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Circannual changes in the expression of vascular endothelial growth factor in the testis of roe deer (*Capreolus capreolus*)

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ABSTRACT

Adult roe deer males show seasonal cycles of testicular growth and involution. The exact timing of these cycles requires endocrine regulation and local testicular control by autocrine/paracrine factors. Recent findings suggest that the vascular endothelial growth factor (VEGF) might have effects on both vascular and germinative cells in testis. Thus, we studied the expression pattern of vascular endothelial growth factor (VEGF) in roe deer testis using quantitative RT-PCR. The strength of VEGF mRNA expression depended on season. It reached its highest level at the peak of spermatogenesis during the pre-rutting period and had its nadir at the end of the rut when involution already began. The results suggested that VEGF may directly affect the regulation of spermatogenesis but may not be involved predominantly in testicular microvasculature as initially expected.

1. Introduction

European roe deer (*Capreolus capreolus*) have evolved numerous adaptations to cope with the seasonally changing conditions in their distribution range such as feeding strategies, locomotion, behavior and reproduction (Fickel et al., 1998, 1999; Duncan et al., 1998). In roe deer the latter is characterized by a distinct seasonal pattern with a fully arrested spermatogenesis in December (Blottner et al., 1996; Schön et al., 2004) and a summer breeding season that lasts only about four weeks from mid-July to mid-August (Semperé et al., 1998). Quantitative studies on seasonal activity changes and histological organization of roe deer testis had demonstrated that spermatogenesis was already fully activated in June (Schön et al., 2004). Thus males have synchronised cycles of testicular involution and recrudescence during the transition between non-breeding and breeding periods (Bronson and Heidemann, 1994). Especially during testis growth, an adequate blood supply is necessary for the provision of nutrients and growth factors, most likely requiring neovascularisation of roe deer testis as was seen in adult golden hamster testes during their seasonal transitions from reproductive quiescence to reproductive activity (Mayerhofer et al., 1979).

Members of the vascular endothelial growth factors' (VEGF) family are major mediators of neovascularisation. They stimulate endothelial cell proliferation and are involved in the alteration of microvascular permeability (Ferrara and Davis-Smyth, 1997). However, beyond its well-known angiogenetic effects the VEGF system appears to participate in the development of male germ cells too because it was detected in different somatic and germinative testis cell types (Ergün et al., 1997; Nalbandian et al., 2003; Guo et al., 2004). In mice over-expression of VEGF isoforms resulted in markedly reduced fertility, suggesting an involvement of VEGF in spermatogenesis (Korpelainen

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et al., 1998; Huminiecki et al., 2001) and thus emphasizing the need to decipher the complex functions of VEGF in the male genital tract (Ebisch et al., 2008).

To study the role of VEGF during the reproductive cycle of roe deer, VEGF gene expression was analysed monthly from March to December by quantitative RT-PCR (qRT-PCR). To investigate the varying energy demand of testis development during the reproductive cycle, the expression of glyceraldehyd-3-phosphate dehydrogenase (GAPDH), an enzyme of the glycolysis pathway, was analysed simultaneously.

2. Materials and methods

2.1. Animals and tissues

Roe deer testis tissue was obtained monthly from March to December by fine needle biopsies of the same five to six free ranging animals immediately after capture as described elsewhere (Göritz et al., 2003). Biopsies were submersed immediately in 20 μl TRIzol Reagent (GIBCO Life Technologies) and snap-frozen in liquid nitrogen.

2.2. Reverse transcription (RT) and PCR-reaction

Total RNA was isolated from biopsies (~10 mg) with the High Pure RNA Tissue Kit (Roche Applied Science) following Wagener et al. (2003). The gene specific primers were taken from Garrido et al. (1993) and Wagener et al. (2000), respectively. As house-keeping genes are most likely expressed seasonally, plasmid-cloned PCR-fragments of VEGF (isoforms 121 and 165) and of GAPDH, respectively (Wagener et al., 2003), were used as standards for quantitative PCR. The purified plasmids (1 μg) were linearized with 10 units *Spe*I (Promega), purified (QIAquick PCR Purification Kit 250; Qiagen GmbH), and then quantified spectrophotometrically at 260 nm. Standard curve dilutions contained 10^6, 10^5, 10^4, 5×10^3, and 10^3 molecules per 5 μl. Dilutions of the plasmids containing the isoform $VEGF_{121}$ and $VEGF_{165}$ were then mixed at a ratio of 1:1. PCRs were carried out as follows: Either 5 μl of the RT reaction, 5 μl of each standard dilution, 5 μl RT reaction mixture without RNA (negative control 1) or 5 μl of water (negative control 2) were used in a 25 μl PCR reaction. The PCRs were performed with 2× Applied Biosystems' (ABI) SYBR®-Green Mastermix and 200 nM of each specific primer on a GeneAmp 5700 Sequence Detection System (ABI). PCRs were initially denatured for 10 min at 95 °C and had a final extension phase of 5 min at 72 °C. Amplification programmes were applied as follows: 40 cycles at 95 °C 30 s, 52 °C (GAPDH) or 61 °C (VEGF) 20 s, 72 °C 40 s. Absence of primer dimers was controlled for by post-PCR melting curve analysis according to the manufacturer's recommendations (ABI). Measured values were analysed using the system integrated GeneAmp 5700 SDS software (ABI).

2.3. Statistical analysis

For each animal and each month, the total number of mRNA-molecules per 10 ng RNA was calculated for VEGF and GAPDH and mean values ±standard error of means (SEM) for the five animals were determined. The repeated measurement ANOVA was applied to estimate the variation among monthly means. We also analysed the expression of VEGF and GAPDH using a contrast analysis by comparing all pairs of subsequent main phases within the annual reproductive cycle of roe deer; phase I: testis growth (March/April), phase II: pre-rutting period (June/July), phase III: late rutting period including onset of testicular involution (August/September), and phase IV: arrest of spermatogenesis (November/December) as described by Wagener et al. (2003). All calculations were performed using the SPSS 9.0 (SPSS Inc., Chicago, IL) software, with the significance level set to 5%.

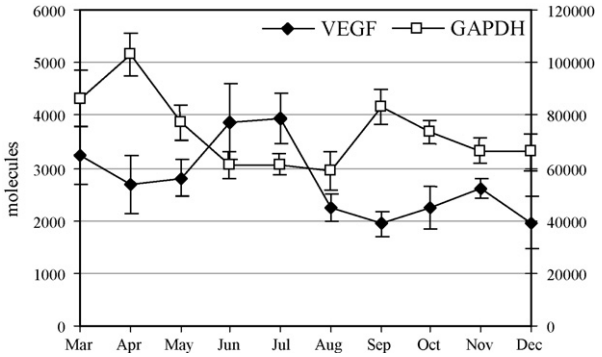

Fig. 1. Absolute expression levels of VEGF (left y-axis) and GAPDH (right y-axis) in the course of the year. Seasonal variation of the means was significant for both mRNAs ($p < 0.05$; ANOVA).

3. Results

Messenger RNAs for VEGF and GAPDH were detected in all biopsies examined at all time points. Based on the standard curve, 10 ng of total RNA was calculated to contain between 2100 and 3800 molecules of VEGF and between 61,000 and 95,000 GAPDH molecules (Fig. 1). According to this estimate the amount of GAPDH mRNA was approximately 25 times higher than that of VEGF mRNA in roe deer testis. VEGF and GAPDH both showed a seasonally changing expression pattern, with a significant seasonal variation of the means ($p < 0.05$; ANOVA). The graph of the VEGF expression throughout the year had its peak in June/July during the pre-rut (Fig. 1). According to the contrast analysis, the relative expression level of VEGF was increased at the beginning of the rutting period and reached its nadir already at the end of the rut (Fig. 2). Its relative expression in the pre-rut was almost two times as high as during the late rut ($p < 0.05$). After involution was complete, VEGF gene expression increased slightly, but did not differ significantly from those of the other phases. The VEGF expression level during testis growth was also not higher and did not differ significantly compared with the other phases. GAPDH showed a different seasonal expression pattern than VEGF. In its annual course GAPDH mRNA expression was highest in April during the phase of testis growth (Fig. 1). According to the contrast analysis, GAPDH was significantly higher expressed during this phase than during others (Fig. 2). The