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Do G-protein coupled estrogen receptor and bisphenol A analogs influence on Leydig cell epigenetic regulation in immature boar testis *ex vivo*?

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ABSTRACT

Organotypic culture of testicular fragments from 7-day-old male pigs (Polish White Large) was used. Tissues were treated with an antagonist of G-protein coupled estrogen receptor (GPER) (G-15; 10 nM), and bisphenol A (BPA), and its analogs (TBBPA, TCBPA; 10 nM) alone or in combination and analyzed using electron and light (stainings for collagen fibers, lipid droplet and autophagy markers) microscopes. In addition, mRNA and protein abundances and localization of molecules required for miRNA biogenesis and function (Drosha, Exportin 5; EXPO5, Dicer, and Argonaute 2; AGO2) were assessed together with calcium ion (Ca^{2+}) and estradiol concentrations. Regardless of GPER blockade and/or treatment with BPA, TBBPA and TCBPA, there were no changes in Leydig cell morphology. Also, there were no changes in lipid droplet content and distribution but there were changes in lipid and autophagy protein abundance. In the interstitial tissue, there was an increase of collagen content, especially after treatment with BPA analogs and G-15 + BPA. Independent of the treatment, there was downregulation of *EXPO5* and *Dicer* genes but the *Drosha* and *AGO2* genes were markedly upregulated as a result of treatment with G-15 + BPA and TCBPA, respectively. There was always a lesser abundance of EXPO5 and AGO2 proteins regardless of treatment. There was markedly greater abundances of Drosha after G-15 + BPA treatment, and this also occurred for Dicer after treatment with G-15 + TCBPA. Immunolocalization of miRNA proteins indicated there was a cytoplasmic-nuclear pattern in control and treated cells. There was an increase of Ca^{2+} concentrations after treatment with G-15 and BPA analogs. Estradiol secretion decreased after antagonist and chemical treatments when

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these were administered alone, however, there was an increase in estradiol secretion after treatment with combinations of these compounds.

1. Introduction

In primates and pigs, three populations of testicular Leydig cells arise sequentially: fetal, infantile and adult (Griswold and Behringer, 2009). Fetal Leydig cells are present in the embryonic testis from shortly after testis cell determination to birth. Infantile Leydig cells contribute to a 'minipuberty' event a few months after birth. These two populations are eventually replaced by adult Leydig cells that expand at puberty. The knowledge on the biology of Leydig cell populations in the immature mammalian testis is limited, in contrast to the adult population, which has been studied extensively (Mendis-Handagama and Ariyaratne, 2001; O'Shaughnessy et al., 2005; Thoma, 2016; Rijntjes et al., 2017; Ye et al., 2017). Noteworthy, based on using genetic tracing in mouse studies, the fetal Leydig cell population was found to persist in adult testes, making up ~20% of the total Leydig cell number (Wen et al., 2016). In addition, findings by Kaftanovskaya et al. (2015) show that genetic ablation of the androgen receptor in fetal Leydig cells affected development of adult lineage. Results of studies in boars treated with an antiandrogen early in life indicated there were negative consequences on Leydig cell morphology and function later in life (Kotula-Balak et al., 2012).

Plasma estrogen concentrations in boars are greater than the estrogen concentrations detected in sows during estrus (Fawcett et al., 1973). Biologically inactive estrone sulphate is the major estrogen in the testicular vein of boars (Mutembei et al., 2009). There is no explanation for this phenomenon. The presence of aromatase along with canonical estrogen receptors (ERs) in boar Leydig cells, Sertoli cells and germ cells indicates there is a source and a target for estrogen functions in boar testes (Fraczek et al., 2001; Rago et al., 2004, 2007; Mutembei et al., 2009). In addition, Krejčířová et al. (2018) confirmed there was G-protein coupled membrane estrogen receptor (GPER) present as a result of assessing the abundances of both mRNA and protein for this receptor in adult boar testes. This finding indicates there is an involvement of rapid, non-genomic estrogen signaling in control of adult male reproductive function. The signaling cascades activated by GPER include the release of intracellular calcium ions (Ca^{2+}) (Prossnitz et al., 2008), an action that can be strictly related to steroid biogenesis (Abdou et al., 2003). Taken together, this information supports a possible function for GPER in Leydig cells.

MicroRNAs (miRNAs) are being recognized as important factors in posttranscriptional regulation of gene expression *in situ*. Exportin 5 (EXPO5) functions in the export of the nuclear pre-miRNA into the cytoplasm after cleavage by Drosha and further cleavage by Dicer into a double-stranded mature species that can bind to Argonaute (AGO) proteins (Gupta et al., 2012).

Although it is likely that estrogens regulate miRNAs by both genomic and non-genomic mechanisms of action (*via* membrane ER or GPER-associated signaling cascades), these pathways only occur at the beginning of investigation (Klinge, 2012). Furthermore, involvement of GPER in miRNA-estrogen regulation in human endothelial cells has been reported (Vidal-Gómez et al., 2018). In mice, estrogen response elements have been identified for specific miRNAs (Bhat-Nakshatri et al., 2009). Gao et al. (2018a), however, reported that bisphenol A (BPA) dysregulates mouse steroidogenic testis function through actions of miRNA.

Considering the findings in these previous studies, the aim of the present study was to elucidate the effects of GPER regulation using xenoestrogens [BPA and its analogs tetrabromobisphenol A (TBBPA), and tetrachlorobisphenol A (TCBPA)] on miRNA biogenesis and function-regulating molecules (Drosha, EXPO5, Dicer, and Argonaute 2; AGO2) in Leydig cells of immature boar testis. Additionally, through measurement of Ca^{2+} concentrations and intra-testicular estrogen concentration, there was an attempt to gain insights into possible mechanisms of action of GPER and BPA derivatives.

2. Materials and methods

2.1. Collection of boar testes and ex vivo culture

The study was approved by the National Commission of Bioethics at the Jagiellonian University in Krakow, Poland (no. 144a/2015). In accordance with Polish legal requirements and in compliance with the Directive 2010/63/EU on the Protection of Animals Used For Scientific Purposes fresh testes of 7-day-old male pigs (Polish White Large) from breeding farm in Malopolska, Krakow, Poland were transported to the laboratory in Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, St Louis, MO, USA) supplemented with 2% Penicillin-Streptomycin solution (Invitrogen, Carlsbad, CA, USA) within 1 h.

A day before the beginning of the experiment, agarose pillars were prepared. Pre-autoclaved agarose (1.5%) was left to solidify in culture dishes at room temperature and then cut into small pillars (approximately 8 mm diameter and 5 mm height), submerged in the Dulbecco's modified Eagle medium (Sigma-Aldrich) overnight before use and transfer into the six well plates (three pillars per well).

Testes were trimmed free of excess fat and connective tissue. After the capsule had been removed, the testes were cut into small pieces (approximately 2 mm) and were placed on top of the agarose pillars, one piece per pillar, and culture medium (DMEM supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and l-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin, without phenol red) in the wells of six well plates so that it reached the edge of the pillar without covering the testicular tissue pieces. Tissue explants were incubated at 32 °C containing 95% air: 5% CO_2. This temperature is important for the maintenance of normal germ cell development and even temporary exposure of the adult testes to abdominal temperature leads to marked disruption of

spermatogenic cells (Yaeram et al., 2006).

Selective GPER receptor antagonist [(3aS*,4R*,9bR*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone; G-15] (Tocris Bioscience, Bristol, UK) was dissolved in DMSO. Bisphenol A (Sigma-Aldrich), TBBPA, and TCBPA (Santa Cruz, CA, USA) were dissolved in absolute ethanol. The concentration of chemicals used was chosen based on preliminary dose-dependent experiments and previous studies (Gorowska-Wojtowicz et al., 2018; Hoffmann et al., 2018). Stock solutions stored at -20 °C were subsequently dissolved in DMEM media to a final concentrations. After a few hours of incubation of testicular fragments, the standard medium was removed and replaced with medium without phenol red supplemented with 5% dextran-coated, charcoal-treated FBS (ThermoFisher Scientific) for 12 h. Cells were subsequently pretreated with G-15 for 2 h and treated with BPA, TBBPA, or TCBPA alone or in combinations for 48 h. The final concentration of the solvent in culture medium was < 0.1% (v/v). Three independent experiments were performed, each in triplicate.

2.2. Tissue topography - scanning electron microscope (SEM)

Pieces of boar testicular tissue (control and experimental) were fixed in a mixture of 2.5% glutaraldehyde with 2.5% formaldehyde in a 0.05 M cacodylate buffer (Sigma; pH 7.2) for several days, washed three times in a 0.1 M sodium cacodylate buffer and later dehydrated and subjected to critical-point drying. These tissues were then sputter-coated with gold and examined at an accelerating voltage of 20 kV or 10 KV using a Hitachi S-4700 scanning electron microscope (Hitachi, Tokyo, Japan).

2.3. Tissue structure

Boar testicular tissue (control and experimental) were fixed in 2.5% (v/v) glutaraldehyde4% (v/v) formaldehyde in a 0.1 M sodium cacodylate buffer (pH 7.0) for several days, washed three times in a 0.1 M sodium cacodylate buffer pH 7 and post-fixed in a 1% (w/v) osmium tetroxide solution for 1.5 h at 0 °C. Dehydration using a graded ethanol series and infiltration and embedding using an epoxy embedding medium kit (Fluka) followed. Semi-thin sections (0.9–1.0 µm thick) were prepared for light microscopy (LM) and stained for general histology using aqueous methylene blue/azure II (MB/AII) for 1–2 min (Humphrey and Pittman, 1974) and were examined using an Olympus BX60 light microscope.

2.4. Tissue histochemistry - connective tissue staining

For histological visualization of collagen I and III fibers in boar testicular tissue (control and experimental) sections Picro-Sirius Red Stain Kit (Connective Tissue Stain) was used according to the manufacturer's protocol.

2.5. RNA isolation, reverse transcription and real-time quantitative RT-PCR

Total RNA was extracted from boar testicular tissue (control and experimental) using TRIzol® reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. The yield and quality of the RNA were assessed using a NanoDrop ND2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Samples with a 260/280 ratio of 1.95 or greater and a 260/230 ratio of 2.0 or greater were used for analysis. Total cDNA was prepared using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions.

The purified total RNA was used to generate total cDNA. A volume equivalent to 1 µg of total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. Total cDNA was prepared in a 20-µL volume using a random primer, dNTP mix, RNase inhibitor and reverse transcriptase (RT). Parallel reactions for each RNA sample were performed in the absence of RT to assess genomic DNA contamination. The RNase-

Table 1
Sequences of forward and reverse primers, TaqMan Gene Expression Assays and RT-PCR reaction conditions.

Genes	Primers (5'–3')	Product size (bp)	Annealing temperature (°C)	Cycles
Drosha	5'-TCCAGATCATCATGAAGGACAC-3' 5'-CGTGGAAAGAAACAAACATTCA-3'	246	60	40
EXPO5	5'-CTCCATCATCAAGATGTGTCGT-3' 5'-ATCTCTTCAGAAAGCCAGATGC-5'	129	60	40
Dicer	Ss04248156_m1	75	60	40
AGO2	5'-GGAAGATGATGCTGAACATTGA-3' 5'-TCCACCTTGAGACCTTTGATTT-3'	172	62	40
GAPDH	5'-TGAACGGGAAGCTCACTGG-3' 5'-TACAGCAACAGGGTGGTGGA-3'	307	62	38
GAPDH	Ss03373286_u1	83	62	40

Abbreviations: Drosha(miRNA endonuclease; Exportin 5 (EXPO5; miRNA transporter; Dicer (miRNA endonuclease; Argonaute 2 (AGO2; GAPDH (Glyceraldehyde 3-phosphate dehydrogenase.

free water was added in place of the RT product.

Real-time RT-PCR was performed using the StepOne Real-Time PCR system (Applied Biosystems) and optimized standard conditions as described previously by Kotula-Balak et al. (2018). Based on the gene sequences in Ensembl database primer sets were designed using Primer3 software or TaqMan Gene Expression Assays (Applied Biosystems) were used (Table 1).

To calculate the amplification efficiency serial cDNA dilution curves were produced for all genes. A graph of threshold cycle (Ct) versus log10 relative copy number of the sample from a dilution series was produced. The slope of the curve was used to determine the amplification efficiency: $\%E = (10^{-1/slope}\text{-}1) \times 100$. All PCR assays displayed efficiency between 94% and 104%.

Detection of amplification gene products was performed with 10 ng cDNA, 0.5 μM primers, and SYBR Green master mix (Applied Biosystems) in a final volume of 20 μL or with 5 ng cDNA and TaqMan Gene Expression Assays (Applied Biosystems) in a final volume of 20 μL. Amplifications were performed as follows: 55 °C for 2 min, 94 °C for 10 min, followed by annealing temperature for 30 s (Table 1) and 45 s 72 °C to determine the cycle threshold (Ct) for quantitative measurement as described previously (Milon et al., submitted). To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis and subsequent agarose gel electrophoresis (not shown). In all real-time RT-PCR reactions, a negative control corresponding to RT reaction without the reverse transcriptase enzyme and a blank sample were conducted. All PCR products stained with Midori Green Stain (Nippon Genetics Europe GmbH, Düren, Germany) were performed on agarose gels. Images were captured using a Bio–Rad Gel Doc XR System (Bio–Rad Laboratories, Hercules, CA, USA) (not shown). The abundance of mRNA in the control group was arbitrarily set as 1, against which statistical significance of experimental groups was analyzed. The relative abundances of mRNA were normalized to reference gene abundances (relative quantification, RQ = 1) with the use of the $2^{-\Delta\Delta Ct}$ method. Three independent experiments were performed, each in triplicate with tissues prepared from different animals.

2.6. Western blot analysis

Lysates of boar testicular tissue (control and experimental) were obtained by homogenization and sonication with a cold Tris/EDTA buffer (50 mM Tris, 1 mM EDTA, pH 7.5), supplemented with a broad-spectrum protease inhibitors (Sigma-Aldrich). The protein concentration was estimated by the Bio-Rad DC Protein Assay Kit with BSA as a standard (Bio-Rad Labs, GmbH, München, Germany). Equal amounts of protein were resolved by SDS-PAGE under reducing conditions, transferred to polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany) and analyzed by Western blotting with antibodies listed in Table 2. The presence of the primary antibody was revealed with horseradish peroxidase-conjugated secondary antibodies diluted 1:3000 (Vector Laboratory, Burlingame, CA, USA) and visualized with an enhanced chemiluminescence detection system as previously described (Bilinska et al., 2018). Specificity of antibodies was assessed with the use of blocking peptide and/or positive control. All immunoblots were stripped with stripping buffer containing 62.5-mM Tris-HCL, 100-mM 2-mercaptoethanol, and 2% SDS (wt:v; pH 6.7) at 50 °C for 30 min, and incubated in antibody against β-actin (loading control). Protein abundance within the control group was arbitrarily set as 1, against which statistical significance of experimental groups was analyzed. Three independent experiments were performed, each in triplicate with tissues prepared from different animals. To obtain quantitative results the bands (representing each data point) were densitometrically scanned using the public domain ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) (Smolen, 1990). Results of ten separate measurements were expressed as mean ± SD.

Table 2
Primary antibodies used for immunohistochemistry, Western blotting and immunofluorescence.

Antibody	Host species	Vendor	Dilution
GPER	Rabbit	Abcam	1:100 (IF)
		cat. no. 39742	1:500 (WB)
Perilipin-1	Rabbit	Cell Signaling Technology	1:200 (IF)
		cat. no. 9349	1:500 (WB)
LC3B	Rabbit	Abcam	1:200 (IF)
		cat. no. ab48394	1:500 (WB)
Collagen 6A1	Rabbit	ThermoFisher Scientific	1:250 (WB)
		cat.no. PA5-29068	
Drosha	Rabbit	Abcam	1:100 (IHC)
		cat. no. ab12286	1:500 (WB)
Exportin 5	Mouse	Abcam	1:100 (IHC)
		cat. no. ab 57491	1:500 (WB)
Dicer	Mouse	Abcam	1:100 (IHC)
		cat. no. ab96040	1:500 (WB)
Argonaute2	Rabbit	Abcam	1:100 (IHC)
		cat. no. ab32381	1:500 (WB)
β-actin	Mouse	Sigma–Aldrich cat. no. A2228	1:2000 (WB)

Abbreviations: G-coupled membrane estrogen receptor (GPER); Perilipin-1 (PLIN); structural protein of autophagosomal membranes (LC3B); Collagen 6A1; Drosha (miRNA endonuclease); Exportin 5 (EXPO5; miRNA transporter); Dicer (miRNA endonuclease); Argonaute 2 (AGO2); β-actin.

2.7. Immunohistochemistry and immunofluorescence

To optimize immunohistochemical staining boar testicular sections both control and experimental were immersed in 10 mM citrate buffer (pH 6.0) and heated in a microwave oven (2 × 5 min, 700 W). Sections were subsequently immersed sequentially in H_2O_2 (3%; v/v) for 10 min and normal goat or horse serum (5%; v/v) for 30 min which were used as blocking solutions. After overnight incubation at 4 °C with primary antibodies listed in Table 2. Biotinylated goat anti-rabbit or horse anti-mouse IgGs; 1: 400; Vector, Burlingame CA, USA) and avidin-biotinylated horseradish peroxidase complex (ABC/HRP; 1:100; Dako, Glostrup, Denmark) were subsequently applied in succession.

For immunofluorescence labeling nonspecific binding sites were blocked with 5% normal goat serum in TBS containing 0.1% Triton X-100 for 30 min. Cells were subsequently incubated overnight at 4 °C in a humidified chamber in the presence of primary antibodies listed in Table 2. On the next day, antibodies goat anti-rabbit Cy™3 (Jackson ImmunoResearch Europe) or Alexa Fluor 488 (Invitrogen; Massachusetts, USA); 1:500 were applied for 45 and 60 min, respectively. After each step in these procedures, cells were carefully rinsed with TBS; the antibodies were also diluted in TBS buffer. The staining for the use of light microscopy was developed using 3, 3′-diaminobenzidine (DAB). Sections were subsequently washed, and were slightly counterstained with Mayer's hematoxylin and mounted using DPX mounting media (Sigma–Aldrich). Cells were examined with Leica DMR microscope (Leica Microsystems, Wetzlar, Germany). Fluorescent staining was protected from light and sections were mounted with Vectashield mounting medium (Vector Laboratories) with 40,6-diamidino-2-phenylindole (DAPI) and next examined with epifluorescence microscope Leica DMR

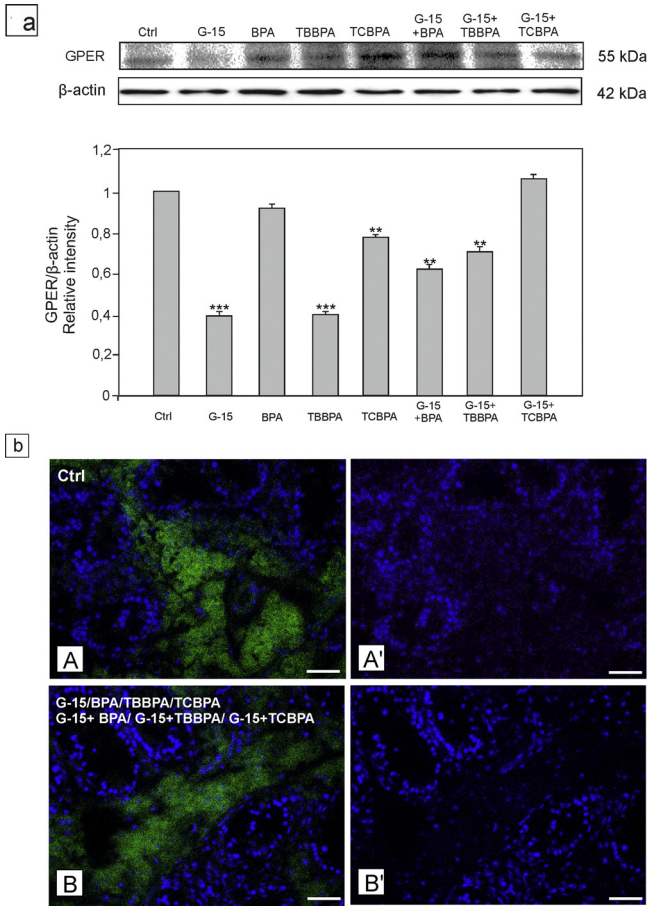

Fig. 1. (a) Representative blots of qualitative abundance and relative abundances (arbitrary units) of GPER protein in control and experimental (treated with G-15, BPA, TBBPA, TCBPA, G-15 + BPA, G-15 + TBBPA, G-15 + TCBPA) immature boar testes. Protein densitometry results are present below the corresponding blots. The relative amount of respective proteins normalized to β-actin. ROD from three separate analyses is expressed as means. From each control and experimental groups at least three samples were measured. Asterisks indicate differences between control and experimental groups. Data are expressed as means. Values are denoted as ** $P < 0.01$ and *** $P < 0.001$. Measurements are from at least three experiments with three replicates of each experimental sample. (b) Representative microphotographs of GPER immunofluorescence localization in control (A) and experimental (B) (treated with G-15, BPA, TBBPA, TCBPA, G-15 + BPA, G-15 + TBBPA, G-15 + TCBPA; microphotograph of G-15 treatment was used as representative for BPA, TBBPA, TCBPA, G-15 + BPA, G-15 + TBBPA, G-15 + TCBPA treatments) boar immature testicular sections. Immunofluorescence with DAPI nuclei counterstaining. Scale bars represent 15 μm. Immunoreaction was performed on cell sections from at least three experiments. (A'B') Negative controls performed with omission of primary antibodies.

(Leica Microsystems) equipped with appropriate filters. Experiments were repeated three times on sections prepared from different animals.

2.8. Ca^{2+} concentration - colorimetric assay

Calcium ion (Ca^{2+}) concentration was estimated in the culture media using Arsenazo III (Sigma–Aldrich, St Louis, MO, USA) according to the modified method by Michaylo and Ilkova (1971). The intensity of the purple complex formed with the reagent was read at 600 nm in a spectrophotometer (Labtech LT-4000MS; Labtech International Ltd., Uckfield, UK) with Manta PC analysis software. The proteins were estimated by modified Lowry et al.'s method (1951). The Ca2+ levels were calculated as μg/mL. Three independent experiments were performed, each in triplicate on media from cultures of tissues from different animals.

2.9. Estradiol concentration - Elisa measurements

Estradiol Enzyme Immunoassay Kit (DRG, Marburg, Germany; cat no EIA 2693) was used for measurement of estradiol secretion by boar testes (control and experimental) according to the manufacturer's instructions. The sensitivity of the assay was 10.60 pg/mL. The absorbance (λ = 450 nm) was measured. Estradiol concentration was calculated from mean ± SD (from three separate measurements) as pg/mL. Three independent experiments were performed, each in triplicate on tissues from different animals.

2.10. Statistical analysis

Each variable was tested by using the Shapiro-Wilk W-test for normality. Homogeneity of variance was assessed with Levene's

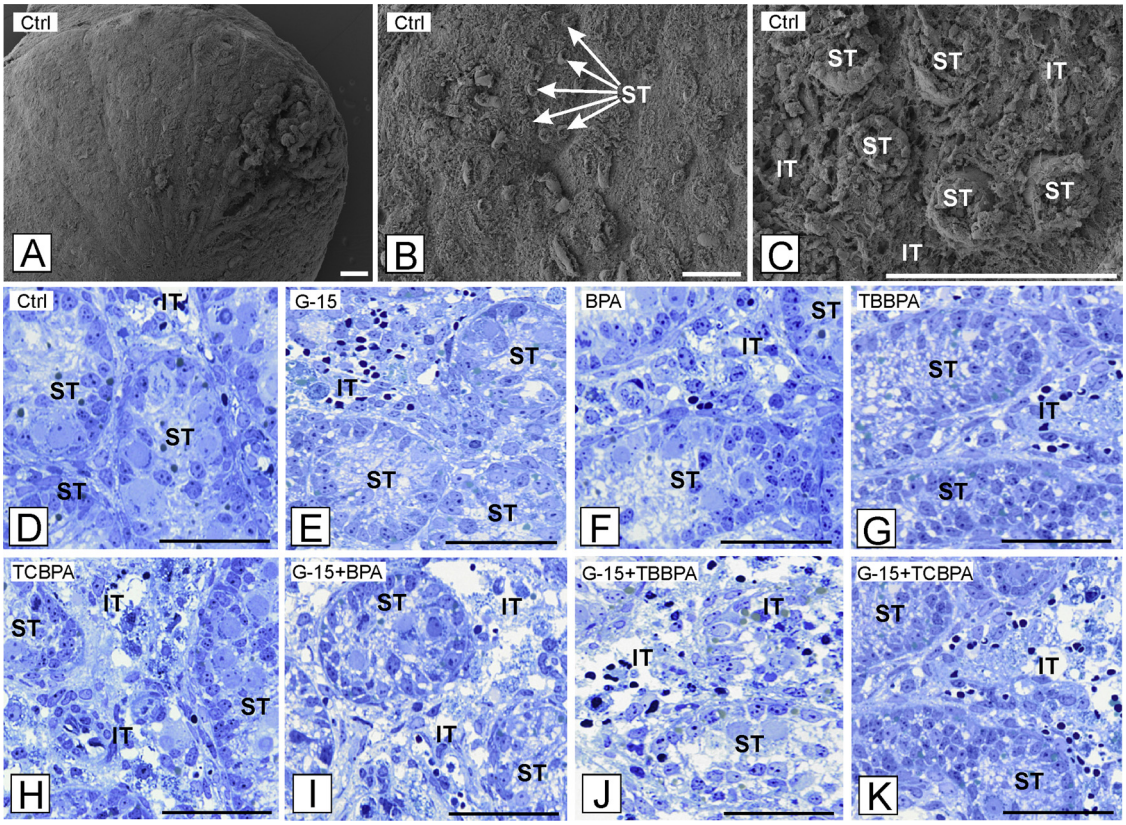

Fig. 2. (A–C) Representative microphotographs of scanning electron microscopic analysis. General structure of control (microphotograph of control tissue was used as representative for tissues treated with G-15, BPA, TBBPA, TCBPA, G-15 + BPA, G-15 + TBBPA, G-15 + TCBPA indicating no histological changes after treatments) boar immature testis fragments after organotypic culture. (B, C) Note abundant interstitial tissue with Leydig cells and a few small seminiferous tubules embedded with interstitial tissue. Bars represent 1 μm. Analysis was performed on three tissue fragments. IT-interstitial tissue, ST-seminiferous tubules.
(D–K) Representative semi-thin sections of immature boar testis fragments control (D) and experimental [treated with G-15 (E), BPA (F), TBBPA (G), TCBPA (H), G-15 + BPA (I) G-15 + TBBPA (J), G-15 + TCBPA (K)]. Note not altered Leydig cell clusters and seminiferous tubules after treatment with G-15 and BPA analogs when compared to control. From each control and experimental group three ultrathin sections were analyzed. Scale bars represent 15 μm. IT-interstitial tissue, ST-seminiferous tubules.

test. Because the distribution of the variables was normal and the values were homogeneous in variance, all statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* comparison test to determine which values differed significantly from controls. The analysis was made using Statistica software (StatSoft, Tulsa, OK, USA). Data were presented as mean ± SD. Data were considered statistically significant at $P < 0.05$. All the experimental measurements were performed in triplicate from material derived from different animals.

3. Results

3.1. Abundance and localization of GPER in Leydig cells of immature boar testis: Effect of GPER blockage and treatment with BPA analogs

Abundance of GPER in Leydig cells of immature boar testis was changed after treatment alone or in combinations with GPER antagonist and BPA analogs (Fig. 1a). Treatment with antagonist and chemicals, with the exception of BPA alone and G-15 + TCBPA which had no effect, decreased GPER abundance ($P < 0.01$, $P < 0.001$). Regardless of treatment used, localization of GPER in Leydig cells did not change, being exclusively membrane-cytoplasmic like in control Leydig cells (Fig. 1b, A, A', B, B').

3.2. Ultrastructure and morphology of immature boar testis: Effect of GPER blockage and treatment with BPA analogs

Electron microscopic and light microscopic examination of testicular tissue sections used for *ex vivo* experiments indicated that in both control [Fig. 2A–C (representative for treated testis as no general histological alterations were found) and Fig. 2D] and treated [Fig. 2E–K G-15 (E), BPA (F), TBBPA (G), TCBPA (H), G-15 +BPA (I) G-15+TBBPA (J), G-15 + TCBPA (K)] (Fig. 2E–K) tissues that there were a few seminiferous tubules (spermatogenically inactive) and a relatively large proportion of Leydig cells between tubules. These latter cells were evenly distributed or clustered. The tubules contained mostly Sertoli cells and a few spermatogonia (Fig. 2D–K). Neither treatment with G-15 or with BPA and its analogs affected tissue morphology (Fig. 2D–K). No somatic cell or germ cell deficiency, aggregation or proliferation were observed when compared to control tissue (Fig. 2D–K).

Treatment with antagonist and chemicals, however, had an effect on the content and distribution of collagen fibers (Fig. 3A–F and G). There were differences particularly after treatment with TBBPA, TCBPA and G-15 + BPA, where there was an increase ($P < 0.001$) in collagen content (Fig. 3D–F and G) compared to control (not shown). In tissues treated with G-15 and BPA, collagen content was decreased ($P < 0.05$, $P < 0.01$) while treatments with G-15 + TBBPA and G-15 + TCBPA did not affect collagen content (Fig. 3A, B, F and G).

Treatment with antagonist and chemicals affected the abundance of lipid droplet protein (perilipin; PLIN) and autophagy protein (LC3) (Fig. 4a and b). Abundance of PLIN increased ($P < 0.05$, $P < 0.01$, $P < 0.001$) after treatment with antagonist and chemicals used alone or in combinations (Fig. 4a). Similarly, abundance of LC3 increased ($P < 0.05$, $P < 0.01$) except when there

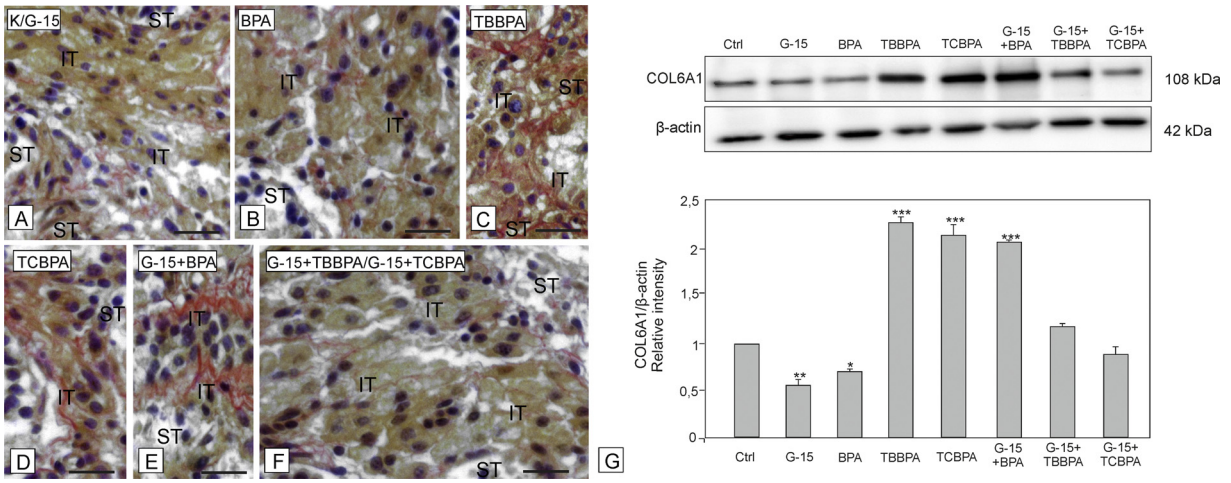

Fig. 3. (A–F) Representative microphotographs of Picro-Sirius Red staining for collagen fibers in immature boar testis control and experimental [treated with G-15 (microphotograph of G-15 treatment was used as representative for Ctrl) BPA, TBBPA, TCBPA, G-15+BPA, G-15+TBBPA treatments (microphotograph of G-15+TBPA treatment was used representative for G-15+TCBPA treatment]. Not differences in collagen content in the interstitial tissue between control and experimental. Scale bars represent 15 μm. Staining was performed on cell sections from at least three experiments.

(G) Representative blots of qualitative expression and relative expression (arbitrary units) of collagen protein in control and experimental (treated with G-15, BPA, TBBPA, TCBPA, G-15+BPA, G-15+TBBPA, G-15+TCBPA) immature boar testes. Protein densitometry results are present below the corresponding blots. The relative amount of collagen was normalized to β-actin. ROD from three separate analyses is expressed as means. From each control and experimental groups at least three samples were measured. Asterisks indicate differences between control and experimental groups. Data are expressed as means. Values are denoted as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Measurements were performed from at least three experiments with three replicates of each experimental sample.

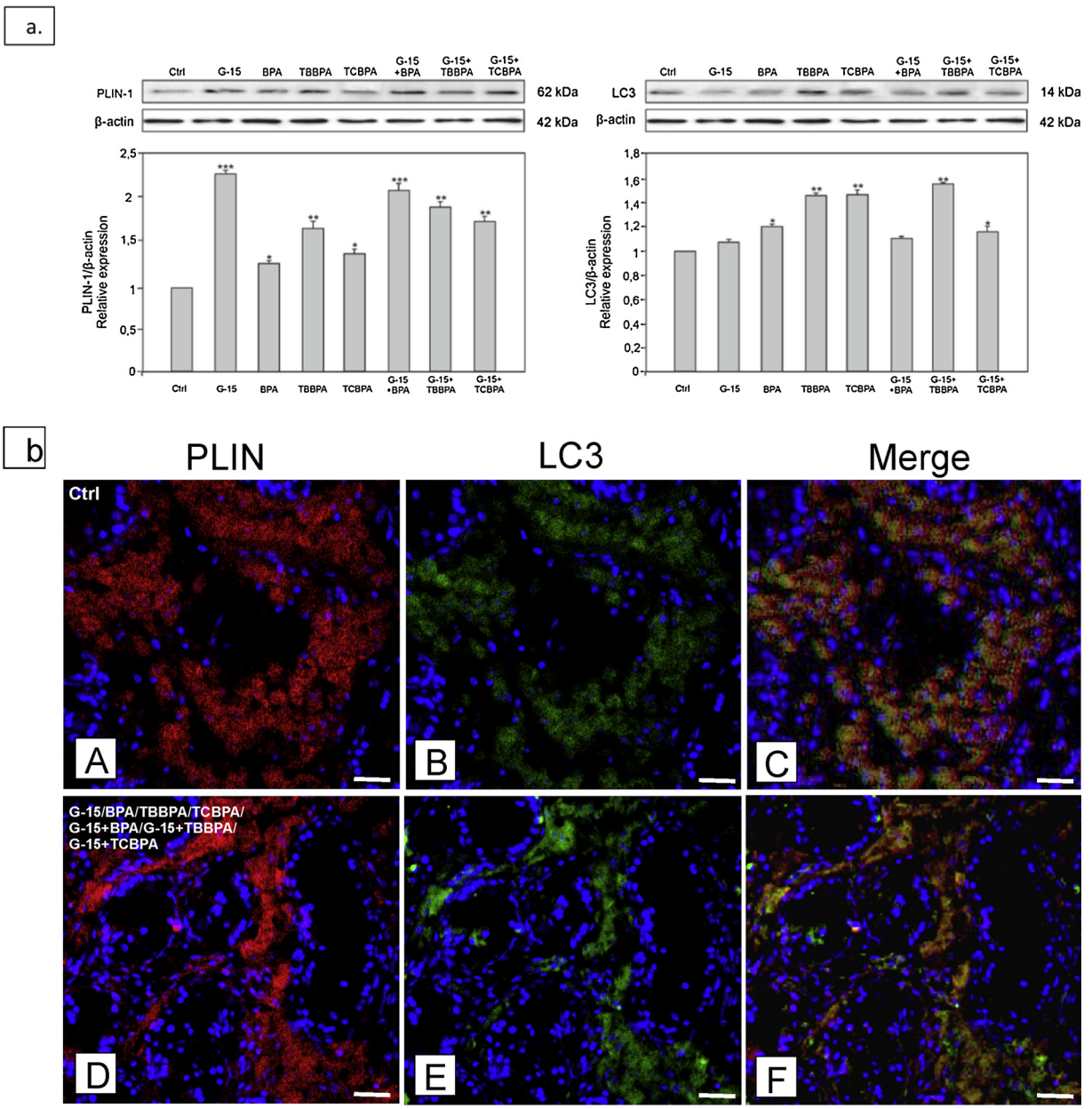

Fig. 4. (a) Representative blots of qualitative expression and relative abundance (arbitrary units) of PLIN and LC3 proteins in control and experimental (treated with G-15, BPA, TBBPA, TCBPA, G-15 + BPA, G-15 + TBBPA, G-15 + TCBPA) immature boar testes. Protein densitometry results are present below the corresponding blots. The relative amount of PLIN and LC3 was normalized to β-actin. ROD from three separate analyses is expressed as means. From each control and experimental groups at least three samples were measured. Asterisks indicate differences between control and experimental groups. Data are expressed as means. Values are denoted as ** $P < 0.01$ and *** $P < 0.001$. Measurements were performed from at least three experiments with three replicates of each experimental sample. (b) (A–F) Representative microphotographs of perilipin (PLIN) and autophagy marker (LC3) immunohistochemical localization and co-localization in control (A, D) and experimental (B, E treated with G-15, BPA, TBBPA, TCBPA, G-15 + BPA, G-15 + TBBPA, G-15 + TCBPA; microphotograph of G-15 treatment was used as representative for G-15, BPA, TBBPA, TCBPA, G-15 + BPA, G-15 + TBBPA, G-15 + TCBPA treatments) boar immature testicular sections. Merge PLIN and LC3 (C, F). Immunofluorescence with DAPI nuclei counterstaining. Scale bars represent 15 μm. Immunoreaction was performed on cell sections from at least three experiments.

was the G-15 and BPA treatments where there were no changes as compared with the control values (Fig. 4a). In Leydig cells after treatment with antagonist and chemicals either alone or in combinations, lipid droplet content and distribution were unchanged when compared to the control (Fig. 4b, A–F).

3.3. Abundances of mRNA and protein of Drosha, Exportin5, Dicer and Argonaute2 in immature boar testis: Effect of GPER blockage and treatment with BPA analogs

The greatest increase ($P < 0.001$) in relative abundance of *Drosha* mRNA occurred after treatment with G-15 + BPA (Fig. 5a). In addition, with TBBPA treatment, there was a slight increase ($P < 0.05$) of relative abundance of *Drosha* mRNA, while in other

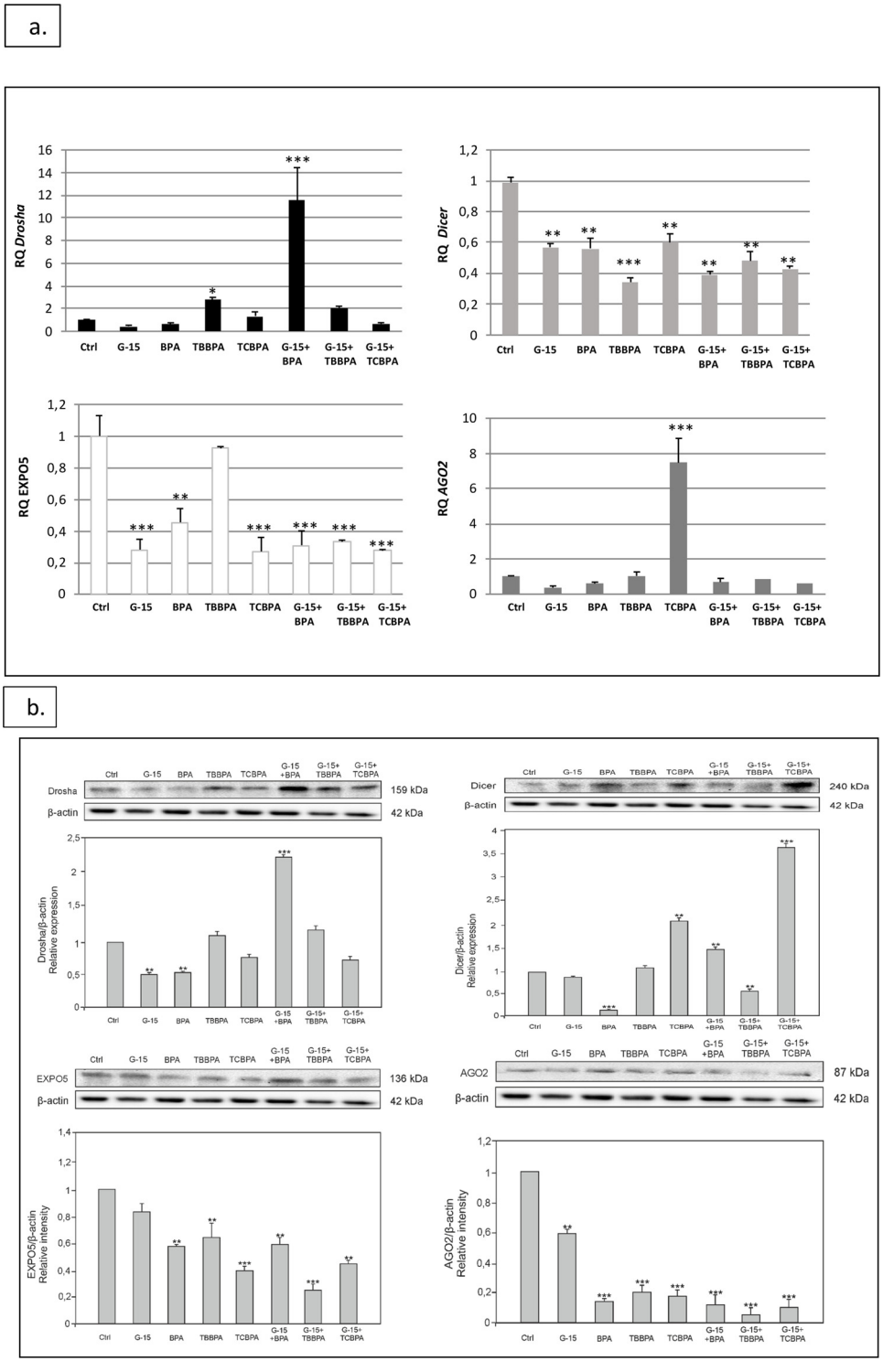

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Fig. 5. (a) Relative abundance (relative quantification; RQ) of mRNA for Drosha EXPO5, Dicer, and AGO2 in control and experimental (treated with G-15, BPA, TBBPA, TCBPA, G-15 + BPA, G-15 + TBBPA, G-15 + TCBPA) immature boar testes using real-time RT-PCR analysis with $2-\Delta Ct$ method. As an intrinsic control, GAPDH mRNA abundance was measured in the samples. From each control and experimental groups at least three samples were measured. Asterisks indicate differences between control and experimental groups. Data are expressed as means. Values are denoted as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Measurements were performed from at least three experiments with three replicates of each experimental sample. (b) Representative blots of qualitative abundance and relative abundance (arbitrary units) of proteins Drosha EXPO5, Dicer, and AGO2 in control and experimental (treated with G-15, BPA, TBBPA, TCBPA, G-15 +BPA, G-15 + TBBPA, G-15 + TCBPA) immature boar testes. The relative abundance of respective proteins normalized to β-actin. ROD from three separate analyses is expressed as means. From each control and experimental groups at least three samples were measured. Asterisks indicate differences between control and experimental immature boar testes. Data are expressed as means. Values are denoted as $^{**}P < 0.01$ and $^{***}P < 0.001$. Measurements were performed from at least three experiments with three replicates of each experimental sample.

experimental groups there were no changes. Relative abundance of *EXPO5* mRNA was decreased in all experimental groups ($P < 0.01$, $P < 0.001$), with the exception of testes treated with TBBPA, where there were no changes as compared with values for the control group. Independently of treatment used, abundances of *Dicer* mRNA were always less compared with abundances in the control group ($P < 0.01$, $P < 0.001$). Inconsistent with this finding, abundance of *AGO2* mRNA was markedly increased ($P < 0.001$) after TCBPA treatment, whereas in other experimental groups there were no changes as compared with values for the control group.

Abundances of proteins regulating biogenesis and function of miRNA changed after treatment either with G-15 or BPA analogs alone or in combinations. Abundance of Drosha protein was less ($P < 0.01$) after treatments with G-15 and BPA (Fig. 5b and supplementary material). In contrast, treatment with G-15 + BPA and G-15 + TBBPA resulted in a greater abundance of Drosha protein ($P < 0.01$, $P < 0.001$). Abundance of EXPO5 protein was less ($P < 0.01$, $P < 0.001$) in all treatment groups as compared with the control group. Similarly to EXPO5, the abundance of AGO2 was less in all treatment groups as compared with the control group ($P < 0.01$, $P < 0.001$). While abundance of the Dicer protein was less ($P < 0.01$, $P < 0.001$) with BPA and G-15 + TBBPA treatments, it was greater ($P < 0.01$, $P < 0.001$) in the other treatment groups but not in G-15 group as compared with the values for the control group.

3.4. Localization of Drosha, Exportin5, Dicer and Argonaute2 in immature boar testis: Effect of GPER blockage and treatment with BPA analogs

Regardless of treatment, localization of Drosha, EXPO5 and Dicer was exclusively cytoplasmic while AGO2 was also present in nuclei of some cells (Fig. 6A–L). In addition, treatment with BPA, TBBPA, TCBPA alone or in combination with G-15 increased the immunohistochemical signal for Drosha and AGO2 when compared to the signal for the control group. With omission of antibodies, there was no positive staining (insert at B, D, I, J).

3.5. Calcium ion concentration in immature boar testis: Effect of GPER blockage and treatment with BPA analogs

There was an increased Ca^{2+} concentration after treatment with antagonist and chemicals (Fig. 7). There was the most marked increase in Ca^{2+} concentration after treatment with G-15 + BPA ($P < 0.001$). There was also an increase ($P < 0.01$) of Ca^{2+} concentration after treatments with G-15, BPA and TBPA.

3.6. Estrogen secretion in immature boar testis: Effect of GPER blockage and treatment with BPA analogs

There was a lesser estradiol secretion after treatment of testicular explants with G-15, BPA, TBBPA and TCBPA ($P < 0.05$, $P < 0.01$, $P < 0.001$). The combination treatments with BPA, TBBPA, and TCBPA with G-15 resulted in greater secretion of estradiol as compared with that of the control group ($P < 0.05$, $P < 0.01$; Fig. 8).

4. Discussion

Recent studies confirmed that estrogen signaling through GPER is involved in the regulation of Leydig cell function in different physiological and pathological conditions (Rago et al., 2011; Kotula-Balak et al., 2012; Vaucher et al., 2014; Zarzycka et al., 2016). In the present study, for the first time, there was found to be an abundance of GPER in Leydig cells of immature boar testis which indicates GPER may have important functions in developing steroidogenic cells. It was previously reported that estrogen had regulatory functions in boar immature testis at this developmental stage (Fraczek et al., 2001). Estrogen was reported to affect fetal Leydig cells differentiation and biochemical characteristics in human and rodents (Pérez-Martínez et al., 1997; Majdic et al., 1996; Delbès et al., 2005) but there has been no detailed study previously on estrogen signaling. Zhang et al. (2015) reported that GPER was present in the gubernaculum of newborn mice indicating GPER had functions in the testicular descent into the scrotum during fetal development. Based on results from the present study, it is suggested that GPER modulates immature boar Leydig cell physiology at both cellular and molecular levels throughout male life. It is proposed that GPER is an important component of a proper boar Leydig cell function.

Interspecies differences in the responsiveness of the reproductive system to BPA have been reported in many studies (N'Tumba-

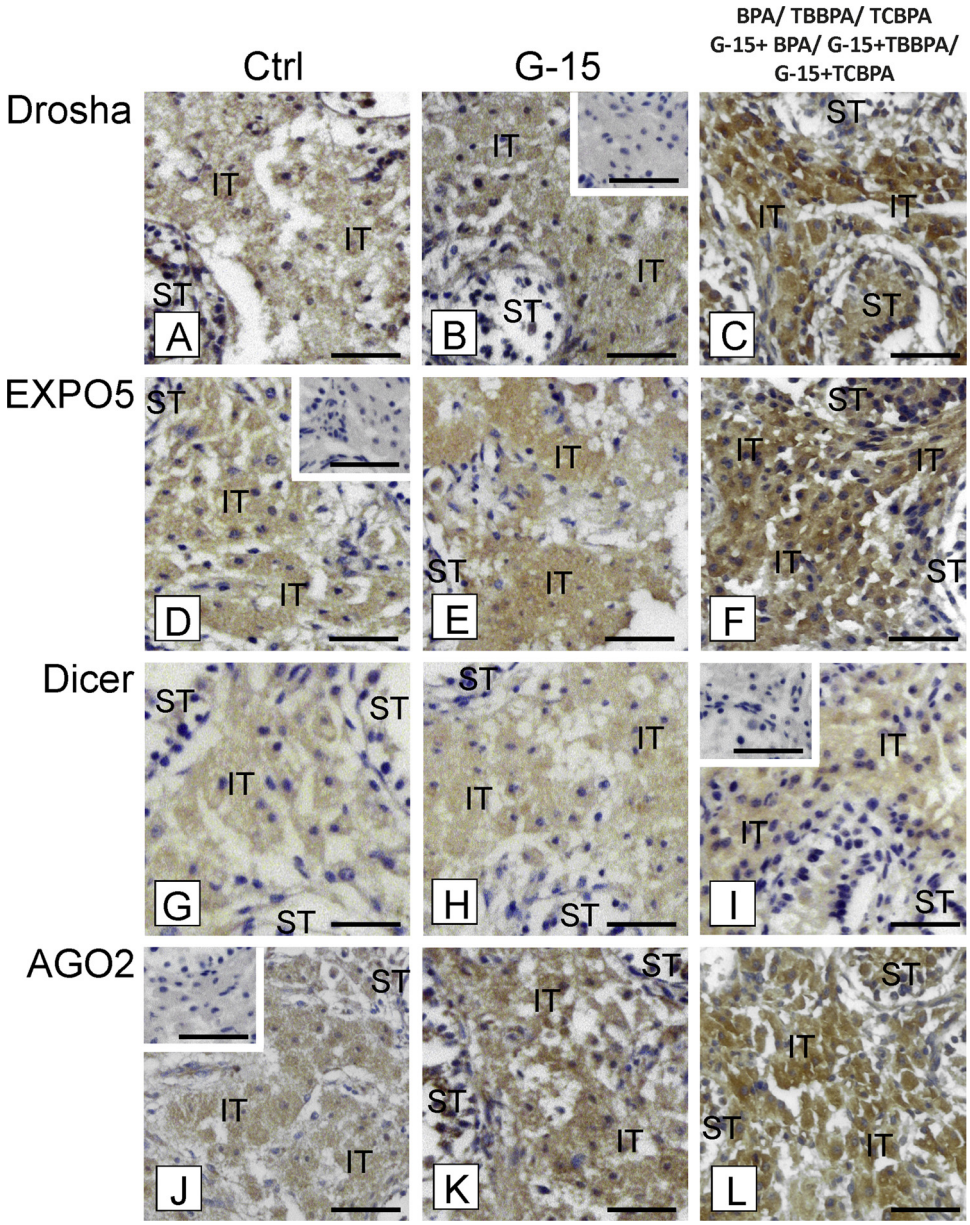

Fig. 6. (A–L) Representative microphotographs of Drosha, EXPO5, Dicer and AGO2 immunohistochemical localization in control and experimental (treated with G-15 and BPA, TBBPA, TCBPA, G-15 + BPA, G-15 + TBBPA, G-15 + TCBPA; microphotograph of BPA treatment was used as representative for BPA, TBBPA, TCBPA, G-15 + BPA, G-15 + TBBPA, G-15 + TCBPA treatments) boar immature testicular sections. Immunostaining with DAB and counterstaining with hematoxylin. Scale bars represent 15 μm. Immunoreaction was performed on cell sections from at least three experiments. Negative controls performed with omission of primary antibodies (Insert at B, D, I, J). IT-interstitial tissue with positive staining in Leydig cells, ST-seminiferous tubule.

Byn et al., 2012; Eladak et al., 2015). Rodents were not considered to be a relevant model for predicting the effect of BPA on the endocrine function of the human fetal testis. Habert's group has developed rodent and human organotypic culture system for fetal testis on floating filters preserving both testis architecture and intercellular communications (Habert et al., 1991; Delbès et al., 2005; Livera et al., 2006). In this previous study, this system was assessed for the first time to examine immature boar testicular tissue and there were specific changes in molecules regulating Leydig cell biology together with steroidogenic/secretory function of this cell. There were, however, no morphological changes in Leydig cells. There were changes in the interstitial tissue in collagen content after treatments with G-15, BPA and its analogs. The opposite effects of G-15 and BPA used alone in comparison to use in combinations indicate the actions of chemicals in mixture can be unpredictable depending on chemical dose and type as well as targeted tissue, process, and molecule (Fent et al., 2006). Similar changes in collagen were noted in boars with malignant Sertoli-Leydig cell tumors (Mabara et al., 1990). Recent findings confirmed changes in GPER activity alter collagen remodeling in the myocardium (Wang et al.,

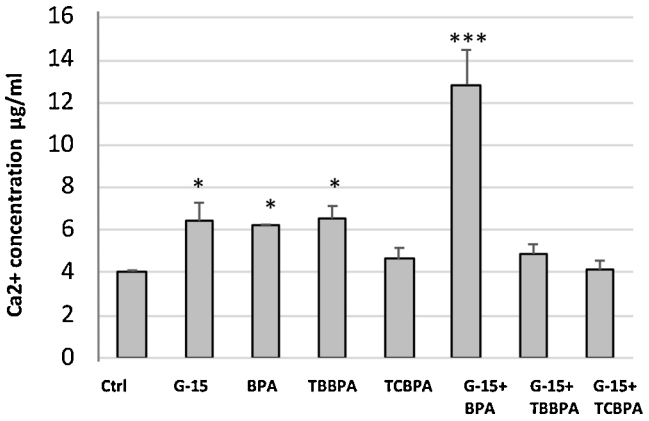

Fig. 7. Calcium ion (Ca2+) concentration in control and experimental (treated with G-15, BPA, TBBPA, TCBPA, G-15 + BPA, G-15 + TBBPA, G-15 + TCBPA) immature boar testes. Asterisks indicate differences between estradiol secretion in control and experimental testes. Data are expressed as means ± SD. Values are denoted as $^{*}P < 0.05$, $^{***}P < 0.01$. Measurements were performed from at least three experiments with three replicates of each experimental sample.

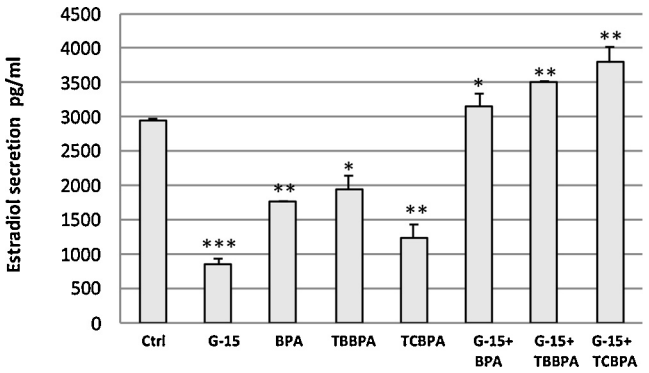

Fig. 8. Estradiol secretion in control and experimental (treated with G-15, BPA, TBBPA, TCBPA, G-15 + BPA, G-15 + TBBPA, G-15 + TCBPA) immature boar testes. Asterisks indicate differences between estradiol secretion in control and experimental testes. Data are expressed as means ± SD. Values are denoted as $^{*}P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.01$. Measurements were performed from at least three experiments with three replicates of each experimental sample.

2018). There were also changes in GPER abundance in immature boar Leydig cells treated with GPER antagonist, and BPA alone, and in combinations. It is possible that there might be other receptors interacting with BPA in Leydig cells (e.g., ER, glucocorticoid receptor, peroxisome proliferator-activated receptor, estrogen-related receptor) and/or changes in function of these receptors (Okada et al., 2008; Rouiller-Fabre et al., 2015; Pardyak et al., 2016; Zhang et al., 2017; Gorowska-Wojtowicz et al., 2018). This multi-receptor and/or molecule cross-talk should be not excluded from further considerations of BPA actions. The initiated responses can be similar or different to those when the usual receptor modulators are used (Gonçalves et al., 2018). In addition, action of BPA via a non-receptor mediated pathway can directly affect target proteins (e.g. collagen) being another possible mechanism of action of hormonally-active chemicals (Fisher, 2004).

In the present study, for the first time, treatment of immature boar testis with BPA chemicals specifically and diversely disrupted protein production that is important for miRNA biosynthesis in Leydig cells. There are possible implications of GPER in some of these regulations related to molecule type and/or site of action and regulation. Because in fetal pig testis there was a marked abundance of ERβ (Knapczyk et al., 2008), it is possible that BPA and its derivatives with various properties might function via GPER and/or ER (Kuiper et al., 1998). This might also be dependent on the specific intracellular circumstances. Some of results from the present study indicate there is an indirect effect of GPER and ER interaction in regard to individual molecule and/or processes and regulatory functions. The greater abundance of Drosha mRNA and protein may be a result of actions of BPA through the GPER but also through the ER. This may also be an effect of an interaction between receptors with Drosha. Abundance of *EXPO5* mRNA was not affected by TBBPA treatment, and treatment G-15 only suppressed abundance of EXPO5, thus TBBPA apparently functions through GPER to have biological actions. Treatment with G-15 together with TBBPA decreased the abundance of EXPO5 to a greater extent than when these molecules were used alone. The TBBPA action, therefore, may be partially modulated via GPER. In addition, BPA and TBBPA can partially function through GPER in modulation of the abundance of *Dicer* mRNA. Dicer protein abundance was not affected by BPA treatments, thus, there is not likely functions of this compound through GPER. There was a marked increase of Dicer protein after TCBPA treatment as a result of a possible action and/or interaction between TCBPA and GPER and/or action through other receptors.

The abundance of *AGO2* mRNA was affected only through TCBPA but neither BPA nor its analogs acted through GPER. The amount of AGO2 protein was affected by treatment with BPA chemicals but not through GPER. In regard to used chemicals, specific changes at both mRNA and protein abundance of the miRNA-regulating molecule can also be related to dynamic changes that occur in Leydig cells of developing testis (Rouiller-Fabre et al., 2015). It is also possible (due to molecule type, mutual interactions, concentrations of regulatory molecules) that G-15 can additionally function in stimulatory/inhibitory manner (Dennis et al., 2009). Chlorine and bromine additives in BPA analogs can additionally effect on properties of other chemicals in the mixture (Zhang et al., 2018).

In the present study, treatment with BPA chemicals changed intra-testicular estradiol concentrations, as well as Ca^{2+} concentrations. Liang et al. (2014) reported that treatment with BPA inhibited the L-type Ca^{2+} channel current and modified the Ca^{2+} release/reuptake kinetics in adult myocytes. It seems likely that steroid production in the fetal boar testis can be regulated by Ca^{2+} signaling and modulated by G-15 and BPA chemicals. Especially G-15 and BPA interactions are involved in increasing Ca^{2+} concentration, indicating BPA does not function via the GPER. Furthermore, BPA and its analogs markedly increased estrogen secretion but not through GPER. It, therefore, is proposed that other regulatory mechanisms besides the Ca^{2+} pathway exist in regulation of GPER cellular and epigenetic actions. The cross-talk of lipid droplet and its controlling proteins and/or effect of some of miRNA-regulating molecules and/or autocrine action of estrogens are possible. Rago et al. (2004) reported that there were rapid responses of changes in Ca^{2+} concentrations to estrogens in spermatozoa that are mediated by both GPER and ER. In men, the greatest concentration of urinary BPA have been associated with elevated estrogen concentrations (Adoamnei et al., 2018). After treatment of mouse Leydig cells (MA-10) with BPA analogs (BPA S, F and TBBPA), marked changes in concentrations of steroid hormones were observed (Roelofs et al., 2015). In mouse Leydig cells (mTCL1), BPA did not change the abundance of steroidogenic enzymes as it did in rat cells (Tanaka et al., 2006; Nanjappa et al., 2012).

Results of numerous studies clearly indicate that different hormones modulate miRNA abundances in many organs (Cortez et al., 2011; Gao et al., 2018b; Ma et al., 2018; Milon et al., submitted). MicroRNAs control several fundamental biological processes, including autophagy (Klinge, 2015). In the present study, the autophagy marker LC3 and PLIN, were present in lipid droplets and there was no modulation of LC3 and PLIN abundances by GPER, with the exception of LC3 after treatments with BPA and TCBPA. These findings indicate, in immature boar Leydig cells, lipid content is well-controlled and can be modulated by steroid synthesis specifically and diversely by G-15 and BPA chemicals and/or other mechanisms.

Estradiol effected miRNA production in mammary and ovarian cells by inhibiting Drosha action (Gupta et al., 2012). Taguchi (2016) reported that there was a variability in miRNA abundance profiles in ER-positive and -negative breast cancers. Data from Davis-Dusenbery and Hata (2010) indicate there is a lack of changes in Drosha and Dicer in breast cancer cells, but there were alterations in EXPO5 and AGO2 after BPA treatment reflecting specific BPA actions in miRNA processing. Not much is known about particular molecules regulating biogenesis and function of miRNA in different tissues. In developing gonads of birds and mammals there were species-, age-, organ/tissue- specific changes in abundance of miRNA in response to BPA treatments (Torley et al., 2011; Cutting et al., 2012; Tao et al., 2016), but molecular mechanisms for these actions are not understood. The alternate regulations by mirtron miRNA involving estrogens, may have an additional effect (Cheloufi et al., 2010).

5. Conclusion

In Leydig cells of the immature boar testis, modulation of abundance of GPER by an antagonist and BPA analogs has a diverse effect on abundances of molecules controlling the biogenesis and function of miRNA as well as estrogen secretion. Involvement of GPER in some of these processes appears to be related to the type of molecule, process and the regulations and concomitantly chemicals having actions on these processes. Specifically selected endogenous interactions (with involvement of ER and/or individual miRNA-regulating molecules and/or interaction of lipid droplets and their regulatory molecules) and exogenous interactions (autocrine estrogen action) may be a useful approach for identifying and developing therapies for morpho-functional Leydig cell alterations leading to testicular dysgenesis syndrome.

6. Author contributions

Authors' contribution to the research described in the paper: P.P., M.D., E.G-W., A.M., B.J.P., performed research. R.T. A.P., W.T., B.J. P., K.K-S., B.B., M. K-B analyzed the data.

M.K.-B. designed the research study and wrote the paper. All authors have read and approved the final version of the manuscript.

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Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2019.06.006.

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