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Steroidogenesis during in vitro maturation of bovine cumulus oocyte complexes and possible effects of tri-butyltin on granulosa cells☆

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Abstract

Steroids are known as important factors on the route of oocytes development and cumulus oocyte complexes (COC) as well as follicular granulosa cells (GC) are suggested to be themselves involved in steroidogenesis. The aim of this study was to characterize such a local sex steroidogenic system during in vitro maturation (IVM) of bovine COCs according to the production of estradiol (E), testosterone (T) and progesterone (P). The expression of two steroid-converting key-enzymes was measured in parallel by quantitative RT-PCR. Furthermore, possible effects of the environmental pollutant tri-butyltin (TBT) were elucidated for the first time on bovine COC and GC in vitro concerning that steroidogenic system.

During IVM of bovine COCs concentrations of P increased continuously, corresponding with steady-state levels of 3-beta-hydroxy-steroid-dehydrogenase (HSD) transcripts. In contrast, E together with P450 aromatase mRNA (ARO) increased in the first hours of IVM but declining thereafter, whereas T reached almost balanced levels. However, TBT showed only slight effects during IVM of COC. In cultured GC, LH caused highest P- and E-production within 24 h and treatment with 50 pM TBT induced a significant decrease of E in contrast to 100 pM TBT and the control. These results indicate, that (1) COCs were able to modulate their steroidogenic environment in vitro and that (2) TBT may possibly influence or disturb steroidogenesis in the cows reproductive tract shown here for GC.

Keywords: Steroidogenesis; Cumulus oocyte complexes; Granulosa cells; Tri-butyltin

1. Introduction

In the last decades important basic research focused on the developmental events during the maturation of mammalian oocytes investigating primordial up to antral and Graafian follicles. Taking advantage of some knowledge of these events in vitro maturation (IVM) of bovine cumulus oocyte complexes (COC) has become a usual technique for commercial production of bovine embryos [1]. It has been recognized that sex steroids fundamentally influence the maturation process in different ways: estradiol (E) may promote alterations in the reactivity of the calcium liberation system during cytoplasmic oocyte maturation [2] influencing the typical calcium oscillation during fertilization [3]. Additionally, the presence of both aromatisable and non-aromatisable androgens during bovine IVM increases oocyte cleavage rates without affecting embryo development. In contrast, progesterone (P) inhibited bovine oocyte maturation leading to reduced embryo development after cleavage [4].

Steroidogenesis within the follicle occurs depending on its developmental stage and gonadotropin status. In preantral follicles, P is produced by cells of the theca interna, indicated by high expression rates of 3-beta-hydroxy-steroid- dehydrogenase (HSD) and cholesterol side chain cleavage enzyme (P450scc), whereby in antral follicles androgens are synthesized and converted by granulosal aromatase P450 (ARO) into 17β-estradiol (reviewed in [5]). Biosynthesis of steroids in GC and cumulus has been demonstrated by several authors for different species: in the cow [6,7], in humans [8], in rats [9] and pigs [10]. Therefore, steroidogenesis should be expected in cultured bovine COCs and GCs as well.

This fine-tuned steroidogenic system, involved in normal sexual differentiation and reproduction, could be dramatically influenced by endocrine disruptors, interfering

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with steroid hormone receptors [11] or interacting with steroidogenic enzymes [12]. Often such disruptors are man-made synthetic chemicals used as pesticides, especially trialkyl organotin compounds. A member of these substances is the environmental pollutant tri-butyltin (TBT), which has been used since the 1950s as a biocide for wood preservation, marine antifouling paints, antifungal action in textiles and industrial cooling systems, paper mills, and breweries [13]. The effects are widespread and depend on the concentration and exposure time. In spite of restrictive usage, the ecological effects of TBT are still present, e.g. impose syndromes (pseudohermaphroditism) in snails after exposure to TBT at concentration lower than 5 pg/ml [14]. Additionally, TBT shows immunotoxicity to human natural killer cells leading to a decreased binding capacity to tumor cells [15], causes apoptosis of rodent thymocytes [16] and induces irritations of the skin as well as lung epithelia [17–19]. Informations about unwanted effects on mammalian reproduction are somehow unavailable.

The aim of the present study was (1) to detect a functional steroidogenic system in bovine COCs during IVM and in granulosa cells (GC) in vitro in response to LH, FSH and FSH + LH in combination. Secondly possible effects of industrial pollutants like TBT on reproductive endocrine systems should be elucidated in that model by recording production of P, T and E in parallel to transcript changes of the steroid-converting enzymes HSD and ARO.

2. Materials and methods

2.1. Cell sampling and culture conditions

Non-cystic bovine ovaries were collected, irrespective to stage of the estrus cycle, at a local abattoir as described by Einspanier et al. [20]. Oocytes were received by aspiration and underwent a common in vitro maturation IVM [21]. COCs were cultured in 400 μl IVM-medium MPM199 containing TCM199 (M-2520, Sigma, GER) supplemented with 10% (w/w) FCS (Seromed, Biochrom KG, GER), 50 μg/ml gentamycin (Selectevet, GER), 0.23 mg/ml pyruvate (15220, Serva, GER), 50 μg/ml ascorbic acid (A-4034, Sigma, GER), 0.55 mg/ml calcium lactate (Merck, GER) and 0.01 U/ml of bovine FSH (NIH B1 activity) as well as LH (NIH LH B10) (Sioux Biochemicals, USA).

GCs were recovered by rinsing antral follicles with PBS (0.24 g/l KH_2PO_4; 8.0 g/l NaCl; 0.2 g/l KCl; 1,44 g/l $Na_2HPO\ 2H_2O$; adust to pH7.4 with NaOH) and a 20G needle (Sterican, Braun, GER), washed three times in TCM199 containing 50 μg/ml gentamycin. After final centrifugation at 800 × *g* for 5 min, washed cells were resuspended in culture medium and seeded into 12-well uncoated tissue culture plates (4×10^5 cells per well) by using 2 ml of IVM-medium. To eliminate the potential influence of the oocyte all cultures were essentially COC-free. Gonadotropin stimulation (onset) was done 1.5 d after seeding when all GCs were attached to the bottom and well proliferating.

2.2. RNA extraction and RT-PCR

Cultured COCs and granulosa cells were washed twice in sterile PBS and introduced to RNA extraction performed by spin columns (NucleoSpin RNA II, Macherey-Nagel, GER) including a DNase 1 digestion to reduce contamination by genomic DNA. RNA was quantified spectroscopically by OD260/OD280 nm absorption (Biophotometer, Eppendorff, GER). Total RNA (COC: 200 ng, GC: 500 ng) was reverse transcribed with 200 U M-MLV-reverse transcriptase (Promega, GER) using 2.5 μM random hexamer primers (Gibco BRL, USA) and 0.5 mM dNTP's (Roche Diagnostics, GER) as described earlier [20].

Primers for target genes, designed according known sequences and generated by MWG Biotech (Ebersberg, Germany), were checked according their optimal annealing temperatures (AT) in a gradient cycler (Eppendorff, GER) using pooled bovine cDNA. Verifying specific gene amplification, PCR-products were isolated based on spin column technique (NucleoSpin Extract, Macherey-Nagel, GER) and sequenced (TopLab, Martinsried, GER). Following primer sets were newly selected according to data base search (EMBL accession) and trimmed for LightCycler PCR according the quantification temperatures (QT) of the PCR-products: ubiquitin (UBI) forward 5′-AGA TCC AGG ATA AGG AAG GCA T-3′, reverse 5′-GCT CCA CCT CCA GGG TGA T-3' (EMBL: Z18245; 198, 426 and 654 bp; AT = 60 °C; QT = 85 °C), aromatase P450 (ARO) forward 5′-CAT CAT GCT GGA CAC CTC TAA C-3′, reverse 5′-ATG TCT CTT TCA CCA ACA ACA GTC-3′ (EMBL: U18447; 457 bp; AT = 62 °C; QT = 80 °C) and 3-beta-hydroxy-steroid-dehydrogenase (HSD) forward 5′-TAC CCA GCT GCT GTT GGA G-3′, reverse 5′-ATG CCG TTG TTA TTC AAG GC-3′ (EMBL: X17614; 322 bp; AT = 60 °C; QT = 84 °C).

2.3. LigthCycler real-time PCR

For LightCycler reaction a 10 μl mastermix (Roche Diagnostics, GER) of the following reaction components was prepared containing the indicated end-concentrations: LightCycler mix (1×), $MgCl_2$ (4 mM), each primer (0.4 mM) and sample cDNA (COC: 0.33 ng/μl, GC: 0.83 ng/μl). QT was set 4.0 °C below melting temperature of specific PCR-product. For specific gene amplification the following LightCycler protocol was used:

(a) *Denaturation*: 95 °C for 10 min.
(b) *Amplification and quantification*:
denaturation at 95 °C for 3 s,
AT (indicated above) for 10 s,
extension at 72 °C for 10 s,
single fluorescent measurement at QT (indicated above) for 3 s.

(c) *Melting curve* (60–99 °C):
with a heating rate of 0.1 °C/s, a continuous fluorescent measurement and finally a cooling step at 40 °C for 60 s.

Relative quantification requires the determination of the crossing points (CP) for each transcripts. They are defined as the points at which the fluorescence signals rise appreciably above the background fluorescence [22]. CPs were analyzed by the LightCycler software 3.5 (Roche Diagnostics, GER).

2.4. PCR-Efficiency and relative quantification

2.4.1. PCR efficiency

The PCR efficiency (E) of each primer set was determined in a distinct detection range (2.7 pg up to 8.33 ng of cDNA). Each PCR efficiency was calculated according to the Eq. (1) [22]:

$$E = 10^{-1/s} \quad (1)$$

A standard curve ($n = 3$) served as basis to create the slope (s) and the regression (r). The following PCR efficiencies were detected: 2.00 for UBI ($s = -3.320$; $r = -0.994$), 2.09 for ARO($s = -3.121$; $r = -0.99$), and 1.95 for HSD ($s = -3.435$; $r = -1.00$).

2.4.2. Relative quantification

A mathematical model adopted from LigthCycler software package [23] served to calculate the relative differences between groups. Relative expression ratios were shown by Eq. (2):

$$\text{ratio} = (\text{efficiency of targetgene})^{\text{CP}_{\text{control}} - \text{CP}_{\text{sample}}} \quad (2)$$

where $\text{CP}_{\text{control}}$ is defined as crossing point of the untreated group at the onset of the experiment and $\text{CP}_{\text{sample}}$ represents the treatment groups.

2.4.3. Intra- and inter-assay variation of LightCycler data

To confirm reproducibility and accuracy of real-time PCR, intra-assay precision was performed three times within one LightCycler run as well as inter-assay variation at three different days by using different batches of LightCycler premixes and standard curve (indicated above). Variation tests were carried out with the ubiquitin primer set and resulting standard curve. Reproducibility ($n = 3$) within one run were calculated by using CP mean variation of 0.35% (absolutely 6.05%). Concerning the inter-assay variation a mean variation of 0.48% (absolutely 8.40%) could be detected. The calculation of test precision and variability is based on the standard deviation of CP mean values additionally converted by $(E^{\text{standard deviation}} - 1) \times 100\%$ into absolute data.

2.4.4. Expression of reference genes during gonadotropin and or TBT stimulation

To evaluate a constant efficiency of the RT-reaction it is requisite to compare all samples due to an endogenous standard. Therefore, mainly non-regulated reference genes or housekeeping genes were applicable [24]. For basic cell functions in nucleated cells the presence of these housekeeping gene is important. While screening for a suitable reference gene, we detected a significant upregulation of 18S rRNA ($P = 0.001$) through the LH application compared to the control group in GC cell culture. In contrast, ubiquitin expression seems not to be significantly influenced by the treatments ($P = 0.092$). Therefore, arithmetical normalization of the expression ratios was not carried out.

2.5. Steroid hormone measurements (EIA)

All corresponding supernatants of cell cultures were measured for hormone levels of P, T and E: therefore a simple direct enzyme immuno assay (EIA) on microtiter plates using the second antibody coating technique and horseradish peroxidase (HRP) as the enzyme label (EIA–HRP) were used for quantification [25,26]. The detection limits of the different EIAs ranged form 0.05 up to 25 ng/ml for P, between 2 and 1000 pg/ml for E, and 10 up to 5000 pg/ml for T. If hormone levels were below standard, the concentrations were set to the half of the lowest standard to enable a correct statistical evaluation.

According to the charcoal treatment of the FCS, the hormone levels of P, T as well as of E were eliminated and situated under the specific detection limits of the EIA (data not shown).

2.6. Statistical analysis

One way ANOVA was used to test main effects of LH, FSH, FSH + LH and TBT treatments. If normality test were passed, differences between groups ($n = 6$) of mRNA-levels as well as steroid hormone levels were identified by pair wise multiple comparison procedure (Student–Newman–Keuls method), when normality was failed, differences were analyzed by Kruskal–Wallis one way ANOVA on ranks (Dunn's method). Analyses were done on raw data and performed with SigmaStat 2.0 (Access Softek Inc.). All hormone data are presented as means ± standard deviation (S.D.). Additional, results of mRNA expression were depicted by the calculated ratios according to the control group as explained before by using REST© software tool [27].

3. Results

Initial transcript experiments using RT-PCR showed remarkable amounts of both ARO and HSD mRNA in bovine COC (Fig. 1). During a 24 h IVM distinct expression changes were found and therefore transcript quantifications, done by real-time RT-PCR (LightCycler), were subsequently initiated.

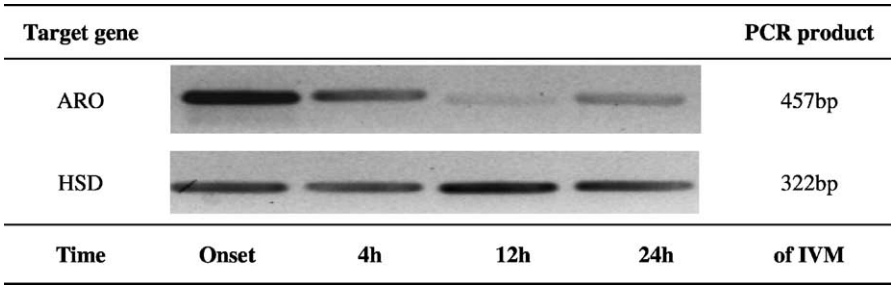

Fig. 1. PCR-products of COC cDNA during IVM, generated by conventional block PCR and separated by 1.5% agarose gel electrophoresis (inversed picture). Target genes: aromatase P450 (ARO), 3-beta-hydroxy-steroid-dehydrogenase (HSD).

3.1. Steroid production of COC during IVM and after TBT treatment

Cultured bovine COCs produced P in a time dependent manner showing highest concentrations after 24 h (Fig. 2a). TBT-treated COCs secreted slightly more P. Corresponding HSD) transcripts significantly declined rapidly and stayed on a lower level until the end of IVM (Table 1) with lowest concentrations after TBT. Concentrations of T in COC supernatants (Fig. 2b) showed a significant in-

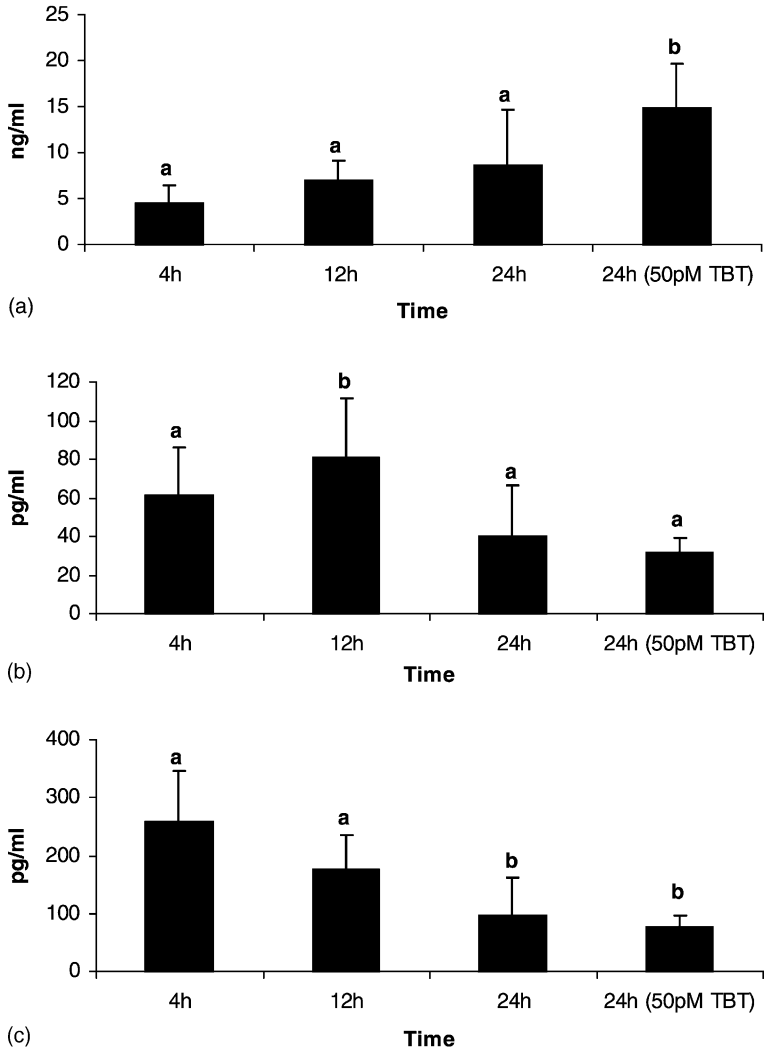

Fig. 2. Steroid hormone concentrations in culture media of COCs during IVM: (a) progesterone, (b) testosterone and (c) estradiol. Data ($n = 6$) are presented as means ± S.D. $P < 0.05$ (significant difference indicated by letters).

Table 1
Ratio of aromatase P450 (ARO) and 3-beta-hydroxy-steroid- dehydrogenase (HSD) mRNA

Target gene	Time (h)				
	0	4	12	24	24 (TBT 50 pM)
Expression ratios (%) to onset (0 h) during COC IVM					
HSD	100 a	42.7 b	36.3 b	49.1 b	32.1 b
ARO	100 a	71.8 a	1.9 b	0.3 c	0.5 c

$P < 0.05$ (significant difference indicated by letters), $n = 6$.

crease after 12 h, but appeared unchanged until 24 h of IVM irrespectively of TBT supplementation. E was found highest within the first hours (Fig. 2c), but declined significantly during 24 h of IVM with comparable low levels after TBT. This continuous decrease of E was accompanied by the significant decrease of ARO expression (Fig. 1 and Table 1).

3.2. Effects of gonadotropins on steroidogenesis granulosa cells culture

Immediately after replacement of the old culture media the GC began to produce P reaching significant increased levels after 4 h under gonadotropins (Fig. 3a). FSH showed the highest stimulus concerning P-secretion followed by LH. After 24 h P reached highest levels when treated with LH compared to the control and FSH. In contrast, co-stimulation with FSH + LH showed a decrease of P. Such P-concentrations were supported by the constant mRNA expression of HSD during the whole experimental period (Table 2).

In the same experiment testosterone (T)-levels (Fig. 3b) did not show significant alterations between the groups after 4 h. Only after 24 h the T-concentration of the FSH stimulated GC cultures was elevated. Obvious differences of E-secretion (Fig. 3c) could be detected when

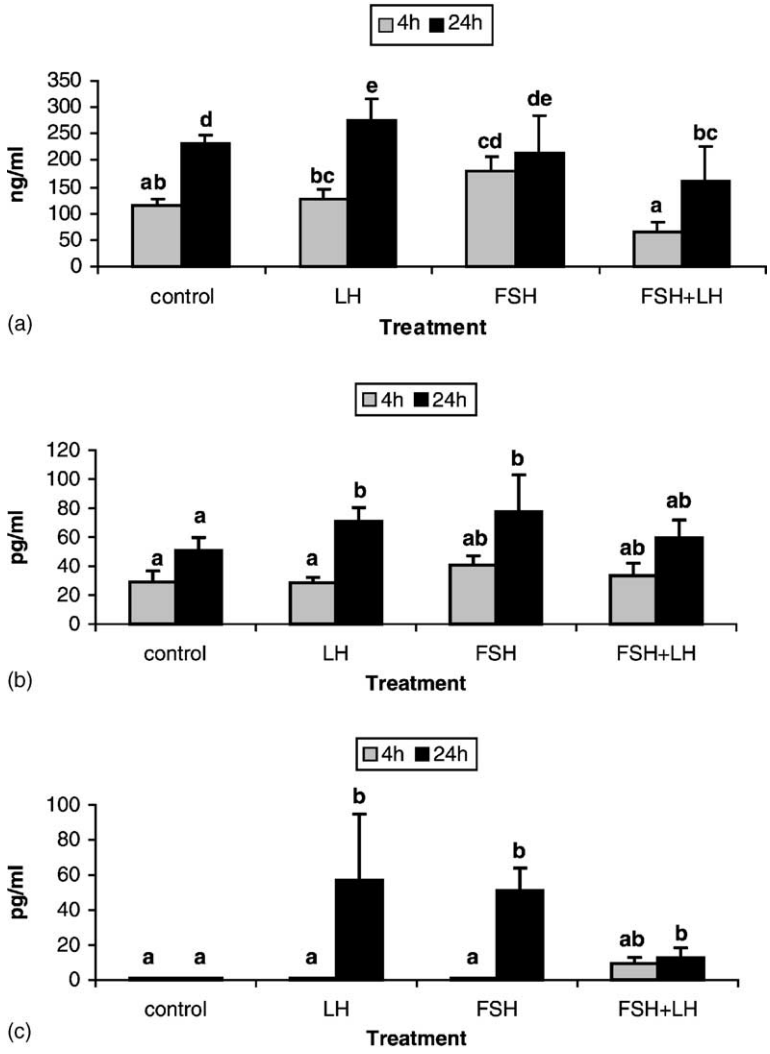

Fig. 3. Steroid hormone concentration in culture media of GC during gonadotropin stimulation: (a) progesterone, (b) testosterone and (c) estradiol. Data ($n = 6$) are presented as means ± S.D. $P < 0.05$ (significant difference indicated by letters).

Table 2
Ratio of aromatase P450 (ARO) and 3-beta-hydroxy-steroid- dehydrogenase (HSD) mRNA according to experimental onset (0 h, 100%)

	Target gene			
	HSD (time (h))		ARO (time (h))	
	4	24	4	24
Expression ratios (%) to onset during cultivation of GC (FSH and/or LH)				
Control	64.6	75.0	134.4	254.0
LH	78.8	73.0	255.3	186.3
FSH	81.9	85.4	317.3	289.8
FSH + LH	51.7	74.6	115.3	395.9

$P < 0.05$ (no significant difference), $n = 6$.

GCs were incubated with all gonadotropins. Whereby the E-concentrations in the controls were always below the detection limit (2 pg/ml), after 24 h E was clearly increased in response to LH and FSH but remarkably lower after a combined treatment. Compared to the aromatase mRNA (ARO) dynamics slightly but not significantly increased transcript levels could be elucidated (Table 2).

3.3. Toxicity of TBT to granulosa cell culture

In order to recover cytotoxic effects of TBT, GC were initially incubated with different TBT-concentration over a wide concentration range (50 pM, 50 nM and 50 μM) and visually characterized. At 50 μM it was obviously that the cultured cells were damaged and detached from the bottom of the dishes (data not shown). Low cellular total RNA concentrations under 50 μM TBT accompanied visual observations. No difference neither in appearance nor total mRNA concentration could be detected in all other GC cultures treated with concentration at or below 50 nM TBT.

3.4. Effects of tri-butyltin on steroidogenesis granulosa cells culture

In the following experiments the possible influence of selected TBT supplementations (50 and 100 pM) on steroidogenesis in cultured GCs should be elucidated.

In contrast to all other tested steroids, P increased significantly after 24 h under 50 pM TBT.(Fig. 4a). However, higher TBT-levels lead to a minor increase of P compared to both gonadotropins applied individually. Accompanying P-levels a slight increase of HSD mRNA (Table 3) could be seen only after 4 h. At the end of the incubation period HSD transcripts in controls were more prominent than TBT-treated samples. As shown previously for gonadotropin stimulations, as well TBT alone had no effect on the T-concentrations (Fig. 4b). Effects of TBT on E-secretion were found contrary to the P measurements: high E contents were significantly reduced under low TBT-concentrations (Fig. 4c). In contrast to low TBT-concentration, 100 pM TBT induced a slight increase in E, but the concentration

Table 3
Ratio of aromatase P450 (ARO) and 3-beta-hydroxy-steroid- dehydrogenase (HSD) mRNA according to experimental onset (0 h, 100%)

	Target gene			
	HSD (time (h))		ARO (time (h))	
	4	24	4	24
Expression ratios (%) to onset during cultivation of GC (TBT)				
Control	51.8	253.5	44.8	321.5
TBT 50 pM	102.2	133.5	787.3	112.5
TBT 100 pM	33.9	69.0	107.5	303.1

$P < 0.05$ (no significant difference), $n = 6$.

Table 4
Ratio of aromatase P450 (ARO) and 3-beta-hydroxy-steroid- dehydrogenase (HSD) mRNA according to experimental onset (0 h, 100%)

	Target gene			
	HSD (time (h))		ARO (time (h))	
	4	24	4	24
Expression ratios in % to onset during cultivation of GC (TBT + FSH)				
Control (FSH)	181.9	331.7	130.1	5.0
TBT 15 pM	680.7	376.6	58.6	1.7
TBT 50 pM	603.4	47.0	21.4	96.3
TBT 50 nM	233.4	226.6	41.0	607.1

$P < 0.05$ (no significant difference), $n = 6$.

remained below the controls. Although E-levels were decreased, ARO expression (Table 3) was increased after 4 h in GC when treated with 50 pM TBT, whereby 100 pM TBT did not cause any significant effects concerning the control.

In order to detect possible synergistic effects between gonadotropins and TBT on GC, additional investigations with FSH and different concentrations of TBT (15 pM, 50 pM and 50 nM were introduced. All three steroids were significantly increased after 24 h of culture under 50 nM of TBT in combination with FSH (Fig. 5). All other TBT treatment groups reacted similarly without significant differences between these each other. Obviously increased ARO mRNA-levels during this experimental setup (Table 4) at 50 nM TBT support directly the measured high E-concentrations in the supernatants.

4. Discussion

Follicular maturation and development are complex processes influenced both by intra- and extra-ovarian events leading to successful ovulation. The LH-surge initially stimulates the secretion of both androgens and estrogens [28], but E-concentrations decline several hours after the LH-surge while P-concentrations are increasing [29]. Furthermore, intra-follicular events such as cumulus expansion may terminate gap junctionial communication between cumulus cells and the oocyte resuming meiosis followed by the germinal vesicle break down. Early experiments demonstrated that

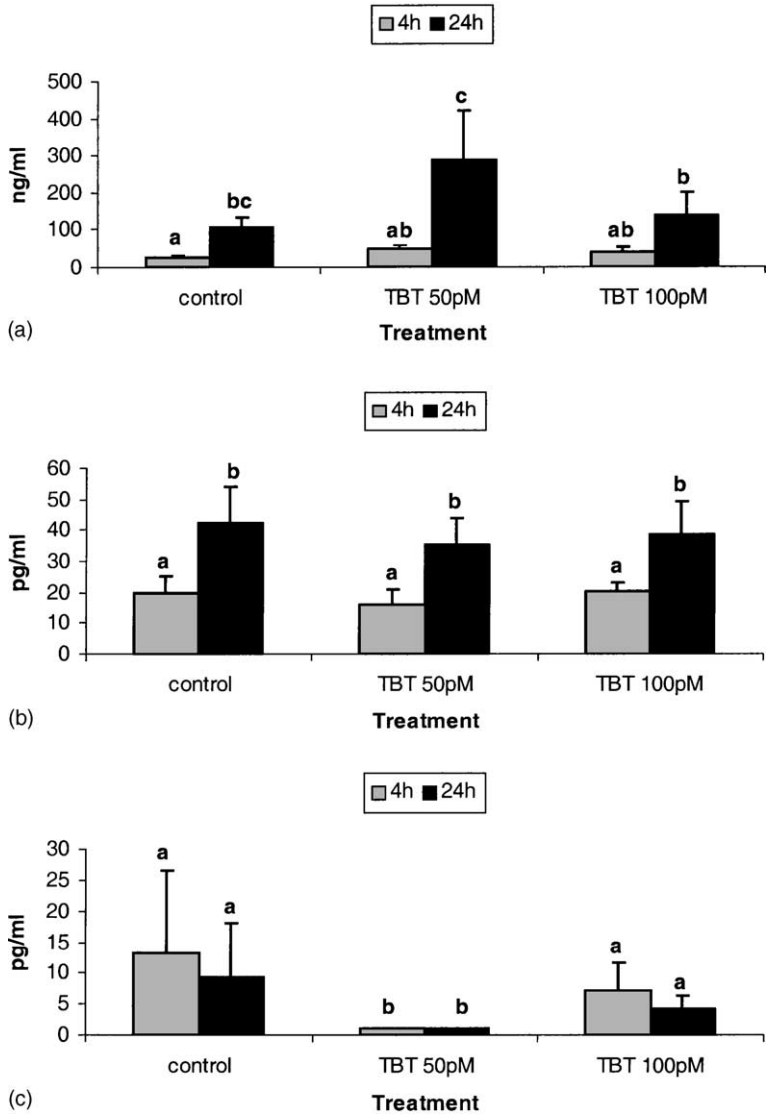

Fig. 4. Steroid hormone concentration in culture media of GC during TBT treatment at different concentrations: (a) progesterone, (b) testosterone and (c) estradiol. Data ($n = 6$) are presented as means ± S.D. $P < 0.05$ (significant difference indicated by letters).

oocytes, removed from the follicle, were also sometimes able to undergo spontaneously resumption of meiosis even without the gonadotropin stimulus [30], a independent local steroid system may contribute to that development.

4.1. Steroidogenesis during IVM of COC

The present results indicate that bovine COCs are able to secrete several steroid hormones during IVM without support of follicular granulosa or theca cells and possess a selection of important steroidogenic enzymes. The observed increase of progesterone is not as high as described for intact follicles after the LH-surge [29]. Such progesterone concentrations in COC culture remained below in vivo data of follicle fluid concentrations, but were comparable to values found in peripheral serum during the estrus cycle [31]. In contrast to progesterone, the secretion dynamics of estradiol were found reversed continuously decreasing. Similar effects have been described for in vivo conditions after the LH-surge [32]. The reduction of estradiol could be seen as a consequence of decreased mRNA expression of FSH receptor (unpublished own data) because FSH receptors are known to activate aromatase or increase cAMP accumulation in granulosa cells [33]. Therefore, cumulus cells, which derived from granulosa cells could still resemble similar reactivity.

4.2. Effects of TBT during IVM of COC

An aberrant composition of local steroids during IVM is known to influence oocyte maturation [7,34] and finally development of the embryo [4,35]. After 24 h of COC

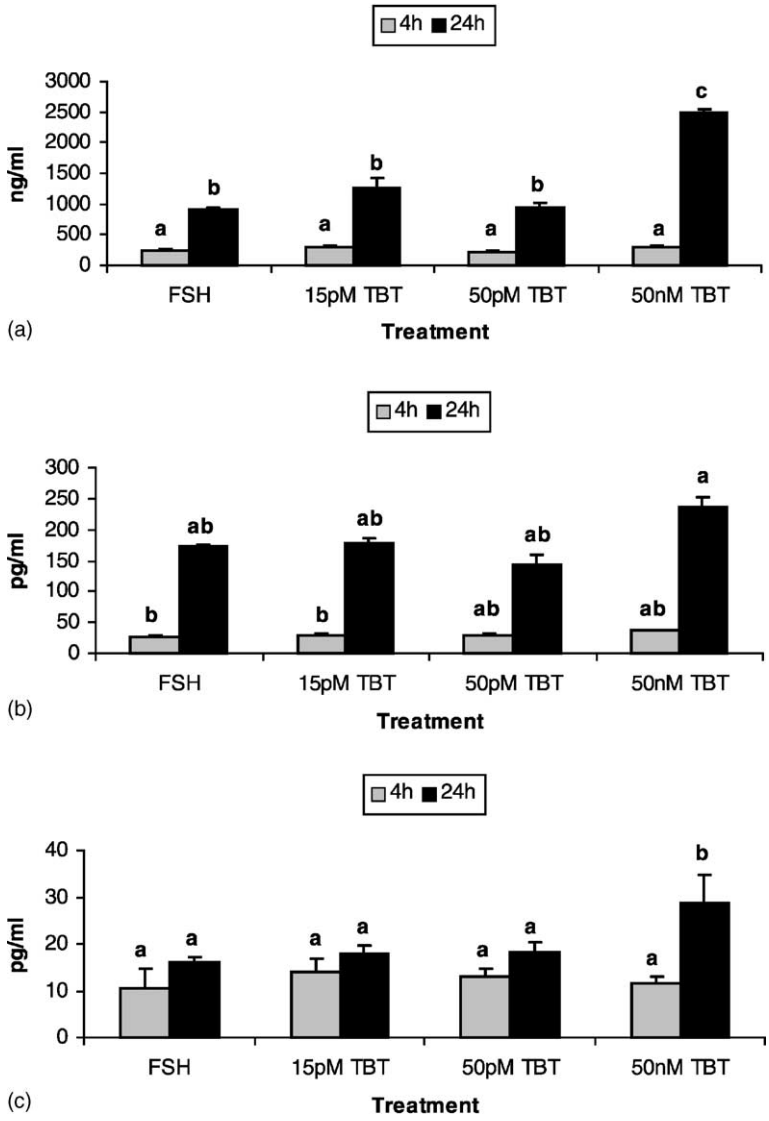

Fig. 5. Steroid hormone concentration in culture media of GC FSH stimulation and simultaneous TBT treatment at different concentrations: (a) progesterone, (b) testosterone and (c) estradiol. Data ($n = 3$) are presented as means ± S.D. $P < 0.05$ (significant difference indicated by letters).

maturation TBT induced either a significant increase in progesterone and a slight reduction in T and E. The decrease of E could be caused either by the reduced mRNA-levels of aromatase or though TBT-induced ARO enzyme-inhibition as previously described for human [36,37]. Furthermore, Whalen and Loganathan [38] described a rapid decrease of intracellular cAMP-levels in human natural killer cells after exposure to TBT. A proposed similar cAMP breakdown could as well happen in the bovine cumulus or granulosa cells, which may lead to a pre-term resumption of meiosis in the oocyte.

4.3. Steroidogenesis during GC culture in response to FSH and LH

A granulosa cell culture was used to further elucidate possible effects of (1) gonadotropins, (2) TBT and (3) gonadotropins and TBT in combination. Although the bovine GCs may undergo luteinisation after removing from the follicle, they showed typical effects after FSH and LH supplementation. Without gonadotropins, the GCs produced P but less estradiol as expected for luteal cells [39]. Although rapid morphological and steroidogenic changes are described for cultured GC [40], our GCs responded well to LH and FSH by increased E beside almost unchanged T- and P-secretion. Supporting such unaffected P-levels, HSD mRNA expression showed no significant changes during gonadotropin treatment.

In contrast, initial ARO mRNA increase caused by FSH and LH led to delayed secretion of E whereby co-treatment induced just slight increased E-levels. These results possible indicate a estrus cycle dependent reactivity of the GCs to different gonadotropin ratios, which were existent in distinct but changing ratios in vivo.

4.4. Steroidogenesis during GC culture in response to TBT

Recent studies would expected that TBT inhibits the conversion of androgens to estrogens [36,37] and therefore T-secretion should increase. However, in our system no significant alteration of the T-levels are induced by TBT possibly indicating that T is not the predominant substrate for the estrogen synthesis. It is known that ARO could also converts androstendione to estrone. Therefore, additional experiments have to elucidate possible effects of TBT on the androstendione pathway.

The most interesting result was that 50 pM TBT blocked completely the production of E although ARO mRNA could be detected at higher concentration as compared to the control GC culture. In contrast, P-concentration were increased by 50 pM TBT despite of lowered HSD mRNA transcripts. These data support the results of Heidrich et al. [37], postulating that TBT blocks ARO through only moderate affecting HSD enzyme activity. However, higher concentrations of TBT (100 pM) led to an increase of E when compared to 50 pM indicating a strict dose-dependant effect of this pollutant in that system.

4.5. Combined effects of TBT and FSH during GC culture

To further enlighten possible interactions between endogenous hormones and the pollutant, a co-treatment was introduced. It was made use of the known stimulatory effect of gonadotropins on the E-production in GC (see this communication and [39]) to simulate an in vivo-like scenery. The main reason for choosing especially FSH as co-stimulator was, that FSH receptor mRNA was much more abundant in GC than the LH receptor (personal data).

To our surprise the highest TBT treatment led to an increase of all steroids in GC cultures additionally provided with FSH, but the previously measured effects of low TBT were erased by simultaneous application of FSH. Distinct dose-dependent and synergistic effects of TBT on bovine GC in cultures have to be taken into account. A possible influence of TBT on the FSH receptor expression may be observed in this content.

Although TBT alone caused lowered E-levels at 50 pM in co-stimulation with TBT such effects were not detectable. Higher concentrations of TBT (50 nM) were necessary to evoke alterations in steroid ratios. Therefore, our results indicate that in vivo effects of TBT could be much more complex as shown in the isolated experiments.

In conclusion, cumulus oocyte complexes were able to produce their own micro-environment according to the three steroids progesterone, testosterone and estradiol, although they are separated from their natural milieu. In addition, it seems that mRNA expressions of aromatase P450 and 3-beta-hydroxy steroid dehydrogensase in parallel reflect the measured steroid products. Our first results concerning COCs under TBT exposure indicate that this potential biocide could influence steroidogenesis of the maturing oocyte in mammals especially through increasing progesterone. In the future cAMP-levels after TBT treatment, potentially modulating meiotic arrest of the oocyte, should be tested. Experiments on bovine granulosa cells supported that gonadotropins clearly elevated levels of estradiol whereby aromatase mRNA data may indicate immediate modulation of transcription. As well as in the cumulus oocyte complexes TBT showed a possible effect onto steroid hormone composition in the culture media. Besides testosterone also changes in androstendione—likewise a substrate of aromatase P450—should be examined as well as short term effects on mRNA expression. There is strong evidence that TBT acts in time—as well as concentration-dependent manner on the steroid hormone producing reproductive cells furthermore interacting with circulating gonadotropins. More efforts are necessary to illuminate the complex action of potent pollutant as TBT. Possible disturbing influences of TBT on the mammalian steroid system within the ovary cannot be excluded.

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