LOCAL REGULATORS OF SEASONAL REPRODUCTION PROCESSES IN UTERUS MASCULINUS OF AN ADULT MALE EUROPEAN BISON (BISON BONASUS, LINNAEUS 1758)

Growth factors, hypoxia-inducible factor 1-alpha (HIF-1α) and klotho protein all have very important functions in the male reproduction; however their role in the regulation of seasonal reproductive processes in the male European bison remains unclear. Similarly, although the uterus masculinus is very frequently found in the bison, its importance and functions remain unknown. It is likely that, this organ may have secretory functions and thus be a target for various regulatory factors. Therefore, the aim of this study was to investigate expression and activity of several factors: vascular endothelial growth factor (VEGF-A), fibroblast growth factor (FGF-2), transforming growth factor β1 (TGF-β1), nerve growth factor (NGF), insulin-like growth factor receptor (IGF-IR β), hypoxia-inducible factor 1 alpha (HIF-1α), and klotho protein in the uterus masculinus, immediately after the season of the reproductive activity (November and December). Our study reveals that the growth factor expression levels are significantly higher in November, when compared to December, while expression of HIF-1α and klotho was higher in December. These results provide novel data on differences in the expression levels of several factors in the uterus masculinus of European bison bulls after the breeding season. The described factors may, therefore, be potent regulators of the seasonal reproduction.

Key words: growth factors, hypoxia-inducible factor 1-alpha, klotho, uterus masculinus, seasonal reproduction, European bison, vascular endothelial growth factor, nerve growth factor
presence and expression of the above-mentioned growth factors, HIF-1α and klotho protein, in *uterus masculinus* tissues of Polish European bison bulls immediately after the breeding season.

**MATERIAL AND METHODS**

**Animals and tissues**

*Uterus masculinus* tissues of European bison (*Bison bonasus*, Linnaeus 1758) bulls were collected from mature adult males (500 – 620 kg body mass) during November (n = 6) and December (n = 6) (time after the breeding season, falling in September and October). Although both months belong to the period post the intense reproductive activity, processes occurring in reproductive tissues may change intensively due to gradual inhibition of spermatogenesis. As the bison population is covered by increased protection, elimination of bison and thus access to *uterus masculinus* tissues are strictly limited. However, it will be a priority in further seasonal analyzes. Animals were culled ‘on the spine’ (a shot from a firearm in the area of 2 – 3 cervical vertebrae causes defragmentation of the spinal cord, the animal completely loses consciousness and does not feel any pain, next, circulation is stopped and death occurs) without the use of anesthetics, during selective eliminations in the Bialowieza National Park and Bialowieza Forest (northeastern Poland), according to the approved Polish legal requirements. The individuals for the analyses were carefully selected, and mechanical injury to the limbs was eliminated in the Bialowieza National Park and Bialowieza Forest (northeaster Poland), according to the approved guidelines for ethical treatment of animals in accordance with Polish legal requirements. The individuals for the analyses were carefully selected, and mechanical injury to the limbs was adopted as the enrolment criterion. For mRNA and protein analyzes, tissues were collected from the *uterus masculinus* (20 mg) containing all tissue layers: endometrium, myometrium and adventitia. The obtained tissues were shock-frozen in liquid nitrogen (–196ºC) and stored at –80ºC, until further analyzes.

**Real time polymerase chain reaction**

RNA was isolated from *uterus masculinus* tissues using a column-based kit according to the manufacturer protocol (11-100, A&A Biotechnology, Poland). One µg of the total RNA was reverse-transcribed into cDNA using the Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer protocol. Bovine TaqMan gene expression assays for VEGF-A (Bt03213282; Entrez Gene ID 281161) and β-actin (Bt03279174; Entrez Gene ID 280979) were performed using the StepOne Plus system (Applied Biosystems, USA) and protocols provided by the supplier. β-actin was used as the reference gene for normalization, and mRNA abundance was quantified using the 2−ΔΔCT method (16).

**Polymerase chain reaction**

cDNA samples used for RT-PCR analyzes were the same as used for the real-time polymerase chain reaction (PCR). Received cDNAs were amplified using primers specific to TGF-β1, NGF and β-actin (Table 1). The reactions were conducted as follows: 5 µl of 2 × PCR TaqNova-RED Master Mix (DNA Gdansk; Gdansk, Poland), 4 µl of primers (2 µl of 1 µM forward and reverse primer each) (Genomed; Warsaw, Poland) and 1 µl of cDNA (10 ng). Amplification of PCR included 35 cycles of denaturing (95ºC for 45 s), annealing (51ºC for TGF-β1, 49ºC for NGF, 55ºC for β-actin) and extension (72ºC for 45 s), and finally extension (72ºC for 10 min). In 2% agarose gel with ethidium bromide, PCR reaction products were visualized by electrophoresis. Using the GelQuantNET software, the relative density was calculated and the results were normalized to β-actin.

**Western blot**

The *uterus masculinus* tissues homogenized in 2% SDS were centrifuged at 15,000 g at 4ºC for 15 min. Protein concentrations were determined using the BCA protein assay according to the manufacturer protocol (Thermo Scientific, USA). Bovine serum albumin (BSA) was used as a calibration standard. 30 µg of proteins were separated by SDS-PAGE and electroblotted onto the PVDF membrane (Thermo Scientific, USA). Membranes were blocked at RT for 1 hour in 1% BSA for VEGF-A, FGF-2, IGF-IRβ, HIF-1α and 3% BSA for klotho protein in TBST (20 mM Tris-HCl pH 7.5, 137 mM NaCl, 0.1% Tween 20). They were incubated overnight in one of the primary antibodies: mouse mononclonal anti-VEGF-A, 1:1000 (#MA1-16626, Thermo Scientific, USA), mouse mononclonal anti-NGF, 1:1000 (#MA1-24682, Thermo Scientific, USA), rabbit polyclonal anti-IGF-IRβ, 1:500 (sc-9038, Santa Cruz, USA), rabbit polyclonal anti-klotho, 1:1000 (#PA5-21078, Thermo Scientific, USA), goat polyclonal anti-HIF-1α, 1:200 (sc-12542, Santa Cruz, USA), or rabbit polyclonal anti-actB, 1:10,000 (#PA1-16889, Thermo Scientific, USA) prepared in 1% BSA in TBST. The membranes were carefully rinsed (4×) with TBST for 5 min and next incubated in RT with secondary anti-rabbit or anti-mouse antibody 1:80,000 in 1% BSA in TBST and finally washed (4×) with TBST for 5 min.

**Statistical analysis**

Data was presented as a mean ± SD. A statistical analysis was conducted using GraphPad Prism 6.0 with the unpaired t-test for

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product length (bp)</th>
<th>T (ºC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>Forward: 5’-GGACACAACTACTGCTTCA-3’ Reverse: 5’-ACCTTCCAAGTGCTG-3’</td>
<td>99</td>
<td>51</td>
</tr>
<tr>
<td>NGF</td>
<td>Forward: 5’-CTGGGAGAGGTGAACATCAAC-3’ Reverse: 5’-TGTTGAACGAGTCTCCCG-3’</td>
<td>115</td>
<td>49</td>
</tr>
<tr>
<td>ACTB</td>
<td>Forward: 5’-CATCGGCAATGAGCCGTTC-3’ Reverse: 5’-CCGTGTGCCGTAGAGG-3’</td>
<td>147</td>
<td>55</td>
</tr>
</tbody>
</table>
comparisons between two independent groups (n = 6) in November and in December. P value of < 0.05 was considered statistically significant, and statistical significances are shown as *P < 0.05, **P < 0.01, and ***P < 0.001.

RESULTS

Growth factor mRNA expression in uterus masculinus of the European bison male

The analysis of VEGF-A mRNA expression in uterus masculinus showed no significant differences between November and December (P > 0.05) (Fig. 1A). Similarly, no differences were found in FGF-2 mRNA expression for the analyzed months (P > 0.05) (Fig. 1B).

The TGF-β1 mRNA expression level was significantly higher in November than in December, by 2.032 times (P < 0.05) (Fig. 2A). Furthermore, there was no statistically significant difference in NGF gene expression between November and December (P > 0.05), although its value in December was clearly higher than in November (Fig. 2B).

Growth factors, hypoxia-inducible factor 1-alpha and klotho expression in uterus masculinus of the European bison

Similarly as in the case of mRNA VEGF-A expression, measurements of the protein levels revealed no variations between the analyzed months (P > 0.05) (Fig. 3A). Similarly, the relative optical density of FGF-2 protein synthesis in uterus masculinus of the European bison did not show any differences between November and December (P > 0.05) (Fig. 3B). However, the expression of IGF-IR β was 3.599 times higher in November when compared to December (P < 0.01) (Fig. 3C). As shown in Fig. 3D, the quantitative analysis of HIF-1α protein revealed its significant increase in December (3.168-fold increase) (P < 0.05).

The relative optical density of klotho revealed that its level was higher in December versus November, for both membrane and secreted forms (P < 0.01) (2.318- and 1.685-fold increases, respectively) (Fig. 4).

DISCUSSION

In the European bison duration of its reproductive season is clearly marked and limited (2). According to our knowledge, this
is the first study showing the expression of mRNA and protein levels for different factors: TGF-β1, NGF, IGF-IRβ, FGF-2, VEGF-A, HIF-1α and klotho in *uterus masculinus* of the European bison after the breeding season. In this paper, we demonstrated the stable levels of FGF-2 and VEGF-A in November and December. As it has been previously proven, VEGF is an important factor responsible for both neovascularization and vascular densities, phenomena directly affecting the seasonality of the reproduction (2, 17). The seasonal changes in VEGF in roe deer testes have been already linked to testicular involution and recrudescence (4). However, authors do not report any differences in VEGF mRNA expression at other seasons, and this could explain the lack of detectable differences in European bison *uterus masculinus* between November and December as observed in our study (4). Furthermore, another theory suggests that the increase in angiogenesis is caused by a decrease in production and secretion of anti-angiogenic factors, including angiostatin, endostatin, thrombospondins, and the platelet factor (18). Additionally, increased expression of VEGF stimulating testosterone secretion from Leydig cells may indirectly regulate the male reproductive function (19). Perhaps similar mechanisms may be found in the European bison. Supposedly, a higher level of VEGF in *uterus masculinus* during and after the reproductive season may stimulate proliferation of reproductive cells in a paracrine manner. Initiation of testosterone production and secretion by Leydig cells in the bison testes may also be a result of paracrine regulation. For NGF, our results show that there were no differences in the level of its expression between analyzed months. According to our assumptions, NGF may be not needed in the post-reproductive season. November and December are months when sperm cells are not produced. However, it is possible that NGF affects the quality of bison semen during the breeding season. Because this neurotrophin is produced in the reproductive tract specifically by the accessory glands, it may indirectly affect quality of the produced semen in the

Fig. 3. Western blot analysis of: A) VEGF-A, B) FGF-2, C) IGF-IRβ1, D) HIF-1α, E) representative immunoblots in *uterus masculinus* of European bison. Bars indicate SD; n = 6; ***P < 0.001, **P < 0.01, *P < 0.05; no indication - no statistical significance (P > 0.05); N, November; D, December.

Fig. 4. Western blot analysis of klotho protein and representative immunoblots in *uterus masculinus* of European bison.; n = 6; ***P < 0.001, **P < 0.01, *P < 0.05; no indication - no statistical significance (P > 0.05); N, November; D, December.
Klotho protein occurs in two forms: membrane and secrete, both of which have variable, but their unknown functions in functional studies. In our analyzes, the level of HIF-1α protein expression was much higher in December than in November. In roe deer testes and epididymides the stable mRNA expression was found during the period after reproductive season. The changes in growth factor expression may result from the fact that development of germ cells is variable, but their unknown functions in such specific sites as the uterus masculinus should also be considered.

In contrast, the presence of HIF-1α was also demonstrated in testes and epididymides of various animal species in normoxic conditions. In our analyzes, the level of HIF-1α protein expression in uterus masculinus was much higher in December than in November. In roe deer testes and epididymides the stable low levels of HIF-1α mRNA expression were found during the pre-rut, rut and post-rut seasons, whereas during the reproductive season, the level of HIF-1α protein expression was high (31). Our results indicating the increase in the levels of HIF-1α protein expression after the reproductive season suggest a protective effect against free radicals, which may appear after the intense biochemical processes. The high ROS levels produced during the intense biochemical processes associated with the reproductive activity, may require activity of the HIF-1α protein in uterus masculinus to maintain the proper oxygen environment after the reproductive season.

Klotho protein occurs in two forms: membrane and secrete, and each of them has a different function. The membrane form is a co-receptor for fibroblast growth factor (FGF-23) responsible for phosphate homeostasis and regulation of vitamin D metabolism (32, 33). The secrete form is a humoral factor and determines phosphate homeostasis and regulation of vitamin D metabolism (34-36). Our results suggest that expression of klotho protein in uterus masculinus of the European bison differs significantly between November and December for membrane and secrete forms alike. In both cases, much higher protein expression was found in December. Current literature reports that klotho can regulate IGF 1 levels (36, 37). Our results also show such dependence. It was observed that expression of klotho protein in uterus masculinus of adult bison, especially secrete form, is significantly higher in December, when compared to November, while the trends for the IGF-IR protein expression were opposite. The obtained protein levels confirm the theory of an inhibitory klotho effect on the insulin/insulin-like growth factor (IGF 1) pathway (35). A relationship between klotho and the reproductive system found may imply that it is highly probable that klotho regulates the normal function of this system (14). The above assumption was confirmed by the results obtained by Miranda et al., where klotho-deficient mice were characterized by disturbed gonadotropin regulation (38). Additionally, it has been proven that klotho protein can act as an enzyme in the steroid hormone synthesis pathway (39). Therefore, klotho protein can act as a humoral or as hormonal factor, as required by target organs (40).

Very different experimental observations in vivo and in vitro, shed more insight into understanding of the role played by various physiological, pathophysiologic factors and mechanisms in control and regulation of gonadal activity, the seasonal reproduction, pregnancy and lactation. The results obtained in this study seem to be of great importance as the regulators of the seasonal activity of the male reproductive system, similarly to other factors e.g. Rho and Rho-associated kinases in pregnant uterus (16), adiponectin in steroidogenesis (41) or the impact of specific maternal high-fat diet on offspring (42).

Taken together, presented findings of this study demonstrate differences suggesting seasonal variation in growth factors, HIF-1α and klotho protein in uterus masculinus of the European bison. Nevertheless, further analyses of these tissues at different times of a year are required to fully understand the influence of a season. Unfortunately, the importance of uterus masculinus is poorly understood, but the seasonal changes in various factors found in this organ imply its important regulatory function in the reproductive processes. However, it would be very desirable to determine in further studies the exact regulatory mechanisms underlying the seasonal reproduction.

**Authors contribution:** A. Tabecza-Lonczynska performed the experiments, carried out data interpretation, wrote the paper, conceived and designed the experiments; J. Mytych performed the experiments, carried out data interpretation; P. Solek analyzed the data; A. Abrachamowicz performed the experiments; M. Welz carried out data interpretation; M. Koziorowski contributed materials, carried out data interpretation. All authors read and approved the final version of this manuscript.

**Acknowledgments:** The authors are grateful to the Management and Personnel of the Białowieża National Park for their assistance in collecting the materials.

Conflict of interest: None declared.

**REFERENCES**

3. Czykier E. Is spermiogenesis common or rare in young male European bison aged 2 and 3 years? Pol J Vet Sci 2011; 14: 239-244.


17. Baltes MM, Ten Broeck RA, Brott RC, Clifton DT, Cupp AS. Vascular endothelial growth factor (VEGF) 165 tends to increase vascular density during testis morphogenesis and may be regulated by the inhibitory isoform VEGF 164b. Biol Reprod 2006: special issue.


Received: August 10, 2018
Accepted: October 30, 2018

Author's address: Dr. Anna Tabecka-Lonczynska, Department of Animal Physiology and Reproduction, Faculty of Biotechnology, University of Rzeszow, 502 Werynia Street, 36-100 Kolbuszowa, Poland.
E-mail: annaurz@wp.pl