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REGULAR ARTICLE

Expression of PTGS2, PGFS and PTGFR during downregulation and restart of spermatogenesis following GnRH agonist treatment in the dog

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Abstract

Prostaglandins (PGs) and prostaglandin endoperoxide synthase (PTGS) are considered to be relevant for spermatogenesis and steroidogenesis. PTGS2, prostaglandin F synthase (PGFS) and PGF receptor (PTGFR) are investigated in the adult male dog using the model of the GnRH-agonist implant downregulated canine testis and its subsequent restart of spermatogenesis following abolition of treatment (3, 6, 9, 12 weeks after implant removal). On the mRNA level (ratio), *PTGS2*, *PGFS* and *PTGFR* expression did not differ between downregulation, different stages of recovery of spermatogenesis and untreated adult controls (CG). On the protein level, Sertoli and Leydig cells in all samples and some peritubular cells stained immunopositive for PTGS2. In the tubular compartment, the percentage of the PTGS2-immunopositive area (PIA) and the mean PTGS2-staining intensity (gray scale, GS) did not differ between groups but in the interstitial compartment, the PIA ($p = 0.0494$) and the GS ($p < 0.0001$) were significantly upregulated during early recrudescence (week 3/6). Comparing downregulation by two GnRH-agonist implants with juvenile controls (JG) and CG, the mRNA expression (ratio) did not differ. In the tubular compartment, the GS ($p = 0.0321$) was significantly higher at downregulation compared to CG and in the interstitial compartment, the PIA ($p = 0.0073$) and the GS ($p = 0.0097$) were significantly higher in JG compared to downregulation/CG. *PTGS2*, *PGFS* and *PTGFR* mRNA and PTGS2 protein are regularly expressed in the adult, juvenile and downregulated canine testis and downregulation and subsequent recrudescence affect PTGS2 protein expression mainly in Leydig cells. PTGS2 expression in the downregulated testis resembles the one in seasonal Syrian hamster but not juvenile canine testis.

Keywords PTGS2 · PGFS · PTGFR · Prostaglandin · Slow release GnRH agonist implant

Introduction

Prostaglandins (PGs) and prostaglandin endoperoxide synthase (PTGS), the key enzyme in the PG pathway, play an important role in inflammation and pain but also many other physiological and pathological processes, such as induction of parturition (Aoki and Narumiya 2012; Kowalewski 2012; Rouzer and Marnett 2009; Sugimoto et al. 2015). The development of PTGS-, formerly called Cox-, deficient mice in the 1990s provided important insights into the reproductive roles of PGs with female Cox-knockout mice being sub- or even infertile (Langenbach et al. 1995; Lim et al. 1997). Interestingly, fertility was not affected in male mice deficient in Cox1 and 2, suggesting that PGs may not be critical for testicular function (Langenbach et al. 1999). However, this conclusion has been revisited recently as the two key somatic cell types in the testis, Leydig and Sertoli cells, express the inducible isoenzyme PTGS2 and produce PGs indicating a potential role for testicular development, steroidogenesis and spermatogenesis for various species (Frungieri et al. 2006; McKanna et al. 1998; Sirianni et al. 2009; Winnall et al. 2007). Obviously, species differences in testicular PTGS2 expression exist with the enzyme being

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regularly expressed in mice (Wang et al. 2005), rats (McKanna et al. 1998) and dogs (Ogawa et al. 2017) but only in case of disturbed spermatogenesis in men (Matzkin et al. 2010; Hase et al. 2003). In Syrian hamsters, expression is associated with season; only reproductively active Syrian hamster testes express PTGS2 (Frungieri et al. 2006). Besides Sertoli and Leydig cells, peritubular cells in humans (Schell et al. 2010) and rats with experimentally induced autoimmune orchitis (Iosub et al. 2006) as well as testicular macrophages in rats (Winnall et al. 2007; Iosub et al. 2006) and infertile human patients (Frungieri et al. 2002) and mast cells in infertile human patients (Frungieri et al. 2002) express PTGS2. Whereas the function of PTGS2 in peritubular cells and immune cells is currently not well understood (Mayerhofer 2013), PGs are considered to modulate glucose uptake in Sertoli cells and steroidogenesis in Leydig cells (Frungieri et al. 2015). In Sertoli cell culture, FSH upregulates PGTS2, as well as PGFS production, through phosphorylation of the extracellular signal-regulated kinases 1/2 (ERK) in rats (Crépieux et al. 2001; Meroni et al. 2004) and Syrian hamsters (Matzkin et al. 2012b). In Syrian hamster Leydig cells, classical genomic and non-genomic androgen receptor signaling is involved in PTGS2 and PG regulation (Frungieri et al. 2006). Binding of testosterone to the androgen receptor stimulates phosphorylation of ERK1/2 via a non-classical signaling pathway resulting in an upregulation of PTGS2 and subsequently an increase in PGF2α production (Matzkin et al. 2009). A negative feedback mechanism has been postulated with PGF2α inhibiting testosterone production via the steroidogenic acute regulatory protein (StAR) and 17β-hydroxysteroiddehydrogenase (HSD17B1) in the Syrian hamster (Frungieri et al. 2006; Matzkin et al. 2009; Matzkin et al. 2012a). The observation that PTGS2 but also PGFS are locally expressed in Leydig cells (Suzuki-Yamamoto et al. 2007) and the knowledge that arachidonic acid is also involved in Leydig cell-derived testosterone production (Moraga et al. 1997; Wang et al. 2003; Wang et al. 2000), strengthen the hypothesis that PTGS2 and the PG pathway are involved in the regulation of transcription of StAR and consequently steroid hormone biosynthesis (Wang et al. 2003) in other species, too.

We could previously show that expression of StAR protein and steroidogenic enzymes but also ERK 1/2 activation are significantly affected by a 5 months treatment with a slow release GnRH agonist implant in the dog (Goericke-Pesch et al. 2009; Gentil et al. 2012; Bulldan et al. 2016). In detail, StAR protein and steroidogenic enzymes' mRNA and protein expression were significantly reduced whereas phosphorylated ERK1/2 protein was upregulated during effective treatment. This period was associated with basal gonadotropin concentrations (Goericke-Pesch et al. 2009; Ludwig et al. 2009) as well as the downregulation of the testicular endocrine and germinative function. Downregulation is characterized by basal testosterone concentrations and an arrest of spermatogenesis on the level of spermatogonia and spermatocytes, the lack of a tubular lumen and a reduced tubular area (Gentil et al. 2012; Goericke-Pesch et al. 2013; Goericke-Pesch et al. 2009). Following withdrawal of the implant, the effects induced are reversible and spermatogenesis is restarted associated with a significant increase of expression of StAR and the steroidogenic enzymes (Gentil et al. 2012). Therefore, we considered the model of the downregulated canine testis and its recrudescence of spermatogenesis suitable to further investigate a potential role of PTGS2 and PGFS/PTGFR in the adult male canine and hypothesized that PTGS2, PGFS and PTGFR expression are affected by downregulation and subsequent restart of spermatogenesis.

Materials and methods

Design of experiment

The experimental design was as previously described (Goericke-Pesch et al. 2009; Goericke-Pesch et al. 2013; Gentil et al. 2012): Sexually mature and clinically healthy male Beagle dogs, with all parameters of semen analysis within the normal range, were included in the study. Thirty dogs were treated s.c. in the paraumbilical area with a GnRH-agonist implant containing 18.5 mg azagly-nafarelin (Gonazon®, Intervet, Angers Technopole, France). Implants were removed 5 months later under local anesthesia at the time of downregulation with basal testosterone levels [$\bar{x}_g$(DF)] at 0.10 (1.24) ng/mL and groups of 3–4 dogs were surgically castrated at 3-week intervals (week 0–24).

Testes from five adult healthy untreated male Beagle dogs (group CG) and three juvenile healthy untreated mixed breed dogs aged 2.5 months (group JG) served as untreated controls.

Three adult male Beagle dogs with normal semen parameters were additionally treated for 5 months with an implant containing 6.3 mg buserelin acetate (Profact® Depot; Sanofi-Aventis, Frankfurt Hoechst, Germany, group PG) to decipher whether the state of downregulation differs between the two implants. In PG, the mean testosterone concentration at full downregulation was basal [$\bar{x}_g$(DF)] 0.09 (1.0) ng/ml] (Goericke-Pesch et al. 2009; Goericke-Pesch et al. 2013).

Formation of groups

Samples of the different castration time points were assigned to two datasets: Dataset 1 corresponded to the different states of recrudescence of spermatogenesis in comparison to the adult untreated controls (CG) (Table 1). In dataset 2, the downregulated testes were compared to the testes of the juvenile (JG) and adult dogs (CG) (Table 2). Due to the fact that all dogs castrated at weeks 15, 18, 21 and 24 had full spermatogenesis being not different from dogs castrated at week 12 and

Table 1 Grouping of dogs during recrudescence of spermatogenesis following removal of a slow release GnRH agonist implant containing 18.5 mg azagly-nafarelin and control group

Group name	Week of castration*	Description	*n*
W0	0	No tubular lumen, most developed germ cells: primary spermatocytes	3
W3	3	Tubular lumen re-established, most developed germ cells: round and elongating spermatids	3
W6	6	Most developed germ cells: elongating spermatids	4
W9	9	Most developed germ cells: elongating and elongated	3
W12	12	Full spermatogenesis	3
CG	–	Adult, untreated control dogs	5

*Week of castration after implant removal

the untreated controls, they were not further included in the statistical evaluation.

Reverse transcriptase polymerase chain reaction and real-time PCR

Total RNA was isolated from paraffin embedded tissue samples using the RNeasy ®FFPE Kit (Cat.No.73504, Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. For purification of the mRNA, three sections of 10 μm thickness of each sample were used. The DNase digestion step using DNase I was included in the FFPE protocol. RNA concentration and quality was assessed using a spectrophotometer (NanoDrop® ND-1000, NanoDrop Technologies, Wilmington, USA). Full-length first strand cDNA synthesis was carried out using 200 ng/μl RNA and the RevertAidFirst Strand cDNA Synthesis Kit (#K1622, Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

To test for the expression of *PTGS2*, *PGFS* and *PTGFR*, identical primer sets for reverse transcriptase polymerase chain reaction (RT-PCR) and real-time PCR (RT-qPCR) were developed using known sequences available from GenBank (Table 3). RT-PCR was performed using the following PCR-cycling conditions: 95 °C for 10 min, followed by 40 cycles of 1 min at 94 °C, 2 min at 56 °C and 1`30 min at 72 °C and finally, 72 °C for 10 min. In a first step, a PCR Pre-Mix with 2 μl $MgCl_2$, 4 μl 10xPCR buffer and 32.75 μl sterile Aqua bidest was prepared. Afterwards, 0.5 μl forward primer (10 pmol/μl), 0.5 μl reverse primer (10 pmol/μl) and 0.25 μl Gold AmpliTaq® (all reagents purchased by Thermo Scientific, Waltham, MA, USA) were added to the PCR Pre-Mix. In a last step, 40 μl of the Primer Mix was pipetted to 10 μl cDNA. Sterile bidest. water was used instead of RNA as a no-template control. The amplification of the reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as a control for RNA integrity. RT-qPCR was performed by adding 2 μl cDNA (dilution 1:10) to 5 μl iQTM SYBR Green Supermix (Roche Diagnostics, Basel, Switzerland), 1 μl of the forward and reverse primer (10 pmol) (Table 3) and 1 μl sterile bidest. water. RT-qPCR conditions were 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s, 72 °C 20 s and melting curve with 65–97 °C. Beta-actin, 18 s ribosomal RNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were tested as reference genes. GAPDH showed the slightest variations in mRNA expression and was therefore used as reference for the semiquantitative evaluation. All samples were run in triplicate using a LightCycler®480 real-time PCR system (Software release 1.5.0, Version 1.5.0.39, Roche Diagnostics) and a non-template control was included in every assay. PCR efficiencies of target and reference genes were calculated with Roche Light Cycler® 480 SW 1.5 software by using a relative standard curve derived from a triplet RT-qPCR run of a twofold dilution series (1:2–1:128) of pooled cDNA samples, whereas the efficiency (E) was $E = 10(-1/m)$ with m being the slope of the linear regression line (Pfaffl 2001). The respective efficiencies are given in Table 3. Evaluation of the RT-qPCR results was an efficiency-corrected relative quantification according to Pfaffl (2001).

The specificity of the primers for *PTGS2*, *PGFS* and *PTGFR* used in RT-PCR and RT-qPCR was checked using BLAST (http://blast.ncbi.nlm.nih.gov) and results were confirmed by

Table 2 Grouping of dogs at the state of downregulation and controls (Goericke-Pesch et al. 2013)

Group name	Abbreviation	*n*
W0	Dogs treated with azagly-nafarelin (gonazon® group) = week 0	3
PG	Dogs treated with buserelin acetate (profact® depot group)	3
CG	Adult untreated control dogs	5
JG	Juvenile, untreated dogs	3

sequencing of PCR products (BeckmanCoulterGenomics, United Kingdom). All primers were synthesized by TAG Copenhagen A/S (Copenhagen, Denmark).

Protein extraction and western blot analyses

Western blot analysis was performed to test for the expression of PTGS2 protein and thereby confirm the specificity of the PTGS2 antibody. For protein extraction, frozen total testicular tissue (0.6 g) was crushed with a mortar, dispersed with cold protease inhibitor cocktail (Roche Diagnostics) and homogenized using an Ultraturrax (IKA®, Staufen, Germany). Afterwards, the mixture was cooked with 0.15 g SDS for 10 min and centrifuged for 10 min at 1200*g* at 4 °C. The protein concentration of the supernatant was determined using a BCA protein assay (Pierce™ BCA Protein Assay Kit, Thermo Scientific) and proteins were stored at − 80 °C.

For Western blot, approximately 150 μg protein was used following denaturation by heating for 3 min at 95 °C in the water bath. Protein separation was achieved in a 4–20% TGX gel (Mini-Protean® TGX™ Gels, Bio-Rad Laboratories, Hercules, CA, USA) and proteins were blotted on a PVDF membrane (Trans-Blot® Turbo™ Transfer Pack, Bio-Rad Laboratories). To block unspecific binding sites, the membrane was incubated for 1 h at room temperature in PBS-Blotto (5 g skimmed milk powder, 1 ml thiomersal, 2%, 100 ml PBS buffer). Afterwards, the membrane was washed in 0.2% PBST (phosphate-buffered saline containing Tween 20) and incubated with the primary antibody (COX-2 Monoclonal Antibody, Clone CX229, Cayman Chemicals, Ann Arbor, Michigan, USA) diluted 1:500 (0.001 mg/ml) in PBS-Blotto overnight at 4 °C. After being washed in PBST, the membrane was incubated at room temperature for 1 h with the secondary antibody (dilution 1:200 in PBS Blotto, biotinylated horse anti-mouse IgG antibody, BA-2000, Vector Laboratories, Burlingame, CA, USA). Signals were visualized by using an immunoperoxidase system (VECTASTAIN PK-6101 Rabbit IgG Elite ABC Kit and Vector Nova-RED Substrate Kit SK-4800, Vector Laboratories) according to the manufacturer's instructions. Following the staining step, the membranes were washed with sterile bidest. water and the results were photographed. Negative controls were set up by using PBS-Blotto only and isotype controls by using an irrelevant antibody (Mouse IgG1, ITC0928, Linaris Biologische Produkte GmbH, Dossenheim, Germany). Cox-2 ovine electrophoresis standard (Cayman Chemicals) served as analytical positive control.

A blocking peptid (Cox2 (human) Blocking Peptide, Cayman Chemicals) was used in conjugation with the primary antibody and the positive control to block the protein-antibody complex formation during western blot analysis and thereby proof of the specifity of the antibody.

Immunohistochemistry and evaluation of PTGS2 staining

Immunohistochemistry of all samples was performed in one experiment with the same treatment for all slides. Sections from Bouin-fixed paraffin blocks were deparaffinized and antigens demasked by pretreatment with cooking citrate buffer (pH = 6). Endogenous peroxidase activity was inhibited by using 3% hydrogen peroxide in methanol. Horse serum (10% in ICC buffer) was used to block unspecific binding sites followed by an incubation with the PTGS2 monoclonal antibody (Clone CX229, Cayman Chemicals, dilution 1:200 corresponding to 0.0025 mg/ml) overnight at 4 °C. Each step was followed by a washing step with ICC buffer (1.2 g Na2HPO4, 0.2 g KH2PO4, 0.2 g KCl, 8.0 g NaCl, 3 ml Triton ad 1000 ml). Treatment with the secondary antibody (BA-2000, Vector Laboratories) and visualization of the signals using an immunoperoxidase system (VECTASTAIN PK-6101 Rabbit IgG Elite ABC Kit and Vector Nova-RED Substrate Kit SK-4800, Vector Laboratories) were performed according to the manufacturer's protocol and as described above. Negative controls were set up by using ICC buffer and irrelevant isotype controls (I-2000, Mouse IgG, Control Antibody, Vector Laboratories) in the respective protein concentration instead of the primary antibodies.

Evaluation of PTGS2 staining was done via a computer-assisted image analysis program ImageTool 3.0, freeware (UTHSCSA, San Antonio, University of Texas, http://ddsdx.uthscsa.edu/dig/itdesc.html) to identify the percentage of the immunopositive area (PIA) and the mean gray scale of the interstitial tissue and the tubules separately as described before (Gentil et al. 2012; Hoffmann et al. 2004). In brief, the staining intensity for PTGS2 (mean gray scale) and PIA were examined in ten randomly selected images at 200-fold magnification per dog. The images were transferred into grayscale pictures and an individual threshold was chosen subjectively for interstitial tissue and the tubules allowing for detection of the weakest immunopositive signal by the computer software. Positive signals above the threshold were counted for calculation of the immopositive area and analyzed for determination of the staining intensity (mean gray scale).

Statistical analysis

For all tests, Graph Pad Prism7 software (GraphPad Software, Inc., La Jolla, USA) was used. Values were considered to be statistically significant at a level of $p < 0.05$.

The Shapiro-Wilk normality test was used to test for normal distribution. Ratio of PTGS2, PGFS and PTGFR was not normally distributed; data are presented as geometric mean and dispersion factor [$\bar{x}_g$ (DF)]. The results of the mean gray scale and PIA were normally distributed resulting in a presentation of data as arithmetic mean and standard deviation ($\bar{x} \pm$

Table 3 Sequences of primers for RT-PCR and RT-qPCR, amplicon length, efficiency and accession number (for = forward, rev = reverse)

	Oligonucleotide sequence (5`-3`)	Amplicon length (bp)	Efficiency	Accession number
GAPDH		228	1.988	NM_001003142
for	GGCCAAGAGGGTCATCATCTC			
rev	GGGGCCGTCCACGGTCTTCT			
PTGS2		88	1.975	NM_001003354
for	GGAGCATAACAGAGTGTGTGATGTG			
rev	AAGTATTAGCCTGCTCGTCTGGAAT			
PGFS		133	2.120	NM_001012344
for	AAGGACCCAGTTCTCAATGC			
rev	AGTTCTCCCGGATTCTCTTC			
PTGFR		91	2.049	NM_001048097
for	CAGTGCCCTGGTAATCACAG			
rev	GCGGATCCAGTCTTTATCGG			

SD). Nonparametric ANOVA (Kruskal Wallis test) was performed to identify significant differences between groups, followed by the Dunn test if $p < 0.05$. Assembling of groups was as follows: Dataset 1 (recrudescence) consisted of the groups reflecting restart of spermatogenesis compared to the untreated adult controls (groups W0, W3, W6, W9, W12 and CG). Dataset 2 (downregulated testis) consisted of the groups reflecting the state of downregulation with two different GnRH agonist implants (W0, PG) compared to juvenile (JG) and adult untreated control testes (CG).

Results

Dataset 1: Restart of spermatogenesis

PTGS2, PGFS and PTGFR mRNA expression

RT-PCR revealed a specific band for *PTGS2*, *PGFS* and *PTGFR* in all groups (not shown). mRNA expression (ratio) for *PTGS2*, *PGFS* and *PTGFR* did not differ significantly between the different time points of restart or compared to the untreated controls (CG), mainly due to large individual variations in all groups but especially W12 (Table 4).

PTGS2 protein expression

Western blot for PTGS2 revealed a specific immunoreactive band using protein homogenate from an adult dog with normal spermatogenesis (Fig. 1, lane 1) and Cox-2 (ovine) electrophoresis standard as positive control (Fig. 1, lane 2); however, the molecular weight of the canine band was slightly higher than that of the positive control (72 kDa). The specific signal was omitted in both cases using the blocking peptide (Fig. 1, lanes 3 and 4). No specific immunoreactive band was visible in the negative (not shown) and isotype controls from canine testis and the Cox-2 electrophoresis standard (Fig. 1, lanes 5 and 6).

Immunohistochemistry revealed a specific immunopositive staining against PTGS2 in the cytoplasm of Sertoli cells and Leydig cells in all samples; additionally, peritubular cells stained immunopositive irregularly (Fig. 2).

In Sertoli cells, the immunopositive signals were diffuse and strongest located to the basal membrane within the Sertoli cell cytoplasm of the downregulated dogs (W0) (Fig. 2a). From W3 onwards and in the adult dogs (CG), a homogenous distribution of immunopositive signals was identified in

Table 4 *PTGS2, PGFS* and *PTGFR* mRNA expression (ratio) during downregulation and recrudescence of spermatogenesis (week 0–12) compared to healthy untreated adult controls (CG). Relative gene expression [ratio, $\bar{x}_g$ (DF)] as obtained by RT-qPCR

	PTGS2	*PGFS*	*PTGFR*
W0	0.77 (2.75)	1.13 (4.39)	1.48 (6.68)
W3	1.81 (1.62)	3.39 (1.56)	3.40 (2.09)
W6	0.89 (1.18)	0.92 (1.29)	0.67 (1.90)
W9	0.34 (2.57)	0.58 (2.23)	1.02 (1.83)
W12	0.18 (16.23)	0.13 (23.07)	0.24 (13.09)
CG	0.82 (2.18)	0.47 (4.16)	0.31 (3.32)
p value	n.s.	n.s.	n.s.

n.s. not significant

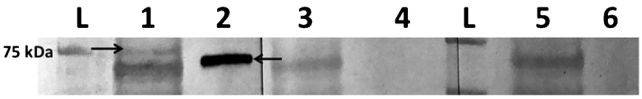

Fig. 1 Western blot analysis for PTGS2. Lane 1, canine total testicular protein; lane 2, PTGS2 ovine standard serving as positive control; lane 3 and 4, PTGS2 antibody conjugated with blocking peptide (canine protein and PTGS2 standard); lane 5 and 6, isotype control for canine protein and PTGS2 standard. Molecular weight markers are expressed in kDa; L ladder

the cytoplasm of the Sertoli cells, reaching from the basal membrane to the lumen. Subjectively, there were no differences in the spreading of the immunopositive staining of the Sertoli cell cytoplasm during recrudescence (W3-W12) and the adult control (CG) (Fig. 2b–f). Using objective quantification of the staining in the tubular tissue, this observation was confirmed (Table 5); there was, however, a trend of a higher

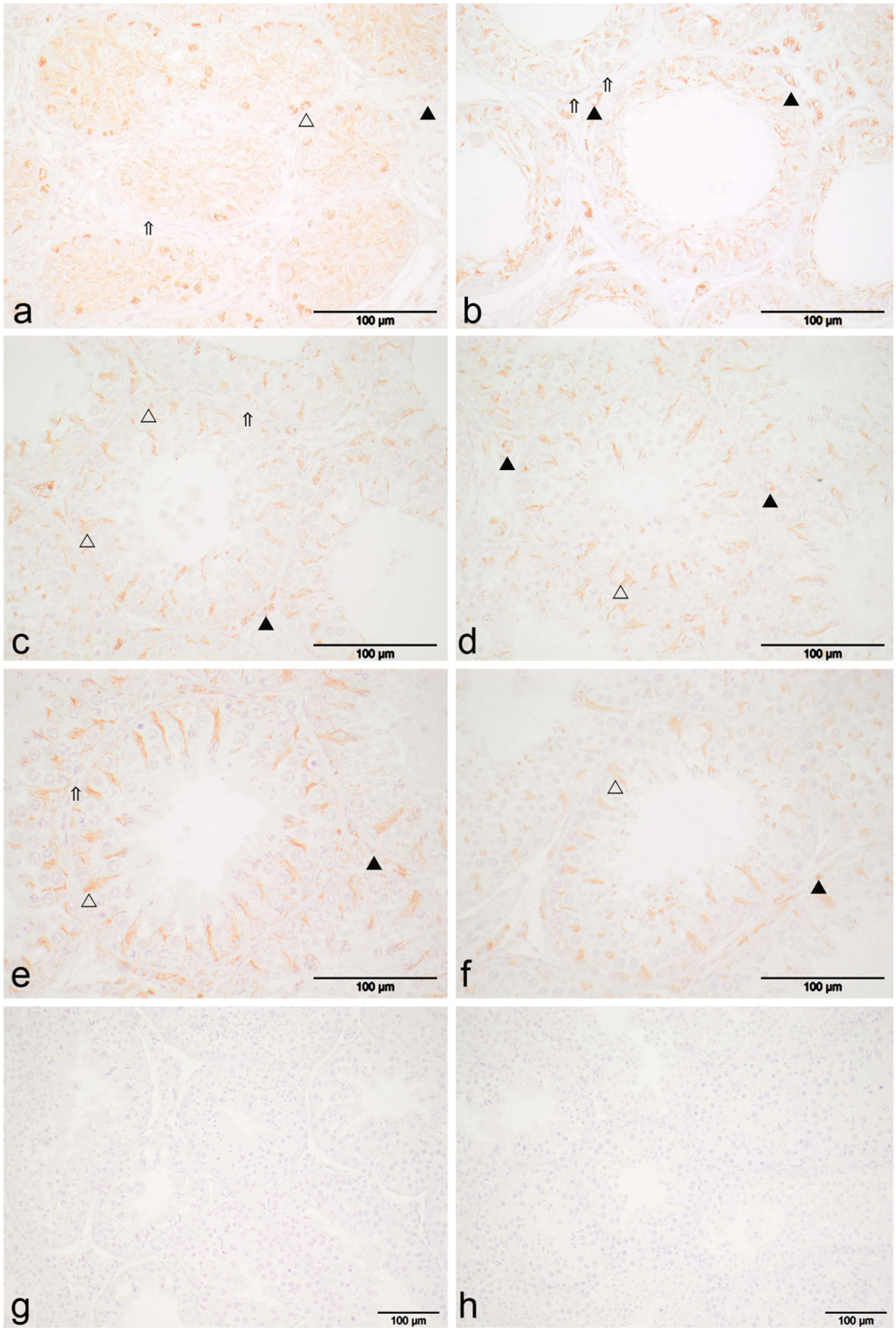

Fig. 2 Immunostaining for PTGS2 in dog testicular tissue (dataset 1). **a** Group W0, **b** group W3, **c** group W6, **d** group W9, **e** group 12, **f** CG (control group), **g** isotype control, **h** negative control (**a–f** magnification × 400; **g**, **h** magnification × 200). ▲Leydig cells, △ Sertoli cells, ⇑ peritubular cells

Table 5 PTGS2 protein expression during downregulation and recrudescence of spermatogenesis (week 0–12) compared to healthy untreated adult controls (CG). Results are presented as the mean gray scale values ($\bar{x} \pm SD$) and the mean percentage of immunopositive area (PIA) ($\bar{x} \pm SD$) within the respective groups in the tubuli and in the interstitium

	Tubuli		Interstitium	
	Mean greyscale	PIA	Mean greyscale	PIA
W0	68.36 ± 1.61	0.97 ± 0.11	55.59 ± 0.57^{a}	0.71 ± 0.44^{a}
W3	67.42 ± 0.47	1.33 ± 0.17	58.32 ± 0.55^{b}	$5.73 \pm 0.69^{a,b}$
W6	67.41 ± 0.48	1.89 ± 0.78	57.11 ± 0.56^{b}	7.09 ± 3.75^{b}
W9	67.03 ± 0.17	0.63 ± 0.04	56.61 ± 0.57^{a}	$3.01 \pm 0.49^{a,b}$
W12	67.13 ± 0.85	1.37 ± 0.84	55.51 ± 0.12^{a}	$4.14 \pm 1.71^{a,b}$
CG	66.18 ± 0.04	0.57 ± 0.10	55.52 ± 0.37^{a}	$5.71 \pm 2.16^{a,b}$
p value	n.s.	n.s.	<0.0001	0.0494

Different superscripts indicate significant differences between groups within the column (Tukey-Kramer test, $p < 0.01$). Data within columns with different superscripts (a, b, c) differ with $p < 0.0001$ to $p < 0.05$

n.s. not significant

staining intensity (mean gray scale) in the downregulated dogs compared to the adult control (CG) (ANOVA, $p = 0.0743$).

Regarding the interstitial tissue, computer-assisted image analysis revealed significant differences between groups for PIA (ANOVA, $p = 0.0494$) and staining intensity (gray scale) (ANOVA, $p < 0.0001$) (Table 5). PIA was significantly lower in W0 compared to W6 (Tukey's test, $p = 0.0382$). The mean staining intensity in the interstitium increased significantly from W0 and was strongest in W3 and W6 during early restart, with the mean gray scale in W3 being significantly higher compared to W0, W9, W12 and CG (Tukey's test, $p < 0.0001$ to $p < 0.05$) and in W6 differing significantly from W0, W12 and CG (Tukey's test, $p < 0.01$ to $p < 0.05$) (Table 5).

Dataset 2: Downregulation

PTGS2, PGFS and PTGFR mRNA expression

RT-PCR revealed a specific band for *PTGS2*, *PGFS* and *PTGFR* in all groups (not shown). The mRNA expression (ratio) for *PTGS2*, *PGFS* and *PTGFR* was highest in the dogs treated with buserelin acetate (PG), with, however, no significant differences between groups (Table 6).

PTGS2 protein expression

As in dataset 1, positive PTGS2 immunostaining was localized in the cytoplasm of Sertoli cells and Leydig cells in all samples (Fig. 3). Additionally, peritubular cells stained immunopositive in the juvenile testes.

Table 6 *PTGS2, PGFS* and *PTGFR* mRNA expression (ratio) during downregulation using different GnRH agonist implants (W0 = Gonazon® implant; PG = Profact® Depot implant) and compared to healthy untreated juvenile (JG) and adult controls (CG). Relative gene expression [ratio, $\bar{x}_g$ (DF)] as obtained by RT-qPCR

	PTGS2	*PGFS*	*PTGFR*
W0	0.77 (2.75)	1.13 (4.39)	1.48 (6.68)
PG	2.40 (1.51)	3.50 (1.40)	6.11 (1.38)
JG	0.37 (3.05)	0.82 (3.25)	2.10 (2.68)
CG	0.82 (2.18)	0.47 (4.16)	0.31 (3.32)
p value	n.s.	n.s.	n.s.

n.s. not significant

The immunopostive signals in the cytoplasm of the Sertoli cells of the downregulated dogs (W0, PG) were mainly located in the basal compartment whereas the strongest staining was adluminal in the juvenile dogs (Fig. 3a–c). Additionally, in the juvenile dogs, some peritubular cells stained immunopositive (Fig. 3c). In the tubular tissue, no significant differences for PIA were observed but the mean gray scale differed significantly between groups of dataset 2 (ANOVA, $p = 0.0321$). The strongest staining intensity was observed in Sertoli cells in week 0 being significantly different from adult controls (CG) (Tukey's test, $p < 0.05$). In the interstitial issue, PIA differed significantly between groups (ANOVA, $p = 0.0073$). PIA was significantly higher in the juvenile dogs (JG) compared to the downregulated dogs (W0/PG, Tukey's test, $p < 0.01$ to $p < 0.05$; Table 7). Furthermore, the mean gray scale was significantly different between the groups of dataset 2 (ANOVA, $p = 0.0097$). Staining intensity (mean gray scale) was highest in juvenile dogs, differing significantly from the buserelin-treated and the control dogs (Tukey's test, $p < 0.01$ to $p < 0.05$; Table 7).

Discussion

Our studies confirm the presence of PTGS2 on mRNA and protein level in the canine testis using a model representing downregulation of spermatogenesis to an arrest on the level of spermatogonia/spermatocytes and subsequent restart to completely normal spermatogenesis. The same has by now only been reported for the Syrian hamster, an animal with photoperiodical spermatogenesis, in which < 12.5 h of light over 3–4 months result in severe testicular regression resembling the changes seen in patients with hypospermatogenesis and germ cell arrest (Sinha Hikim et al. 1988; Rossi et al. 2014). Furthermore, we could for the first time prove the expression of PGFS and PTGFR on the mRNA level in the healthy canine juvenile and adult testis as well as during downregulation due to GnRH agonist slow release implant treatment and subsequent recrudescence of spermatogenesis.

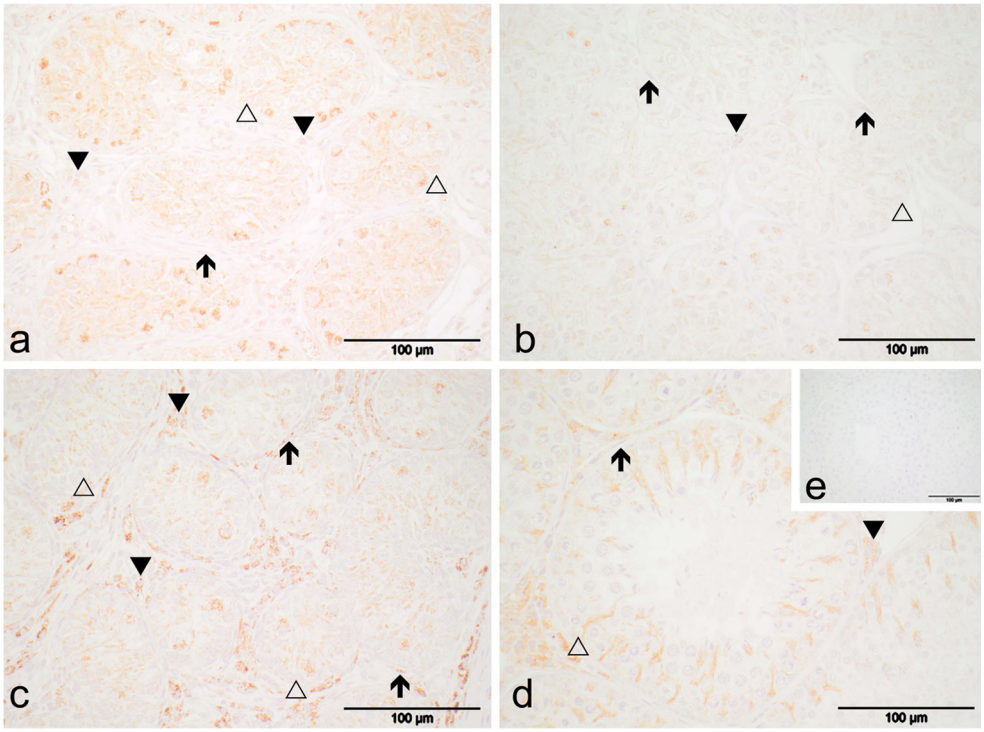

Fig. 3 Immunostaining for PTGS2 in dog testicular tissue (dataset 2); **a** group W0 (Gonazon® group), **b** PG (Profact® Depot group), **c** JG (juvenile), **d** CG (control group), **e** negative control as insert (all magnification × 400). ▲ Leydig cells, △ Sertoli cells, ↑ Peritubular cells

Due to the lack of commercially available antibodies against PGFS and the lacking cross-reactivity of available PTGFR antibodies against the respective canine proteins, our investigations on the protein level are restricted to PTGS2 expression.

PTGS2 protein expression was identified in all groups, at downregulation, during recrudescence, in juvenile prepubertal and adult canine testes, in Leydig and Sertoli cells as has been described before in Syrian hamster (Frungieri et al. 2006; Matzkin et al. 2012a, b), mouse (Balaji et al. 2007), rat (Wang et al. 2005; Chen et al. 2007) and infertile men (Matzkin et al. 2010). Additionally, PTGS2 protein was expressed in some peritubular cells. To the best of our knowledge, PTGS2 expression has by now only been described in peritubular cells of infertile men where the expression had been associated with fibrosis and contractility (Schell et al. 2010; Mayerhofer 2013) and rat peritubular cell lines (Iosub et al. 2006). Our investigations shed some new light into the presence of PTGS2 indicating a possible role of PTGS2 and PGs as paracrine mediators between peritubular cells, Sertoli and Leydig cells in the juvenile canine testis.

Table 7 PTGS2 protein expression during downregulation (W0 = Gonazon® implant; PG = Profact® Depot implant) and compared to healthy untreated juvenile (JG) and adult controls (CG). Results are presented as the mean gray scale ($\bar{x} \pm SD$) and mean percentage of immunopositive area (PIA) ($\bar{x} \pm SD$) within the respective groups in the tubuli and in the interstitium

	Tubuli		Interstitium	
	Mean greyscale	PIA	Mean greyscale	PIA
W0	68.36 ± 1.61^{a}	0.97 ± 0.11	$55.59 \pm 0.57^{a,b}$	0.71 ± 0.44^{a}
PG	$66.06 \pm 0.57^{a,b}$	0.14 ± 0.12	55.17 ± 0.42^{a}	2.28 ± 1.64^{a}
JG	$66.22 \pm 0.12^{a,b}$	0.42 ± 0.17	56.78 ± 0.18^{b}	8.83 ± 2.83^{b}
CG	66.18 ± 0.04^{b}	0.57 ± 0.10	55.52 ± 0.37^{a}	$5.71 \pm 2.16^{a,b}$
p value	0.0321	n.s.	0.0097	0.0073

Different superscripts indicate significant differences between groups within the column (Tukey-Kramer test, $p < 0.01$). Data within columns with different superscripts (a, b, c) differ with $p < 0.001$ to $p < 0.05$

n.s. not significant

No significant differences in mRNA expression for *PTGS2*, *PGFS* and *PTGFR* have been identified and large individual variations, as well as small group size, are for sure a limitation of the study. The results of the immunohistochemistry, however, indicate differences in PTGS2 protein expression in the tubular and interstitial compartment. It seems possible that existing differences between the compartments on the mRNA level were not identified as the used whole testicular homogenates do not take the variable testicular morphology into account: Spermatogenesis is arrested on the level of spermatogonia and/or primary spermatocytes at downregulation and the area of the tubular compartment is significantly reduced resulting in a relative enrichment of Sertoli and Leydig cells, probably also affecting data on mRNA expression. Regarding protein expression, there was no difference in the percentage of immunopositive area (PIA) in the tubules among the groups during recrudescence of spermatogenesis and only a trend for a higher staining intensity in week 0

compared to the adult controls indicating that PTGS2 protein expression was not significantly affected in the Sertoli cells. Different to this, immunohistochemistry revealed significant differences for PTGS2 protein expression in the interstitial compartment. As the Leydig cells—that stained immunopositive against PTGS2—are the predominant cell population in the interstitium, the stained area (PIA) and the staining intensity (gray scale) are considered to correlate directly to PTGS2 protein expression. PIA was lower in the downregulated testis compared to the samples obtained during recrudescence of spermatogenesis and the mean gray scale was significantly higher in weeks 3 and 6 compared to the downregulated dogs, the later stages of recrudescence and the untreated adult controls, CG. A similar observation was made in Syrian hamster testes out of season where PTGS2 expression was significantly lower compared to active testes in season (Frungieri et al. 2006). It has been previously postulated that LH and/or androgens are involved in the testicular, or more precisely Leydig cell-derived, PTGS2 protein expression in the Syrian hamster (Matzkin et al. 2009; Frungieri et al. 2015; Frungieri et al. 2006; Chen et al. 2007) and the rat (Chen et al. 2007) and that PTGS2 and the PGs are involved in the physiological modulation of steroidogenic function in seasonal breeders (Frungieri et al. 2006). This postulate goes along well with the observation that the downregulated canine testis—as the reproductively non-active Syrian hamster testes—is associated with basal peripheral LH and testosterone concentrations (Goericke-Pesch et al. 2013; Goericke-Pesch et al. 2009; Ludwig et al. 2009). We showed that the loss of steroidogenic activity is related to a reduced availability of both, the StAR protein and steroidogenic enzymes, on the mRNA and protein level (Gentil et al. 2012; Goericke-Pesch et al. 2013; Bulldan et al. 2016), which supports the essential role of LH for StAR synthesis (Amory and Bremner 2001; Stocco and McPhaul 2006). Following abolition of treatment by implant removal, StAR and the respective steroidogenic enzymes were rapidly upregulated by week 3 (Gentil et al. 2012) with StAR being considered the bottleneck as the increased availability of StAR protein allowed for the re-establishment of the transduceosome, crucial for the first step for the steroidogenic pathway (Rone et al. 2009). Although not different on the mRNA level, PTGS2 protein expression increased dramatically in week 3, too. Whereas the mean gray scale for PTGS2 in the interstitial tissue was highest in week 3, PIA even further increased in week 6 indicating that the upregulation of protein synthesis in individual Leydig cells occurs faster than reactivation of resting cells. The same observation (PIA, gray scale) has been described for StAR protein and P450side chain cleavage enzyme protein expression in our animal model (Gentil et al. 2012). Matzkin et al. (2009) described an indirect stimulatory effect of LH (via testosterone) on PTGS2 and PGF2α expression in Syrian hamster Leydig cell culture (Matzkin et al. 2009). This regulatory loop where LH via StAR-regulated testosterone biosynthesis stimulates PTGS2 Leydig cell expression (Matzkin et al. 2009) could possibly explain our observations in the canine testes with the highest PTGS2 interstitial protein expression (PIA) occurring slightly later (week 6) than the highest StAR protein expression and LH secretion (week 3). Due to the lack of a suitable PGFS antibody, it can only be speculated if the reason that StAR protein expression did not further increase from week 6/9 onwards was due to an increased PGF2α secretion. It has been well described in the Syrian hamster (Frungieri et al. 2006; Matzkin et al. 2009) and rat (Romanelli et al. 1995; Saksena et al. 1973; Didolkar et al. 1981; Sawada et al. 1994) that PGF2α exerts a negative feedback on peripheral testosterone concentrations via StAR protein and HSD17B1. On the other hand, PGF2α injections induce an increase of peripheral testosterone in the Rhesus monkey (Kimball et al. 1979) indicating again possible species differences.

In Syrian hamster Leydig cells, testosterone stimulates PTGS2 expression and PG production via the classical androgen receptor signaling and a non-classical androgen pathway involving phosphorylation of ERK1/2 (Frungieri et al. 2015). This is, however, obviously different in the canine model as phosphorylated ERK1/2 was significantly upregulated at downregulation with basal LH and testosterone (Bulldan et al. 2016). pERK 1/2 was fourfold increased from week 0 to 6 compared to untreated controls and decreased thereafter reaching normal concentrations in week 24 (Bulldan et al. 2016).

In conclusion, our data show that the downregulation of testicular function by the application of a GnRH-agonist affects PTGS2, PGFS and PTGFR expression. Whereas PTGS2 protein expression in the tubular compartment, namely in Sertoli cells, is only slightly affected, downregulation and subsequent restart of spermatogenesis have a significant impact on PTGS2 protein expression in Leydig cells with the reported changes corresponding well to earlier observations regarding StAR protein and steroidogenic enzymes expression. Therefore, we believe that our observations further support the role of PTGS2 as a local modulator of steroid biosynthesis, likely via regulation of StAR protein. Future studies should consider the use of laser-assisted cell picking to purify mRNA from pure interstitial and tubular tissue, respectively, to allow for identification of possible differences in mRNA expression of the respective genes in the individual testicular compartments. Additionally, studies should focus especially on the first 3 weeks following implant removal to obtain further insights into the sequence of events.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Statement of the welfare of animals All applicable national and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

Animal experimentation had been approved by the respective authority (permit no. AZ V54-19c20/15c GI18/14, Regierungspräsidium Gießen).

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