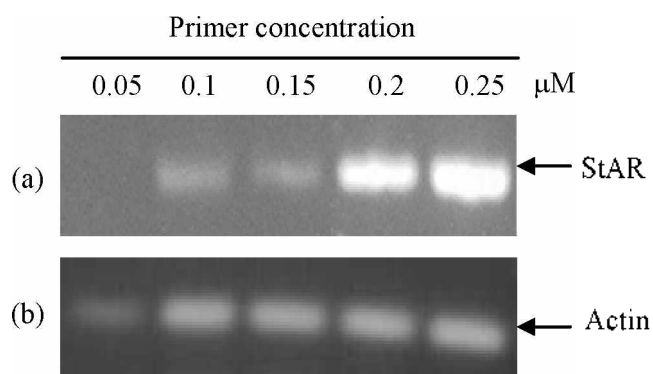


Figure 1. Effect of primer concentration on StAR gene amplification: PCR was performed in 1.5 mM MgCl₂ concentration at 58°C (annealing temperature for StAR) and 60°C for 30 cycles with increasing concentration of primers from 0.05 μM to 0.25 μM. No amplification of StAR was detected at a primer concentration of 0.05 μM. Expression of StAR mRNA increased from 0.10 μM to 0.25 μM while there was no significant change in expression of β actin from 0.05 μM to 0.25 μM primer concentrations.

attachment and viability. MEM containing FBS was removed and the cells were washed with plain media. A concentration of 100 ng/ml recombinant IGF-I (Sigma, #I3769) alone and 100 ng/ml IGF-I with 100 ng/ml FSH (0-17 potent, NIADDK) was prepared in plain media and the cells were treated for 12 to 18 h. Plain media was replaced by 2 ml of MEM containing IGF-I in some wells, while in some plain media containing IGF-I and FSH was added. To remaining wells, considered as control 2.0 ml of plain media was added and the plates were kept for incubation at 5% CO₂ for a minimum period of 12 h. After 12 to 18 h of treatment, cells were observed under microscope for growth and viability. Suspended media was removed and cells were extracted with 1.0 ml of TRI reagent. Total RNA was isolated from this cellular homogenate as described earlier.

Statistical analysis

Statistical analysis was performed using SYSTAT software.

RESULTS

Effect of primer concentration

Figure 1(a) represents the electrophoretic profile of amplification of StAR gene with increasing primer concentration from 0.05 to 0.25 μM. 0.05 μM showed no amplification of StAR gene, while 0.1 μM primer concentration showed a light intensity band corresponding to 307 bp, representing the amplified StAR gene. Thereafter, expression pattern of StAR mRNA increased consistently from 0.2 to 0.25 μM. The primer concentration of 0.2 μM for StAR amplification was considered as optimal. In a

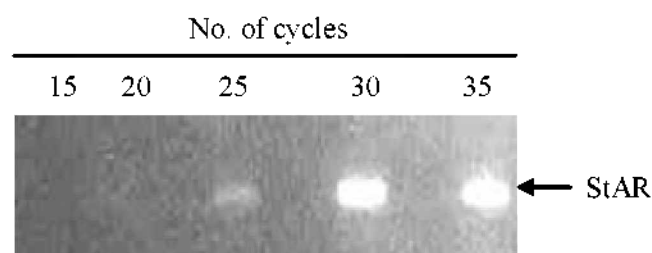


Figure 2. Determination of exponential range for amplification of StAR: The analysis was performed in varying cycle number from 20 to 35 in 1.5 mM MgCl₂ concentration at 58°C (annealing temperature). The intensity of amplification product increased from 25 to 30 and reached a plateau at 35. No StAR mRNA was detected upto 20 cycles.

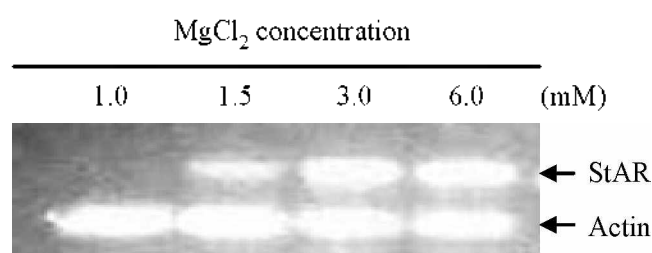


Figure 3. Control for competition between StAR and β-actin amplification: Reactions were performed at 58°C (annealing temperature) for 30 cycles with varying MgCl₂ concentration for amplification of both StAR and β-actin genes. 1.0 mM MgCl₂ concentration showed that primers for StAR compete off with β-actin primers. Increase in MgCl₂ concentration to 1.5 mM eliminate the competition i.e. intensity of StAR band increased with increase in MgCl₂ concentration. At 3.0 mM concentration, amplification of both StAR and β-actin genes were found optimum with both the bands of equal intensity.

similar manner, the house-keeping gene, β-actin was amplified using different primer concentration. Since the annealing temperature for each set of primer was different, accordingly changes were made in standard amplification parameters for PCR. Figure 1(b) represents the electrophoretic pattern of amplified β-actin gene, using different primer concentrations. Results showed a 243 bp amplification product at all concentrations and intensity of amplification consistently increased from 0.05 to 0.25 μM primer concentration.

Exponential range or effect of cycle number on amplification

It is well known that amplification under PCR is initially exponential but reaches a plateau when activity of enzyme declines and when any of the reagents become limiting in the reaction mixture. At plateau, RNAs initially present at high level may give products of equal intensity to low abundant RNAs. In the present study, the number of cycles ranging from 20 to 35 was tested. Figure 2 showed

(a)

	1	15 16	30 31	45
Buffalo	TCTTCTTGGTTCTCA	GCTGGAAGACCCTCT	CTACAGCGACCAAGA	
Bovine	TCTCCTAGGTTCTCA	GCTGGAAGACCCTCT	CTACAGCGACCAAGA	
Sheep	TCTCCTAGGTTCTCA	GCTGGAAGACTCTCT	CTACAGCGACCAAGA	
Porcine	TCTTCTTGGTTCTCA	GCTGGAAGACTCTCT	CTACAGTGACCAAGA	
Human	TCTACTCGGTTCTCG	GCTGGAAGAGACTCT	CTACAGTGACCAGGA	
	46	60 61	75 76	90
Buffalo	GCTGGCTTATATCCA	GCAGGGAGAGGAGGC	CATGCAGAGGGCCCT	
Bovine	GCTGGCCCATATCCA	GCAGGGAGAGGAGGC	CATGCAGTGGGCCCT	
Sheep	GCTTGCC TATATCCA	GCAGGGAGAGGAGGC	CATGCAGAGGGCCCT	
Porcine	TCTGGCCTATATCCA	GCAGGGAGAGGAGGC	CATGCAGAGGGCGCT	
Human	GCTGGCCTATCTCCA	GCAGGGGAGAGGAGGC	CATGCAGAAGGCCT	
	91	105 106	120 121	135
Buffalo	GGGCATCCTCAAAGA	CCAGGAGGGCTGGAA	GAAGGAGAGCCGGCA	
Bovine	GGGCATCCTCAAAGA	CCAGGAGGGCTGGAA	GAAGGAGAGCCGGCA	
Sheep	GGGCATCCTC AAAGA	CCAGGAGGGCTGGAA	GAAGGAGAGCCGGCA	
Porcine	GGGCATCCTCAGCAA	CCAGGAGGGCTGGAA	GAAGGAGAGCCGGCA	
Human	GGGCATCCTTAGCAA	CCAAGAGGG CTGGAA	GAAGGAGAGTCAGCA	
	136	150 151	165 166	180
Buffalo	GGCCAATGGGGACGA	GGTGCTGAGTAAAGT	GATCCCTGACGTGGG	
Bovine	GGCCAATGGGGACGA	GGTGCTGAGTAAAGT	GATCCCTGACGTGGG	
Sheep	GGTCAATGGGGACGA	GGTGCTGAGTAAAGT	GATCCCTGACGTGGG	
Porcine	GGAGAATGGGGACGA	GGTGCTGAGTAAAGT	GATCCAGATGTGGG	
Human	GGACAATGGGGACAA	AGTGATGAGTAAAGT	GGTCCCAGATGTGGG	
	181	195 196	210 211	225
Buffalo	CAAGGTGTTCCGGTT	GGAGGTGGTGGTGGGA	CCAGCCCATGGAGAG	
Bovine	CAAGGTGTTCCGGTT	GGAGGTGGTGGTGGGA	CCAGCCCATGGAGAG	
Sheep	CAAGGTGTTCCGGTT	GGAGGTGGTGGTGGGA	CCAGCCCATGGAGAG	
Porcine	CAAGGTGTTCCGGCT	GGAGGTGGTGGTGGGA	CCAGCCCATGGAGAG	
Human	CAAGGTGTTCCGGCT	GGAGGTGGTGGTGGGA	CCAGCCCATGGAGAG	
	226	240 241	255 256	270
Buffalo	GCTTTATGAAGAGCT	TGTGGAGCGCATGGA	AGCCATGGGCGAGTG	
Bovine	GCTTTATGAAGAGCT	TGTGGAGCGCATGGA	GGCCATGGGCGAGTG	
Sheep	GCTTTATGAAGAGCT	TGTGGAGCGCATGGA	GGCTATGGGCGAGTG	
Porcine	GCTTTATGAAGAGCT	TGTGGAGCGCATGGA	GGCCATGGGCGAGTG	
Human	GCTCTATGAAGAGCT	CGTGGAGCGCATGGA	AGCAATGGGGGAGTG	
	271	285 286	300 301	
Buffalo	GAATCCCAATGTCAA	GAAGATCAAGATCCT	GCAGAAG 307	
Bovine	GAATCCCAATGTCAA	GGAGATCAAGGTCTCT	GCAGAAG 307	
Sheep	GAACCCCAAGTGTCAA	GAAGATCAAGGTCTCT	GCAGAAG 307	
Porcine	GAACCCCAAGTGTCAA	GAAGATCAAGATCCT	GCAGAAG 307	
Human	GAACCCCAATGTCAA	GGAGATCAAGGTCTCT	GCAGAAG 307	

(b)

LLGSQLEDPLYSDQELAYIQQGEEAMQRALGILKDQEGWKKESRQANGDEVLS
 KVIPDVGKVFRLVWVDQPMERLYEELVERMEAMGEWNPVKKIKILQK

Figure 4. (a) Sequences of partial StAR cDNA from the buffalo ovary are compared with corresponding cDNA sequences of the same regions of bovine (ACCESSION No. Y17259, S 79908), porcine (ACCESSION No. NM_213755), sheep (ACCESSION No. NM_001009243), Human (ACCESSION No. AY 411003) and variant sequences are shown (dark). The numbers refer to the position of the nucleotide. The nucleotide sequence of PCR product was 307 nucleotide in length and possessed 98%, 96%, 94%, and 89% homology with the sequence of bovine, sheep, porcine, and human StAR gene, respectively. (b) Amino acid sequences coded by partial StAR cDNA from the buffalo ovary.

that there was increase in StAR mRNA expression from cycle number 20 to 35. The amplification reached a plateau at 35 cycles. Therefore, in exponential phase 30 cycles were selected as an optimal amplification cycle number.

Competition analysis with different sets of primers

Magnesium chloride is an essential factor for Taq polymerase function, but the efficiency of amplification with specific primers is strictly sequence dependent. In a

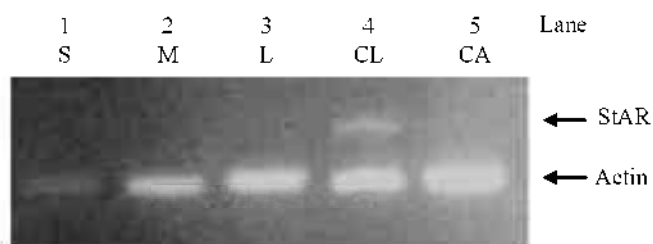


Figure 5. Expression of StAR mRNA during estrous cycle: PCR was carried out in 3.0 mM MgCl₂ concentration at 58°C (annealing temperature) for 30 cycles. Upper band represents StAR mRNA while lower bands showing a consistent expression of β -actin gene as a control. No expression was observed in lanes 2, 3 and 4, which represent RNA from whole follicles small, medium and large, respectively and in lane 5, which represents amplified DNA of corpus albicans. RNA sample from corpus luteum showed StAR expression (lane 4). Experiments were repeated at least three times.

competition analysis performed to assess amplification qualitatively for both StAR and control gene together, cDNA was amplified with different concentration of MgCl₂. StAR cDNA was not amplified at 1.0 mM MgCl₂ concentration (Figure 3). An optimal amplification was found in 3.0 mM MgCl₂ concentration.

Sequencing of StAR specific RT-PCR product sub-cloned into the PCR II vector

The nucleotide sequence of the StAR specific RT-PCR product from buffalo ovary was determined and was compared with reported sequences of bovine, porcine, sheep and human StAR (Figure 4). The nucleotide sequence of PCR product was 307 nucleotides in length and possessed 98%, 96%, 94% and 89% homology with the sequence of bovine, sheep, porcine and human StAR gene, respectively.

Localization of StAR expression during estrous cycle

Figure 5 showed the expression pattern of StAR mRNA from antral follicles of different size, corpus luteum and corpus albicans. No detectable StAR mRNA expression was observed in whole follicles (small, medium and large) as represented in Figure 6. However, there was a significant high expression in corpus luteum. Corpus albicans, on the other hand, showed no StAR message. Expression of StAR mRNA in granulosa cells isolated from different size antral follicles has been shown in Figure 5. StAR gene was expressed in granulosa cells from antral follicles greater than 9 mm in diameter (preovulatory). However, StAR message was not detected in granulosa cells from small and medium sized follicles. In both sets of above experiments, expression of control gene was consistent.

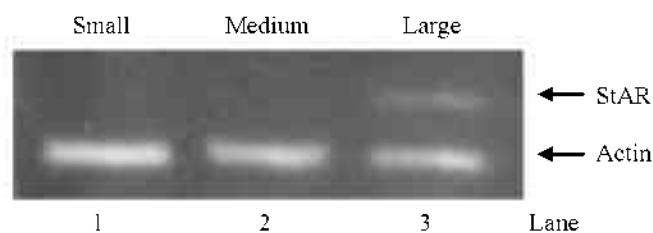


Figure 6. Expression of StAR mRNA in granulosa cells isolated from different follicles: The reaction was carried out in 3.0 mM MgCl₂ concentration 58°C (annealing temperature) for 30 cycles. Upper band represents the StAR mRNA and the lower bands represent amplification of β -actin as the control gene. StAR mRNA expression was localized to granulosa cells isolated from large follicles (preovulatory, lane 3). Lane 1 and lane 2 represent RNA isolated from granulosa cells of small and medium follicles respectively. Experiments were repeated at least three times.

Effect of FSH alone or along with IGF-I on StAR mRNA expression

To assess whether StAR mRNA is hormonally regulated, primary culture of buffalo granulosa cells were treated with FSH (100 ng/ml) alone or along with IGF-I (100 ng/ml) for 12 to 18 h as described in materials and methods. As shown in Figure 7, both FSH and FSH plus IGF-I enhanced the StAR expression versus control. Effect of IGF-I plus FSH treatment for 12 to 18 h on StAR expression in cultured granulosa cells was found highly significant ($p < 0.01$) in comparison to cells treated with FSH alone. β -Actin was used as a control gene. Both, FSH (100 ng/ml) and FSH (100 ng/ml) plus IGF-I (100 ng/ml) treatment increased the StAR mRNA expression (Figure 7, Lane 2 and 3, respectively) as compared to control.

DISCUSSION

In the present study, expression, localization and hormonal regulation of StAR mRNA in buffalo ovary were analyzed by semi-quantitative RT-PCR. Semi-quantitative RT-PCR is a highly sensitive and specific method useful for the detection of rare transcripts or for analysis of samples available in limiting amount (Erich, 1989; Carding et al., 1992; Souza et al., 1996). However, as for all quantitative techniques, great care must be taken in all optimization steps and the necessary controls to ensure a quantitative (semi-quantitative) analysis (Souza et al., 1996).

Despite the greater accuracy of recently developed techniques, semi-quantitative RT-PCR methods are still widely used and appropriate for many purposes (Carding et al., 1992; Ali et al., 1997). When choosing quantitative or semi-quantitative RT-PCR protocol to determine RNA expression, many parameters must be taken into consideration (Freeman et al., 1999) including MgCl₂

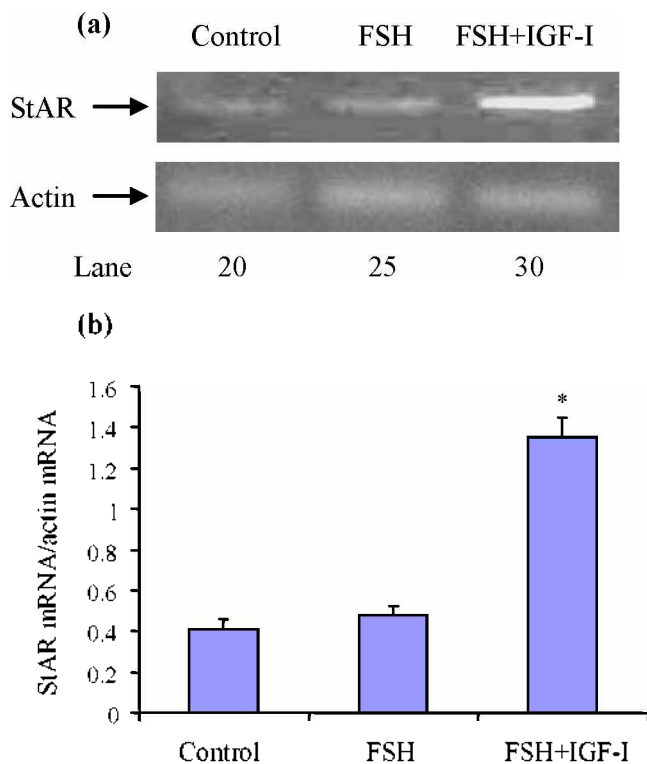


Figure 7. Effect of FSH (100 ng/ml) and FSH (100 ng/ml) plus IGF-I (100 ng/ml) on StAR mRNA expression in cultured granulosa cells treated for 18 h: Reaction was carried out in 3.0 mM MgCl₂ concentration at 58°C (annealing temperature) for 30 cycles. Both FSH (lane 2) and FSH plus IGF-I (lane 3) treatment increased StAR mRNA expression as compared to control (lane 1). Intensity of band in granulosa cells treated with FSH plus IGF-I was significantly (*; $p < 0.01$) higher as compared to FSH treatment. IGF-I synergized the FSH stimulated StAR mRNA expression in cultured granulosa cells. Lower bands represent β -actin as control gene which was constitutively expressed in control and treated cells. Experiments were repeated at least three times.

concentration, cycle number, primer concentration, etc. Likewise, the detail procedure was standardized for analysis of StAR mRNA level. The amplified RT-PCR product was sub-cloned in PCR vector II (*In vitro*) and sequenced to confirm the sequence of band (Figure 7). β -Actin was used as internal control to normalize for sample variation in total RNA content and for reaction efficiency. Reaction conditions in the reverse transcription step are mostly dependent on the enzyme and the primer of choice. The reverse transcription of total RNA was done with preferred hexamers so that PCR analysis could be performed in the same cDNA sample. β -Actin has been used as an internal control because it is expressed constitutively at moderately abundant levels in almost all cell types and, in general, its expression is not influenced by any hormone treatment (Foss et al., 1998).

In our system, the pattern of control gene in all samples

of RNA studied was found normal, though in certain cases, it was seen that β -actin amplifies a non-specific band of 200 bp (not shown). This could be probably because β -actin gene is expressed at a very high level, hence its amplification profile reaches a plateau much earlier than the relatively low abundant mRNA (StAR in the present case). It is also reported that β -actin primers can amplify certain pseudo genes (Dirnhofner et al., 1995; Raff et al., 1997; Mutimer, 1998) In order to rule out this problem, glyceraldehyde 3-phosphate dehydrogenase has been tested as a control gene, which worked best and did not affect the results (unpublished data).

StAR is an important factor in the acute regulation of steroidogenesis (King et al., 1995). In mammalian ovary, its expression is related to enhanced steroidogenic activity as a result of trophic hormone stimulation during follicular development and in the luteal phase. In bovines, expression of StAR has been shown to be limited to theca cells of non-atretic antral follicles (Bao and Garverick, 1998) including corpora lutea tissue (Pescador et al., 1996). In sheep, only faint expression of StAR mRNA was observed in preovulatory follicles by *in situ* hybridization technique (Logan et al., 2002). Spatio-temporal expression pattern of StAR in rat ovary has shown its expression confined to granulosa cells of preovulatory follicles (Ronen-Fuhrmann et al., 1998), which was later confirmed by isolation of two StAR mRNA transcripts corresponding to 3.6 and 1.6 kb (Minegishi et al., 2000). However, using semi-quantitative RT-PCR technique, in the present study, we were unable to detect StAR gene in whole follicles of various sizes in buffalo ovary and most of the expression was confined to corpus luteum. Present study also confirms the absence of detectable StAR gene in regressing corpus luteum (corpus albicans) in accordance with the previous study conducted by Hartung et al. (1995) in bovine. Pollack et al. (1997) also observed a similar result by immunostaining, where corpora albicans was not stained at all.

In concordance with the studies conducted on StAR gene expression during follicular development in rat ovary, the overall expression of StAR was shown to be localized in granulosa cells of preovulatory follicles rather than the non-ovulating follicles (Ronen-Fuhrmann et al., 1998). Present study confirms its expression in preovulatory granulosa cells. No expression of StAR gene was observed in granulosa cells isolated from follicles less than 9 mm diameter. Expression pattern for StAR gene in granulosa cells showed that during follicular development, before luteinization, the preovulatory stage becomes responsive to trophic hormone resulting in increased steroidogenesis simultaneously contributing towards increased synthesis of StAR gene. Since the theca cells of follicles are tightly attached to the basement membrane during aspiration of follicular fluid, it is obvious that they may escape the fluid.

Hence, present results on the expression of StAR mRNA in granulosa cells during follicular development in buffalo was an added confirmation to the sheep in which hybridization technique showed the expression of StAR in granulosa cells of large follicles greater than 4.0 mm in diameter (Logan et al., 2002).

The present study further elucidates the regulatory mechanism underlying the synergism exerted by FSH and IGF-I in stimulating StAR gene expression in differentiating granulosa cells. A number of previous studies have shown that several growth factors like GH, insulin and IGF-I (Adashi et al., 1985) and hormones like FSH (Pescador et al., 1997) and LH (Luo et al., 1998) play an important role in intraovarian regulation of follicular development in a species specific manner. When stimulated by FSH, granulosa cells express StAR mRNA and protein in primary culture (Balasubramanian et al., 1997). It could be attributed to the fact that upon trophic hormone stimulation, there is increased synthesis of estradiol and progesterone in granulosa cells in porcine (Pescador et al., 1997) and hen (Johnson et al., 2002) during follicular development. FSH stimulation elevated StAR messages in luteinized porcine granulosa cells over 6 h of treatment (Pescador et al., 1997).

Present investigation extends to study the effect of FSH alone or along with IGF-I, the two major first messenger molecules involved in maturation and differentiation of granulosa cells. Although, IGF-I is necessary for normal FSH receptor expression in the ovary (Zhou et al., 1997), it was observed that IGF-I augmentation of FSH-mediated StAR activation resulted in enhanced expression. IGF-I stimulated FSH induced StAR expression up to 6-fold, whereas treatment with FSH or IGF-I alone had a small consistent stimulatory effect in porcine granulosa cells (Balasubramanian et al., 1997).

Hormonally untreated sparingly differentiated pig granulosa cells revealed little detectable basal expression of StAR mRNA and protein (Balasubramanian et al., 1997). This finding was consistent with the observations in unstimulated bovine granulosa cells (Hartung et al., 1995). During culture, it was observed that untreated cultured granulosa cells also yield a low StAR message. Though, in our culture system, there was no case of luteinization, there are chances of cells becoming luteinized after 24 h of culture in media containing fetal bovine serum. This was reported during study of hormonal regulation in cultured bovine granulosa and theca cells by Mamluk et al. (1999).

Buffalo granulosa cells treated with FSH alone for 12 to 18 h yield a low StAR message, which was significantly increased in cells treated with FSH plus IGF-I, indicating a prominent synergism of IGF-I on FSH stimulated StAR mRNA expression. The similar synergistic action of IGF-I on FSH stimulation of StAR gene was demonstrated at the promoter level by LaVoie et al. (1999). Though a study of

Eimerl and Orly (2002) demonstrated only a modestly increased effect of IGF-I on FSH stimulation of StAR mRNA in rat ovarian granulosa cells, it was later confirmed that IGF-I mediates the effect of FSH on follicular development, granulosa cell apoptosis and steroidogenesis in goat ovary (Yuansong et al., 2003). Further, it was shown that FSH and IGF-I stimulate the porcine StAR gene promoter activity (LaVoie et al., 2004). The total RNA content in culture was found consistent, since cultured granulosa cells with or without hormonal treatment showed a prominent and consistent expression of housekeeping gene. Thus, present investigation confirmed the synergistic action of IGF-I on FSH-stimulated StAR mRNA expression in cultured buffalo granulosa cells by a semi-quantitative RT-PCR technique.

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