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# EXPRESSION OF ANDROGEN RECEPTORS AT mRNA LEVEL IN BOVINE PLACENTOMES DURING 50-150 DAYS OF PREGNANCY

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Bovine placenta is a classical target and source of many steroid hormones which are involved in the growth, differentiation and development of placenta. Placental trophoblasts are an important source of endocrine, paracrine and autocrine acting hormones. Androgens are considered male sex hormones; however, they are also secreted and have regulatory functions in females and play a predominant role in the development and physiological functions in both sexes. The androgen receptor plays a key role in androgen action. To study the expression of androgen receptor in bovine placentome at mRNA level the placentomes were collected at a local slaughterhouse of Giessen Germany from healthy cows between 50 to 150 days of gestation. RNA was prepared in laboratory with trizol method and reversed transcribed. Conventional PCR agarose gel mRNA and the taqman method of detection was used for expression of androgen receptors. GAPDH was used as housekeeping reference gene. AR-specific mRNA expression was detected in all RNA preparations from placentomes of early pregnancy. Significant AR expression suggests that besides progesterone and estrogens also androgens may be active products of placental steroidogenesis in cattle.
**Keywords:** Cattle, placenta, androgen receptor, trophoblast

## INTRODUCTION

Bovine placenta is a classical target and source of many steroid hormones which are involved in the growth, differentiation and development of placenta (Vanselow *et al*., 2008; Schuler *et al*., 1999; Ali *et al*., 2012). The foremost placental steroids are estrogen (E) and progesterone (P). In most animal species, the circulating levels of progesterone begin to decline prior to labor, while the circulating levels of estrogen increase. During bovine pregnancy the higher amounts of estrogens are produced throughout pregnancy predominantly in sulfoconjugated form i.e. estrone sulfate (E1S) (Hoffmann *et al*., 1997). Sites of estrogen production are the fetal cotyledons (Hoffmann *et al*., 1979; Schuler *et al*., 1994), which together with the maternal caruncular tissue, forms the multiple discrete sites of placentation, the placentomes (Leiser *et al*., 1994). Placental trophoblasts are an important source of endocrine, paracrine and autocrine acting hormones (Vanselow *et al*., 2008).

Androgens are considered as male sex hormones; however they are also secreted and have regulatory functions in females and play a predominant role in the development and physiological functions in both sexes. In addition to estrogen and progesterone, high levels of androgens are also present in the uterine environment during early pregnancy, a seemingly common phenomenon among mammals, including humans, pigs and rats (Bonney *et al*., 1984; Stefanczyk-Krzymowska *et al*., 1998; Warshaw *et al*., 1986; Qureshi *et al*., 2013). In the pig, elongating blastocysts and placenta are an additional source of androgens and may influence some processes occurring during pregnancy (Cardenas and Pope, 2003).

Reproductive functions of androgens are mediated through coordination of diverse physiological processes ranging from brain functions to specific cell proliferation and apoptosis. Androgens regulate the trophic environment and architecture of the uterus via a gene expression program. Development of sex-specific phenotype depends on the presence or absence of androgens during the phase of organogenesis of the urogenital tract (Sajjad *et al*., 2004). At the molecular level, most of these regulatory influences are exerted by altered expression of appropriate genes by the androgen receptor (AR) (Arun *et al*., 2001). AR is one of the transcription factors which are known to be essential for embryonic differentiation.

The androgen receptor plays a key role in androgen action; its main function is a DNA binding transcription factor which regulates gene expression (Haelens *et al*., 2001). Androgen activity is supposed to be the sum of a direct effect mediated by AR and an indirect effect of estrogens produced by aromatization of androgens (Culig *et al*., 1994). Androgen receptor has been reported to be expressed in variety of species during pregnancy, menstrual and estrus cycle. In women, androgen receptors are expressed in healthy and pathological conditions such as adenomyosis,

endometriosis and endometrial carcinoma (Hobisch *et al*., 1998). AR in the pig endometrium during the window of maternal receptivity for implantation and the functional, albeit complex, interactions of androgen and estrogen in the regulation of uterine endometrial gene expression and cell growth in vitro has been reported (Kowalski *et al*., 2004). Inmunostaining is also demonstrated in the uterus of female fetuses on day 90 as well as in the uterus of day-old piglets (Slomczynska *et al*., 2008).

In contrast to the well-documented roles of estrogen receptors and progesterone receptors, no information is available about the physiological role of androgens and the regulation of AR during bovine pregnancy. The objective of the present experiments is to examine the relative amounts of AR in the bovine placentome during early pregnancy at mRNA levels using reverse transcription and real-time-polymerase chain reaction (RT-PCR) techniques.

## MATERIAL AND METHODS

***Sample collection*:** Placentomes were collected at a local slaughter house from 6 healthy cows between 50 to 150 days of gestation. Gestational age was estimated according to fetal crown-rump length (Rexroad *et al*. 1974). From each animal, medium sized placentomes were removed from the mid-region of the horn that contained the fetus and trapezoid pieces encompassing complete height of the interdigitation zone. For RNA extraction, complete placentomes obtained from living animals were snap-frozen in liquid nitrogen and stored at -80°C until analysis. Placentomes collected at the slaughter house were wrapped in aluminum foil and snap-frozen on dry ice. Sample collection from living animals was approved by the responsible authority, the regional council in Giessen (record token II25.3-19c20/15cGI18/14).

***Preparation of total RNA*:** Coarse pieces of deep-frozen (-80°C) tissues wrapped in aluminum foil were broken up using a clean hammer. The tissue fragments were quickly placed in a pre-chilled mortar and powdered by a pestle under liquid nitrogen. 100 mg of tissue powder were immersed in 1 mL Trizol solution (Invitrogen, Karlsruhe, Germany) and further homogenized by three 60-120 seconds bursts on ice using an ultra turrax T25 (IKA-Werke GmbH & Co KG, Staufen Br., Germany). 200 µl chilled (-20°C) chloroform was added to the homogenized tissue, after short gentle shaking (up and down) it was kept on ice for 5 minutes followed by centrifugation at 20160 x g at 4°C for 15 minutes. After centrifugation, the uppermost of the resulting three liquid phases was taken into a fresh 2 ml round Eppendorf tube (Sarstedt, AG & Co., Nümbrecht, Germany) and 200 µl chloroform was added. After centrifugation at 20160 x g at 4°C for 15 minutes, the supernatant was taken into a fresh 2 ml Eppendorf tube and lower portion was discarded. 400 µl chilled (-20°C) isopropanol was added to the supernatant and incubated for 1 hour at -20°C. This was followed by centrifugation at 20160 x g for 10 minutes at 4°C. The supernatant was discarded and the pellet was redissolved in 500 µl chilled (-20°C) 70% ethanol. After incubation for 10 minutes on ice followed by centrifugation at 20160 x g at 4°C for 10 minutes the ethanolic phase was discarded. This washing step was repeated. After complete draining of the alcohol, the pellet was allowed to dry at 37°C. After complete drying, the pellet was solubilized in 50 µl diethylpyrocarbonate (DEPC) treated water and kept in a water bath at 70°C for 10 minutes, followed by vortexing for complete solubilization. 50 U of RNAse inhibitor (Fermentas, Sankt Leon-Rot, Germany) was added and the total concentration of RNA was measured in a BioPhotometer (Eppendorf AG, Hamburg, Germany) at 260 nm in disposable cuvettes (UVette®, Eppendorf AG Hamburg, Germany) containing 2 µl of RNA stock solution and 98 µl double distilled water ($ddH_2O$) using 100 µl $ddH_2O$ as a blank. Working solution aliquots adjusted to an RNA concentration of 100 ng/µl were prepared from stock solution by dilution with $ddH_2O$ and stored at -20°C until use. The remaining RNA stock solution was stored at -80°C till further use.

***Conventional reverse transcription (RT)-polymerase chain reaction (PCR)*:** For RT-PCR, the GeneAmp RNA PCR Kit (Perkin Elmer, Foster City, CA) was used. 0.3 µg of total RNA was reverse transcribed at 42°C for 15 min in a total volume of 10 µl containing 2.5 $mmol.l^{-1}$ random hexamers, 4 mM dNTPs, RNase inhibitor (1 U per reaction) and reverse transcriptase (2.5 U per reaction). The reaction was stopped by heating to 99°C for 5 min. The cDNA mix was amplified in a Personal Cycler (Biometra GmbH, Göttingen) after addition of 20 µM primers and 1.25 units of AmpliTaq DNA polymerase per reaction in a 50 µl reaction volume containing 1 $mM.l^{-1}$ $MgCl_2$. Primers (forward: 5´-CAGATGGCAGTCATTCAG-3´; reverse: 5´-CTTGGTGAGCTGGTAGAAG-3´ were designed to yield a 386 bp fragment from bovine AR sequence data (XM_0012253942). A negative control was set up using $H_2O$ instead of RNA in the RT reaction mix. The program comprised 40 cycles of denaturation at 94 °C for 1 min, annealing at 56°C for 1 min, and extension at 72 °C for 1 min. The products were analyzed on a 2% agarose gel stained with ethidium bromide and visualized under UV transillumination.

***Real time RT-PCR for relative quantification of AR expression*:** Relative levels of mRNA specific for AR were determined by real-time RT-PCR (taqman method) using GAPDH as house-keeping gene in one placentome of each of 6 cows (days 50-150). Total RNA was prepared and reverse transcribed as described above. To eliminate genomic DNA, isolated RNA was treated with DNase (Roche Molecular Biochemicals, D-68305 Mannheim)

following the manufacturer's instructions prior to reverse transcription.

PCR amplification was performed on an automated fluorometer (ABI PRISM 7000 Sequence Detection System, Applied Biosystems, D-64293 Darmstadt, Germany) using 96-well optical plates. Each sample was analyzed in duplicate. For PCR cDNA corresponding to (100 ng) total RNA was used in a 23 µl PCR reaction mixture containing TaqMan_qPCR Master Mix (Eurogentec, B-4102 Seraing Belgium), 300 nM of each primer and 200 nM probe. Primers and probes (listed in Table 1) were designed using the Primer Express software (version 2.0, Applied Biosystems, Foster City, CA). Primers and probe specific for bovine androgen receptor were located in exon 6, which codes for a part of the hormone binding domain. Probes were labeled at the 5'-end with the reporter dye 6-carboxyfluorescin (FAM) and at the 3'-end with the quencher dye 6-carboxytetramethyl-rhodamine (TAMRA). Amplification conditions were the same for the target and the reference gene: denaturation for 10 min at 95 °C followed by 45 cycles at 95°C for 15s and 60°C for 60s. The results were calculated using the comparative CT method (ΔΔCT method) according to the instructions of the manufacturer of the ABI PRISM 7000 system and were reported as n-fold differences in comparison to the sample with the lowest amount of the respective target gene transcripts (calibrator) after normalizing the samples referring to the housekeeping gene GAPDH.

## RESULTS

***Detection of AR-specific mRNA by conventional RT-PCR*:** In order to confirm AR expression in the bovine placentomes at the mRNA level, RNA samples were prepared from complete placentomes of 6 different animals at different stages of gestation (day 50 to 150). mRNA levels were analyzed by conventional RT-PCR by using bovine specific primers specifically designed for this purpose (Table 1). Androgen receptor mRNA was expressed in all of the tested bovine placental tissues (Fig.1), a single band of the expected size (387 bp) was detected in placentomes from all animals irrespective of gestational age, no further amplifications could be detected in any of these samples.

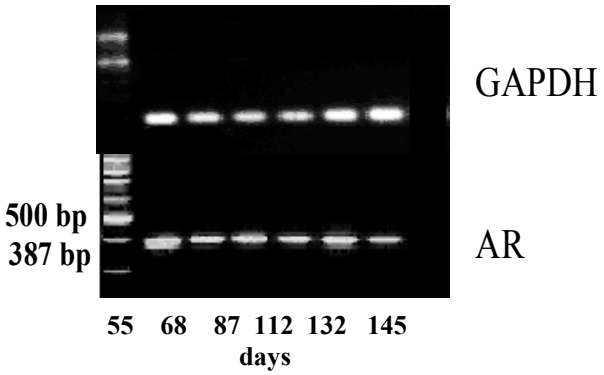


**Figure 1**. **Analysis of RT-PCR products on agarose gels (2%) stained with ethidium bromide and visualized under UV illumination for the presence of androgen receptor-specific mRNA in samples prepared from complete placentomes of cows at different stages of early pregnancy.** Expected sizes of the amplicons are 387 bp. GAPDH was used as positive control. Negative control is product from RT-PCR in the absence of RNA.

***Detection of AR-specific mRNA by Real-Time RT-PCR*:** Relative mRNA levels for AR and for the housekeeping gene GAPDH were determined in one placentome of each of 6 cows. Taqman method of detection was used for quantitative measurement of AR-mRNA. The titration curves prepared to determine linear ranges and amplification efficiencies of GAPDH and androgen receptor gene.

AR-specific mRNA expression was detected in all RNA preparations from placentomes. Mean relative expression levels ranged 1 to 41.2 in various pregnant animals, the highest expression was found in 50 days pregnant cow, whereas, lowest expression was in 150 days pregnant cow.

**Table 1. Primer sets and probe sequences for real time RT-PCR and the corresponding amplicon lengths (base pairs, bp) and their positions (nucleotides, nt) in published mRNA sequences**

| Gene | Primer/Probe | Amplicon length (bp) | Accession No. |
|---|---|---|---|
| AR | forward: 5'-CAC CTC TCC CAA GAA TTT GG-3'<br>reverse: 5'-TGC CTT CAT GCA CAG GAA T-3'<br>probe: 5'-TGG CTC CAA ATC ACC CCC CAG G-3' | 65 | XM_001253942<br>(nt 2278-2340) |
| GAPDH | forward:5′-GCG ATA CTC ACT CTT CTA CCT TCG A-3′<br>reverse: 5′-TCG TAC CAG GAA ATG AGC TTG AC-3′<br>probe: 5′-CTG GCA TTG CCC TCA ACG ACC ACT T-3′ | 82 | U85042<br>(nt 827-908) |

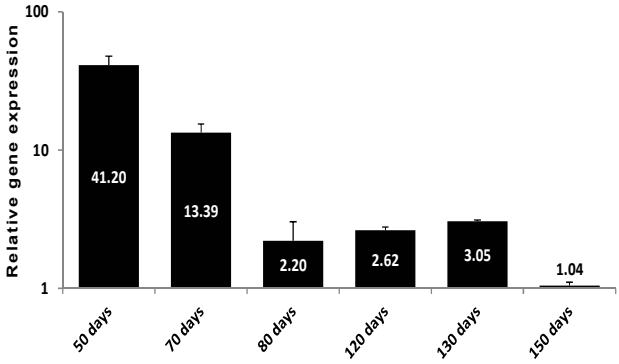


**Figure 2. The AR mRNA levels measured by real-time PCR normalized to GAPDH in placentomes from cows at different stages of gestation (day 50-150).**

## DISCUSSION

Results of the present study demonstrated the highest to moderate expression of AR in the bovine placentomes during early and mid-pregnancy. AR expression in bovine placentome has not been detected before. These results support the AR expression reported in pig during pregnancy (Stefanczyk-Krzymowska *et al*., 1998). AR immunostaining was demonstrated in the uterus of the female fetuses on day 90 as well as in the uterus of day-old piglets. Physiological roles of androgens in the fetal and maternal parts of bovine placentomes on the first view are unexpected. However, the expression of AR in human placenta has been suggested already in early studies using ligand binding assays (Hirota *et al*., 1981; McCormick *et al*., 1981), and was later confirmed by immunohistochemistry in human decidua and trophoblast (Horie *et al*., 1992; Uzelac *et al*., 2010). In bovine placentomes, expression of AR was unequivocally demonstrated on the mRNA-level by conventional RT-PCR and by the Taqman-based quantitative real-time RT-PCR method, where in addition to specific primers further specificity is coming from the gene specific probe.

Thus, in bovine trophoblast cells androgens and AR may be elements of an intracrine mechanism involved in the control of TGC differentiation, and AR may be up-regulated by increasing androgen levels in differentiating trophoblast cells. A dependency of AR expression on the presence of androgens has been previously demonstrated in various cell types of the male genital tract (Zhu *et al*., 2000). On the other hand, up-regulation of AR in the aromatase (Schuler *et al*., 2006) and ERß (Schuler *et al*., 2005) expressing TGC may also be stimulated by estrogens. The up-regulation of AR by estrogens has been demonstrated in endometrial stroma cells of macaques (Slayden and Brenner, 2004).

Since the discovery of steroid receptors, the concept of their roles has significantly changed from monospecific ligand activated receptors to components of the general transcription machinery, which among various other functions, also possess a steroid activated transcriptional activity. Thus, other functions besides effects from the classic interaction of AR with androgens must also be considered, e.g. the cross-talk of various signal cascades initiated by growth factors which may target AR (Zhu and Kyprianou, 2008). Accordingly, the expression of various relevant growth factors such as fibroblast growth factors (Pfarrer *et al*., 2006a), vasoendothelial growth factor (Pfarrer *et al*., 2006b) or transforming growth factor β (Ravelich *et al*., 2006) has been demonstrated in bovine placentomes, and the up-regulation of AR induced by placental steroids may be a prerequisite to enable their full spectrum of effects.

In conclusion, the detection of significant AR expression suggests that besides progesterone and estrogens, androgens may also be active products of placental steroidogenesis in cattle. Moreover, this concept suggests a new function for the predominant production of inactive estrogen sulfates in bovine placentomes which might serve the control of androgen activities.

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