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—Original Article—

The combined effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and the phytoestrogen genistein on steroid hormone secretion, AhR and ERβ expression and the incidence of apoptosis in granulosa cells of medium porcine follicles

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Abstract. Low doses of endocrine disrupting chemicals (EDCs) used in combination may act in a manner different from that of individual compounds. The objective of the study was to examine *in vitro* effects of low doses of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 100 pM) and genistein (500 nM) on: 1) progesterone (P_4) and estradiol (E_2) secretion (48 h); 2) dynamic changes in aryl hydrocarbon receptor (AhR) mRNA and protein expression (1, 3, 6, 24 and 48 h); 3) dynamic changes in estrogen receptor β (ERβ) mRNA and protein expression (1, 3, 6, 24 and 48 h); and 4) induction of apoptosis in porcine granulosa cells derived from medium follicles (3, 6 and 24 h). TCDD had no effect on P_4 or E_2 production, but potentiated the inhibitory effect of genistein on P_4 production. In contrast to the individual treatments which did not produce any effects, TCDD and genistein administered together decreased ERβ and AhR protein expression in granulosa cells. Moreover, the inhibitory effect of TCDD on AhR mRNA expression was abolished by genistein. The treatments did not induce apoptosis in the cells. In summary, combined effects of low concentrations of TCDD and genistein on follicular function of pigs differed from that of individual compounds. The results presented in the current paper clearly indicate that effects exerted by low doses of EDCs applied in combination must be taken into consideration when studying potential risk effects of EDCs on biological processes.
Key words: Aryl hydrocarbon receptor (AhR), estrogen receptor β (ERβ), Genistein, Porcine granulosa cells, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)

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Endocrine disrupting chemicals (EDCs) comprise of a diverse group of compounds known to interfere with normal hormone signaling and action. EDCs include natural (such as plant derived phytoestrogens, e.g., genistein) or industrial (such as dioxins, e.g., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: TCDD) compounds found in the environment and in the diet of humans and animals. Many of EDCs have been shown to induce estrogenic and/or anti-estrogenic effects, and therefore affect reproduction in females and males [1]. The isoflavone genistein is the most abundant soy-derived phytoestrogen present in the diet of humans and animals, including pigs. Genistein was found to influence reproductive processes, along with steroid hormone secretion in humans [2], rats [3], rabbits [4] and pigs [5]. Previous studies indicate that one of the mechanisms of genistein action in cells involves the activation of estrogen receptors (ERs), especially estrogen receptor β (ERβ) [6]. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent congener of compounds known as polychlorinated dibenzo-*p*-dioxins (PCDDs). TCDD is widespread and highly persistent in the environment and accumulates in living organisms, especially in fat tissue. The dioxin is known to adversely affect reproduction in part due to its ability to alter hormone levels in many species, including pigs [7, 8]. Previous studies indicate that most of TCDD effects are mediated through the aryl hydrocarbon receptor (AhR), a ligand-activated member of the bHLH-PAS family of transcription factors [9]. Therefore, genistein and TCDD are simultaneously present in the environment and may be capable of affecting endocrine cell functions in humans and pigs.

Usually, humans and animals are exposed to multiple EDCs present in the environment in relatively low concentrations. It was demonstrated that low concentrations of EDCs administered in mixture were able to induce effects different from those provoked by single compounds [10, 11]. Therefore, it is justified to assume that particular EDCs can act together amplifying adverse effects on

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human and animal health and reproduction. However, studies exploring mechanisms of the interactions in reproductive cells are scarce.

Porcine granulosa cells express both AhR [12] and ERβ [5], which make these cells responsive to TCDD as well as genistein. Studies in cell lines treated with different AhR and ER ligands, indicated possible interaction pathways between these two receptors, described as AhR/ER 'cross-talk' [13, 14]. It is not known, however, whether genistein affects AhR and TCDD activates ER in granulosa cells of the pig, a species particularly vulnerable to both phytoestrogens, due to their presence in the diet, and dioxins, due to their ability to accumulate in fat tissue. Therefore, the goal of this study was to establish the influence of low doses of TCDD and genistein used in combination on porcine granulosa cells *in vitro*. Since extensive dose dependent studies on porcine granulosa cells have been already performed individually for genistein [5, 15, 16] and TCDD [7, 8, 17], the current study was focused on the examination of the combined effects of the low but environmentally relevant doses of the two treatments. Thus, the specific objectives were to examine the effects of TCDD (100 pM) and/or genistein (500 nM) on: 1) progesterone (P_4) and estradiol (E_2) production; 2) dynamic changes in expression of AhR (mRNA and protein); 3) dynamic changes in expression of ERβ (mRNA and protein); and 4) induction of apoptosis in porcine granulosa cells derived from medium (3–6 mm) porcine follicles. Since examination of the receptor expression only in one time point may not allow to determine the dynamic changes in AhR and ERβ levels, five time points (1, 3, 6, 24 and 48 h) were employed in the expression studies. Moreover, due to lack of ERα protein in porcine granulosa cells [5], only estrogen receptor β was investigated in the current study.

Materials and Methods

Materials

In the current study the following reagents were used: [2,4,6,7-^{3}H]-estradiol and [1,2,6,7-^{3}H]-progesterone (Amersham, Little Chalfont, UK); TaqMan Gene Expression Assay and TaqMan® 2 × Universal PCR Master Mix (Applied Biosystems, Faster City, CA, USA); FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA); calf serum and phenol-free Eagle's medium (Biomed, Kraków, Poland); trypan blue dye (Chemapol, Praha, Czech Republic); anti-P_4 and anti-E_2 antibodies (Department of Animal Physiology, University of Warmia and Mazury in Olsztyn, Poland); polyclonal rabbit anti-AhR antibodies (EnzoScientific, Farmingdale, NY, USA); random hexamers $(dNT)_6$ (GenPandora, Gdańsk Poland); bovine serum albumin (BSA; ICN Biomedicals, Santa Ana, CA, USA); RNase OUT™ Recombinant Ribonuclease Inhibitor and TRIzol Reagent (Invitrogen, Carlsbad, CA, USA); gentamycin (KRKA, Novo Mesto, Slovenia); Omniscript RT Kit (Qiagen, Hilden, Germany); oligo$(dT)_{15}$ primers (Roche, Basel, Switzerland); monoclonal mouse anti-ERβ antibodies (Serotec, Kidlington, UK); genistein, nystatin and propidium iodide (Sigma, St. Louis, MO, USA); 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD; Supelco, Bellefonte, PE, USA); goat anti-rabbit biotinylated secondary antibodies, horse anti-mouse biotinylated secondary antibodies and FITC conjugated streptavidin (Vector Laboratories, Burlingame, CA, USA).

Porcine ovaries with medium antral (3–6 mm in diameter) follicles, were collected from cycling pigs in a local slaughterhouse (Biskupiec, Poland). The ovaries were transported (45 min) to the laboratory in cold buffered physiological saline (PBS; 4 C) supplemented with gentamycin and nystatin. Morphology of ovaries and size of follicles were evaluated according to classification of Akins and Morrissette [18]. All experimental procedures were approved by the Local Ethics Committee in Olsztyn, Poland.

Cell cultures and experimental design

Granulosa cells were isolated from the follicles as previously described [19]. Cell viability was determined by trypan blue dye exclusion, and it was always greater than 90%. Following isolation, the cells were resuspended in incubation medium: Eagle's phenol red-free medium with 10% calf serum (CS) and L-glutamine (2 mM), 0.05 mg/ml gentamycin and 60 U/ml nystatin. The granulosa cells were cultured in: 1/ 24-well plates (1.5×10^5 cells/1 ml medium) for 48 h to measure steroid hormone secretion; 2/ 6-well plates (2×10^6 cells/3 ml medium) to measure mRNA expression as well as the incidence of apoptosis, and 3/ 8-well plates (1×10^5 cells/ 0.3 ml medium) to analyze protein expression. Following 40 h of pre-culture (37 C, 95% air/5% CO_2), medium was exchanged (Eagle's phenol red-free medium, 5% CS, L-glutamine (2 mM), 0.05 mg/ml gentamycin and 60 U/ml nystatin), and cells were cultured with TCDD (100 pM; CAS number 1746-01-6) and/or genistein (500 nM; CAS number 446-72-0) for additional 1, 3, 6, 24 or 48 h depending on the examined endpoint. Solvents for TCDD (0.025% dimethyl sulfoxide) and genistein (0.005% ethanol), used separately or in combination, did not affect the examined parameters. The effects of a wide range of TCDD and genistein doses were described in numerous papers [5, 7, 8, 16, 19, 20]. To be able to properly address the aims of the current study, the selected treatment doses were: 1/ based on concentrations that were reported as human daily intake [21], 2/ found in biological fluids/tissues of humans and animals [22–24], and 3/ previously reported to be either ineffective [16, 20] or borderline effective [5, 17] in cell culture models. Moreover, since five time points investigated in the mRNA and protein expression experiments produced a large number of data, it was possible to test only three treatments: one dose of TCDD, one dose of genistein, and a combination of these two treatments.

The effects of TCDD and genistein on steroid hormone secretion (radioimmunoassay)

For estimation of P_4 and E_2 secretion, granulosa cells were cultured for 48 h with TCDD (100 pM) and/or genistein (500 nM). Control samples were cultured without treatments. Concentrations of steroid hormones were measured by radioimmunoassay (RIA), validated and described previously [25–27]. Intra- and inter-assay coefficients of variation for P_4 were 3.64% and 3.63%, respectively. Sensitivity of the assay was 5 pg/ml. Intra- and inter-assay coefficients of variation for E_2 were 5.74% and 3.74%, respectively, and sensitivity of the assay was 2 pg/ml. All samples were run in triplicate. Serial dilutions of medium samples showed parallelism with the standard curves of P_4 and E_2. The correlation coefficients between added and determined amounts of unlabeled steroids were higher than 0.990. Exogenous treatments did not interfere with RIAs.

Table 1. Sequences of primers and probes used for Real-time PCR, the length of PCR products and GenBank accession numbers

Gene	Primer sequences (sense and antisense)	Probe sequences	Accession number	Size of PCR products (bp)
AhR	5'TGGAAGACCAGATTATATCATTGCAACTC 3' 5'GCGTTTTCGTAGATGTTCTTTTCCT 3'	TTCATCTGTGAGAGGTCTCT	EU155082	75
ERβ	5'ATGTGGCGCTCCATCGA 3' 5'CAGAACGAGGTCTGGAGCAAA 3'	CCGGCAAGCTCAT	NM_001001533	58
β-actin	5'GCTCTTCCAGCCCTCCTT 3' 5'GTTGAAGGTGGTCTCGTGGAT 3'	CTGGGCATGGAGTCCT	U07786	60

The effects of TCDD and genistein on AhR and ERβ mRNA expression (Real-time PCR)

The effects of TCDD and/or genistein on dynamic changes in AhR and ERβ mRNA expression were measured in granulosa cells cultured with TCDD (100 pM) and/or genistein (500 nM) for 1, 3, 6, 24 or 48 h. After culture, total RNA was isolated from granulosa cells using TRIzol following the manufacturer's recommendations. After extraction, RNA concentration was determined spectrophotometrically (A_{260}; NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE, USA). The integrity of RNA was estimated electrophoretically. First strand cDNA was generated from total RNA (1 μg) using the Omniscript RT kit. Total volume of working solution (20 μl) contained: 0.1 μM oligo$(dT)_{15}$ primer, 0.1 μM random hexamers $(dNT)_6$, 10 U RNase OUT™ Recombinant Ribonuclease Inhibitor, 10 × RT buffer, 4 U reverse transcriptase and RNase/DNase free water. Reverse transcription reaction was performed at 37 C for one hour (GeneAmp PCR System 2400, Perkin Elmer, Waltham, MA, USA).

Quantitative Real-time PCR was used to establish dynamic changes in AhR and ERβ mRNA expression in TCDD and genistein treated granulosa cells. Specific primers and probes for AhR, ERβ and β-actin (a housekeeping gene) were designed (Table 1) according to the porcine sequences of the AhR (access no: EU155082), ERβ (access no: NM_001001533) and β-actin (access no: U07786) available in GenBank. Real-time PCR was performed using TaqMan Gene Expression Assay and TaqMan® 2 × Universal PCR Master Mix. Each Real-time PCR reaction mixture (25 μl) consisted of: 1/ 1 × TaqMan Universal PCR Master Mix; 2/ the probe (250 nM); 3/ both primers (each: 900 nM); and 4/ a particular cDNA template (1 μl). Real-time reactions (7300 Real-time PCR System, Applied Biosystems) for each sample were carried out in duplicate and non template control (NTC) was included with each run. The relative mRNA expression levels were calculated by the comparative cycle threshold (C_T) method [28], normalized to β-actin expression and expressed as arbitrary units.

The effects of TCDD and genistein on AhR and ERβ protein expression (immunofluorescence)

Because AhR protein level in granulosa cells was too low to be detected by Western blot (WB), the protein level in the study was determined by fluorescent immunocytochemistry. Similar to WB, this method enables to measure the level of fluorescently labeled proteins in a semi-quantify manner. The effects of TCDD and/or genistein on dynamic changes in AhR and ERβ protein expression were measured in granulosa cells cultured (LabTek Chamber Slide Systems, Nunc, Denmark) with TCDD (100 pM) and/or genistein (500 nM) for 1, 3, 6, 24 or 48 h. Following culture, the cells were fixed in 4% paraformaldehyde and incubated (12 h) with primary mouse monoclonal antibodies against ERβ (1:50) or primary rabbit polyclonal antibodies against AhR (1:100). In negative control samples, the primary antibodies were omitted. Next, the cells were incubated (1 h) with respective secondary antibodies (horse anti-mouse biotinylated secondary antibodies or goat anti-rabbit biotinylated secondary antibodies; 1:100), and then treated with FITC (fluorescein isothiocyanate) conjugated streptavidin (1:50). Fluorescence intensity of stained cells was determined using the NIS-Elements 3.0 Imaging System (Nikon). In each of the four independent experiments, all treatments were run in duplicate. To ensure the objectivity of the procedure, six images (in duplicate) were consistently taken from the same six precisely defined areas of the well. Each cell was selected as a region of interest (ROI) and the mean fluorescence intensity of all selected cells on the image was calculated.

Data were expressed as an arbitrary units representing the intensity of staining of granulosa cells.

The effect of TCDD and genistein on apoptosis (flow cytometry)

The effect of TCDD and genistein on the incidence on apoptosis was measured in granulosa cells cultured with TCDD (100 pM) and/or genistein (500 nM) for 3, 6 or 24 h. After culture, the cells were detached from plates by trypsinization and stained using FITC Annexin V Apoptosis Detection Kit following the manufacturer protocol. Cells were immediately analyzed by flow cytometry on a MoFlo™XDP instrument (Beckman Coulter, Brea, CA, USA). The FITC signal of Annexin V was detected at 530 nm and propidium iodide fluorescence was detected at 650 nm. Cells showing up as Annexin V^-/PI^- were defined as live, Annexin V^-/PI^+ were recognized as necrotic, those showing up as Annexin V^+/PI^+ were defined as late apoptotic, whilst Annexin V^+/PI^- cells were considered as early apoptotic cells [8, 29].

Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis (Statistica program StatSoft, Tulsa, OH, USA) was performed using one-way ANOVA for repeated measurements followed by least significant difference (LSD) post hoc test for multiple comparisons. Differences with a probability of $P < 0.05$ were considered significant. RIA raw data were log transformed before statistical analysis and are expressed as a percentage of control culture (100%). Flow cytometry data expressed as a percentage of the number of stained cells were arcsine transformed prior to the statistical analysis.

Results

The effects of TCDD and genistein on steroid hormone secretion

Genistein inhibited ($P < 0.05$) P_4 and E_2 secretion by granulosa cells (Fig. 1AB). In contrast, TCDD did not affect P_4 and E_2 secretion, however it potentiated ($P < 0.05$) the inhibitory effect of genistein on P_4 production (Fig. 1A). Moreover, genistein in combination with TCDD also significantly decreased E_2 secretion by porcine granulosa cells compared to control (Fig. 1B).

The effects of TCDD and genistein on AhR and ERβ mRNA expression

Genistein alone and genistein plus TCDD increased ($P < 0.05$) AhR mRNA expression in the cells after 3 h of culture (Fig. 2B). TCDD decreased ($P < 0.05$) AhR mRNA expression in granulosa cells after 48 h of culture, and genistein abolished ($P < 0.05$) this suppressive effect of TCDD (Fig. 2E). Other treatments did not affect AhR mRNA expression in the examined cells (Fig. 2).

TCDD and genistein applied separately or in combination did not affect ERβ mRNA expression in porcine granulosa cells at the examined time points in comparison to the control group (Fig. 3). However, granulosal expression of ERβ mRNA was lower ($P < 0.05$) in the presence of TCDD and genistein in comparison to that of TCDD alone after 6 hours of culture (Fig. 3C).

The effects of TCDD and genistein on AhR and ERβ protein expression

Semiquantitative densitometric analysis revealed that TCDD as well as TCDD plus genistein decreased ($P < 0.05$) the intensity of AhR immunostaining in porcine granulosa cells after one and 48 h of culture (Fig. 4AE). After 24 h of culture, TCDD in combination with genistein decreased ($P < 0.05$) AhR immunostaining intensity in comparison to single treatments and controls (Fig. 4D). Representative images of AhR immunostaining in porcine granulosa cells cultured for one hour in the absence (Fig. 5A) or in the presence of TCDD (Fig. 5B), genistein (Fig. 5C) and TCDD plus genistein (Fig. 5D) are depicted in Fig. 5. Green fluorescence (FITC) indicated the presence of AhR protein in the cells. Propidium iodide (PI) staining (red fluorescence) was used for better visualization of the cells (Fig. 5).

Semiquantitative densitometric analysis revealed that, in contrast to single treatments, TCDD in combination with genistein decreased ($P < 0.05$) the intensity of ERβ immunostaining after 48 h of culture (Fig. 6E). In addition, after 3 h of culture, the intensity of ERβ immunostaining in the cells was lower ($P < 0.05$) in the presence of TCDD and genistein in comparison to that of TCDD alone (Fig. 6B). None of the treatments affected ERβ immunostaining in the remaining time points (Fig. 6). Representative images of immunostaining intensity of ERβ expression in porcine granulosa cells cultured for 48 h in the absence (Fig. 7A) or in the presence of TCDD (Fig. 7B), genistein (Fig. 7C) and TCDD plus genistein (Fig. 7D) are presented in Fig. 7.

The effect of TCDD and genistein on apoptosis

TCDD and genistein used alone and in combination did not affect the percentage of live, necrotic, early and late apoptotic granulosa cells after 3, 6 and 24 hours of culture (data not shown). Representative dot plots of Annexin V-FITC and propidium iodide double stained granulosa cells cultured for 24 h in the absence (Fig. 8A) or in the presence of TCDD (Fig. 8B), genistein (Fig. 8C) and TCDD plus genistein (Fig. 8D) are presented in Fig. 8.

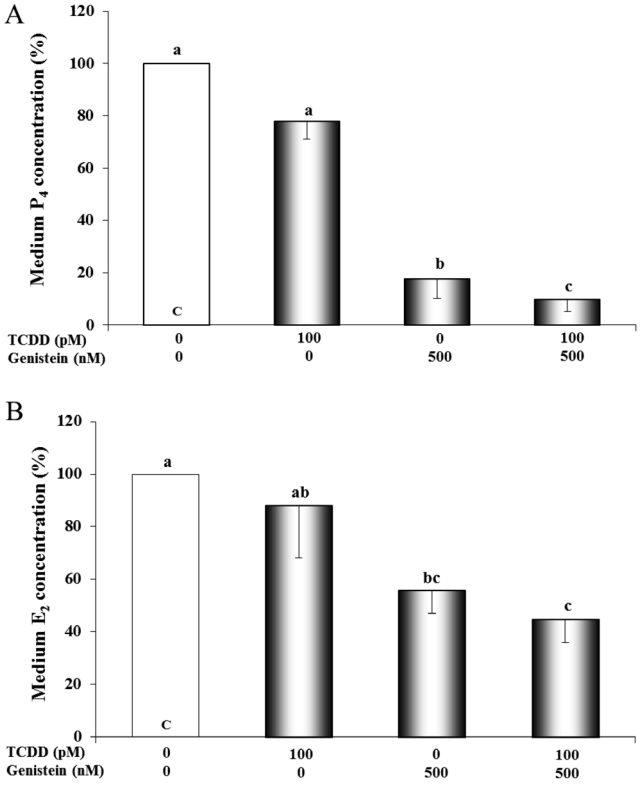

Fig. 1. The effects of TCDD and/or genistein on A) progesterone (P_4) and B) estradiol (E_2) secretion (mean ± SEM) by porcine granulosa cells harvested from porcine medium follicles (n = 6–7 independent experiments). Following pre-culture (40 h), the cells (1.5×10^5 cells/ml Eagle's medium, 5% calf serum) were cultured for 48 h with TCDD (100 pM) and/or genistein (500 nM). Log transformed data were submitted to one-way ANOVA for repeated measurements followed by the LSD post-hoc test. Data are shown as a percentage of control (C; untreated) cells (100%). The same letters over the bars indicate a lack of significant differences between the groups ($P < 0.05$). TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Discussion

In the current study, we demonstrated that *in vitro* P_4 production by pig granulosa cells was affected by low, environmentally relevant doses of TCDD and genistein in a manner dependent on whether the compounds were administered separately or together. TCDD itself had no effect on either P_4 or E_2 production, but potentiated the inhibitory effect of genistein on P_4 secretion. To our best knowledge, this is the first study examining the effect of dioxins and phytoestrogens applied in combination on secretion of ovarian steroid hormones. Kotwica *et al*. [30] demonstrated that genistein prevented the stimulating

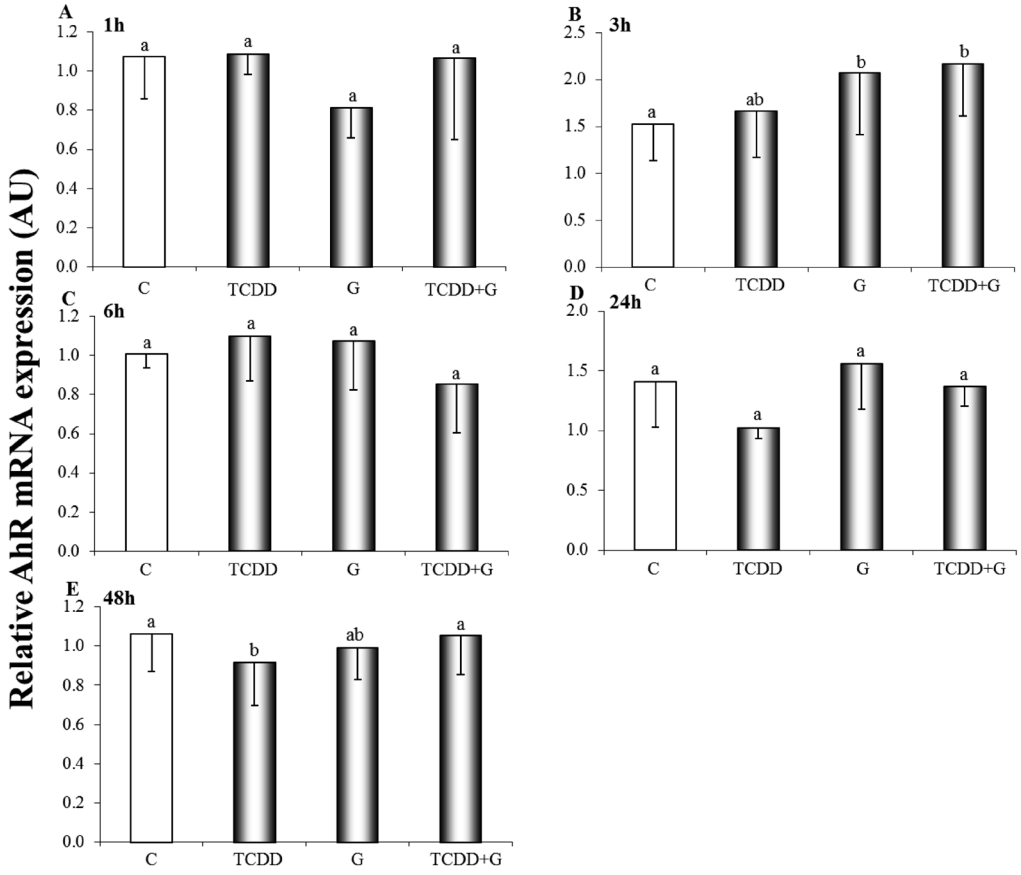

Fig. 2. The effects of TCDD and/or genistein on relative AhR mRNA expression (mean ± SEM) in cultured granulosa cells isolated from porcine medium follicles (n = 4 independent experiments). Following pre-culture (40 h), the cells (2×10^6 cells/ 3 ml Eagle's medium, 5% calf serum) were cultured with TCDD (100 pM) and/or genistein (500 nM) for A) one; B) three; C) six; D) 24 and E) 48 h. One-way ANOVA for repeated measurements was followed by the LSD post-hoc test. The same letters over the bars indicate a lack of significant differences between the groups ($P < 0.05$). AhR: aryl hydrocarbon receptor; AU: arbitrary units; C: control; G: genistein; TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

effect of different congeners of polychlorinated biphenyls (PCBs) on oxytocin secretion by bovine granulosa and luteal cells. Moreover, TCDD alone, but not a mixture of dioxins containing TCDD, affected steroid hormone secretion by placental tissue [31]. Although those results are difficult to compare with data presented in the current paper, they imply the importance of efforts to study the combined effects of EDCs on reproductive processes.

Our previous studies revealed that the mechanism of the combined action of TCDD and genistein does not involve modulation of steroidogenic enzyme activity [20]. Other unexplored mechanisms may include interactions between the intracellular pathways of ER and AhR and/or modulation of steroid hormone metabolism. In the current study, the expression of AhR and ERβ was examined at five time points (1–48 h) after exposure to TCDD and/or genistein. TCDD decreased AhR protein expression after one and 48 h of culture, while AhR mRNA expression was affected only after 48 h. Similarly, TCDD induced rapid depletion of AhR protein without visible effects on mRNA expression in several cell lines [32, 33]. The rapid AhR depletion, observed in our study one hour after the exposure to dioxin, may be the result of proteolytic degradation of the receptor in response to TCDD [32–34]. The longer exposure to TCDD (48 h) resulted in down regulation of AhR mRNA and protein expression. The changes observed in AhR mRNA and protein level in porcine granulosa cells are probably a part of the mechanism responsible for the regulation of cellular sensitivity to AhR ligands.

Genistein alone increased AhR mRNA expression in granulosa cells after 3 h of culture, but had no effect on AhR protein. It was previously reported that feeding female rats with soy protein isolate caused an increase in AhR mRNA and a decrease in AhR protein expression in liver and mammary gland [35]. The authors suggested that the decrease in AhR protein resulted in anticancer properties of soy products containing genistein and other phytoestrogens. Other studies performed in various cell lines indicated that genistein might act as an AhR agonist or antagonist, and potentiate or diminish the effect of typical AhR ligands such as TCDD [14, 36].

Genistein in combination with TCDD decreased AhR immunostaining after one and 48 h of incubation, however, this action did not differ from that of TCDD alone. In contrast, after 24 h, the effects of TCDD and genistein applied separately were significantly different from those caused by the treatments applied in combination. Additionally,

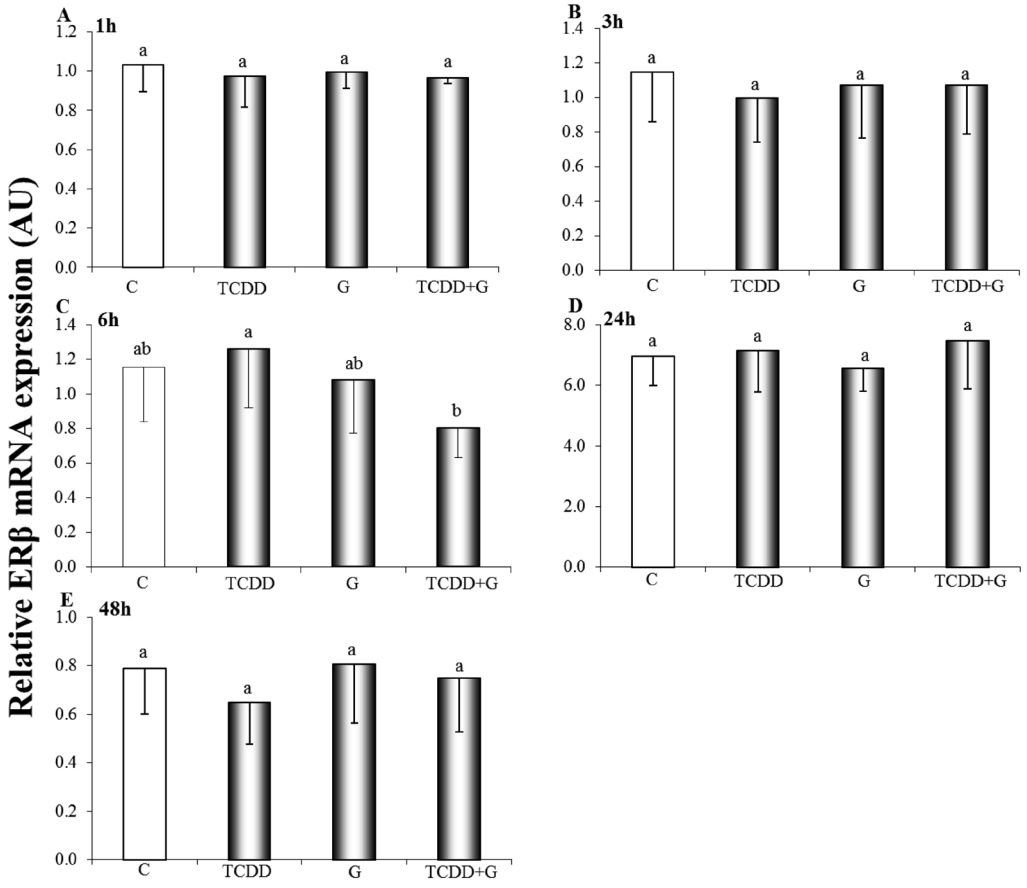

Fig. 3. The effects of TCDD and/or genistein on relative ERβ mRNA expression (mean ± SEM) in cultured granulosa cells isolated from porcine medium follicles (n = 4 independent experiments). Following pre-culture (40 h), the cells (2×10^6 cells/ 3 ml Eagle's medium, 5% calf serum) were cultured with TCDD (100 pM) and/or genistein (500 nM) for A) one; B) three; C) six; D) 24 and E) 48 h. One-way ANOVA for repeated measurements was followed by the LSD post-hoc test. The same letters over the bars indicate a lack of significant differences between the groups ($P < 0.05$). AU: arbitrary units; ERβ: estrogen receptor β; C: control; G: genistein; TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

genistein abolished the inhibitory effect of TCDD on AhR mRNA expression after 48 h of culture. The results of our study indicated that genistein interacted with TCDD action on AhR expression in porcine granulosa cells. This interaction may be at least partially responsible for the differences in steroid hormone secretion.

TCDD had no effect on ERβ mRNA and protein expression in porcine granulosa cells regardless the time of exposure. Similarly, TCDD had no effect on ERβ mRNA and/or protein expression in human breast cancer cell lines [37, 38] and rat ovaries [39]. However, Dasmahapatra *et al*. [40] reported an increase in ERβ mRNA expression in rat granulosa cells treated with nanomolar doses of TCDD. In contrast, *in vivo* studies have shown decrease in ERβ mRNA in the ovary and uterus of mice treated with TCDD [41]. It appears that TCDD's influence on ERβ expression varies and depends on tissue, species and experimental design.

In the current study, we were not able to demonstrate the effects of genistein alone on granulosal expression of ERβ mRNA and protein. Similarly, genistein did not affect ERβ protein or mRNA expression in the uterus of ovariectomized or pubertal rats, respectively [42, 43]. In contrast, results of our previous study indicated an increase in ERβ mRNA and protein expression in porcine granulosa cells treated with genistein [5]. This discrepancy might result from differences in culture conditions and techniques used to measure mRNA and protein expression. Interestingly, some authors reported biphasic action of genistein. Low doses of genistein increased and high doses decreased ovarian ERβ mRNA expression in mice [44]. In addition, these changes in ERβ mRNA expression were observed after 5, but not after 12 or 19 days following genistein injection [44]. This data proves that time of exposure, as well as doses of the phytoestrogen are significant factors influencing the overall outcome after genistein treatment.

In contrast to separate treatments, TCDD in combination with genistein decreased ERβ immunostaining, but not mRNA expression after 48 h of culture. In addition, expression of ERβ mRNA was lower in the presence of TCDD and genistein after 3 h of culture in comparison to that of TCDD alone. The present study is the first investigating the effects of TCDD and genistein on ERβ expression. Interestingly, Tanaka *et al*. [45] have shown inhibitory effects of genistein and 7,12-dimethylbenz[a]anthracene (DMBA, another AhR ligand) on the expression of ERβ and ERα protein in

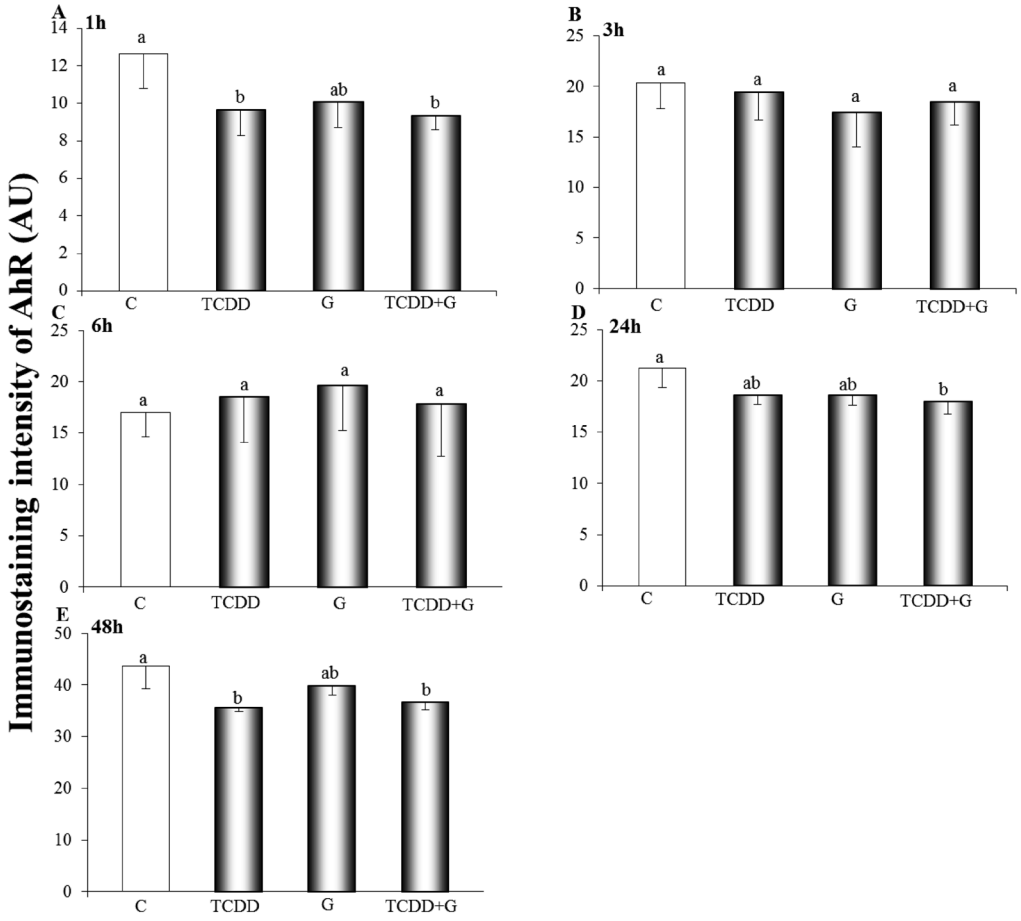

Fig. 4. The effects of TCDD and/or genistein on the intensity of AhR immunostaining (mean ± SEM) in cultured granulosa cells isolated from porcine medium follicles (n = 4 independent experiments). Following pre-culture (40 h), the cells (1×10^5 cells/ 0.3 ml Eagle's medium, 5% calf serum) were cultured with TCDD (100 pM) and/or genistein (500 nM) for A) one; B) three; C) six; D) 24 and E) 48 h. One-way ANOVA for repeated measurements was followed by the LSD post-hoc test. The same letters over the bars indicate a lack of significant differences between the groups ($P < 0.05$). AhR: aryl hydrocarbon receptor; AU: arbitrary units; C: control; G: genistein; TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

rat adenocarcinoma. This effect was not observed in animals treated with DMBA only. The authors, however, did not study the effect of genistein alone. Moreover, it was found that AhR activation by TCDD in human breast cancer cells caused proteolytic degradation of ERα [34]. Also, ligand-activated AhR has been shown to promote the proteolysis of ERα through assembling a ubiquitin ligase complex [46]. It is possible that in cells expressing ERβ as a dominant isoform of estrogen receptors, AhR/ER 'cross-talk' engages ERβ. Additional experiments are needed to understand and explain mechanisms responsible for ERβ depletion observed in our studies.

In the current study we also tested whether the inhibitory effect of TCDD and genistein on steroid hormone production in porcine granulosa cells resulted from proapoptotic actions. TCDD (100 pM) did not affect the percentage of live, necrotic, early and late apoptotic granulosa cells after 3, 6 and 24 h of culture. Similarly, TCDD (100 pM; 100 nM) had no effect on viability and apoptosis of porcine granulosa cells after 48 h of culture [8]. Higher doses of TCDD (3.1 nM; 3.1 μM) induced apoptosis in human luteinized granulosa cells after 24 and 48 h of culture [47]. However, apoptosis in antral and preantral follicles was not affected in rats exposed to TCDD in utero and lactationally [48]. Discrepancies observed in the influence of TCDD on apoptosis may be caused by differences in the sensitivity of cells and/or species to TCDD.

Genistein alone had no effect on the percentage of live, necrotic, early and late apoptotic granulosa cells after 3, 6 and 24 h of culture. Previous studies have shown that genistein (0.05; 0.5; 5 μM) did not influence viability of porcine granulosa cells after 48 h of culture [5]. A cytotoxic effect of genistein on porcine granulosa cells was only observed when high (50 and 100 μM) doses were used [5, 16]. Studies performed on different cell lines revealed proapoptotic action of high, pharmacological doses (> 10 μM) of genistein [49, 50]. Interestingly, lower (< 10 μM) doses of genistein increased proliferation of MCF-7 cells, while higher doses (10–20 μM) induced apoptosis of this cell line [51, 52]. It seems that the action of genistein on apoptosis and/or proliferation of cells depends on the dose of phytoestrogen and origin of cells.

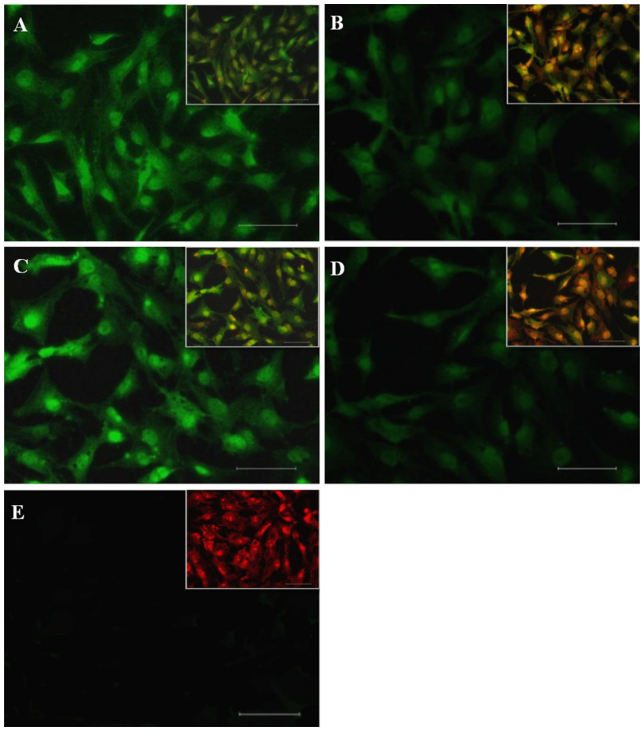

Fig. 5. Exemplary images of AhR immunostaining determined by immunofluorescence (FITC, green color) in porcine granulosa cells originated from medium follicles. Following pre-culture (40 h), the cells (1×10^5 cells/ 0.3 ml Eagle's medium, 5% calf serum) were cultured with TCDD (100 pM) and/or genistein (500 nM) for one hour. A) cells without treatments (control); B) cells treated with TCDD; C) cells treated with genistein; D) cells treated with TCDD and genistein; E) negative control; please note a lack of AhR staining. To better visualize the cells, the insets containing the merged images of both green (AhR) and red fluorescence (propidium iodide) filters, are presented in the figure. Scale bar: 20 µm.

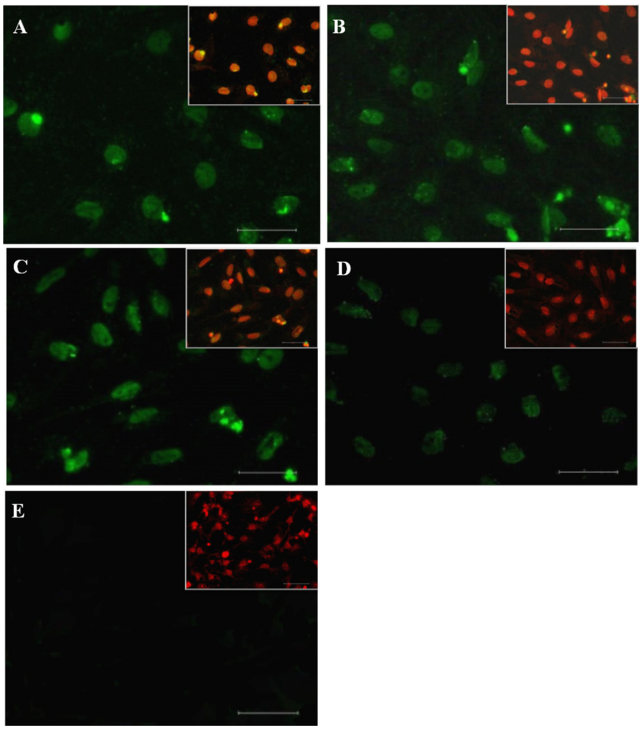

Fig. 7. Exemplary images of immunostaining of ERβ determined by immunofluorescence in porcine granulosa cells originated from porcine medium follicles. Following pre-culture (40 h), the cells (1×10^5 cells/ 0.3 ml Eagle's medium, 5% calf serum) were cultured with TCDD (100 pM) and/or genistein (500 nM) for 48 h. A) cells without treatments (control); B) cells treated with TCDD; C) cells treated with genistein; D) cells treated with TCDD and genistein; E) negative control: please note a lack of ERβ staining. To better visualize the cells, the insets containing the merged images of both green (ERβ) and red fluorescence (propidium iodide) filters, are presented in the figure. Scale bar: 20 µm.

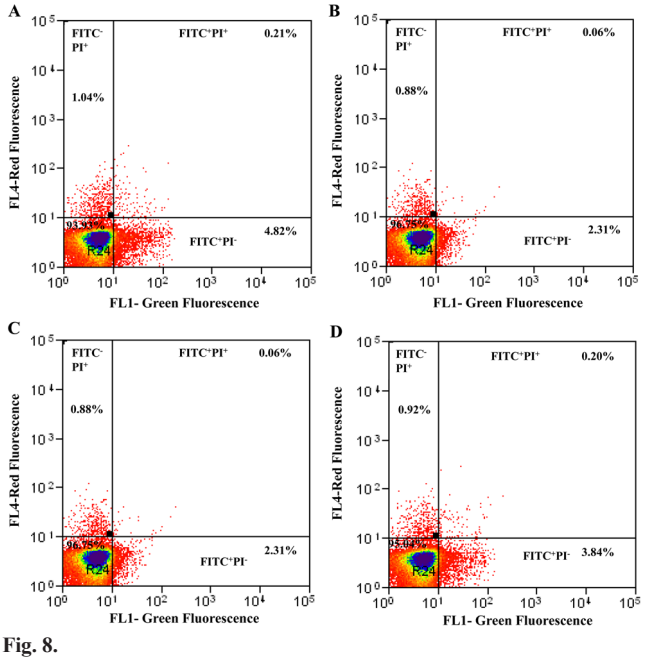

Fig. 8.

Similarly to individual treatments, TCDD and genistein in combination did not change the percentage of live, necrotic, early and late apoptotic granulosa cells after 3–24 h of culture. There are no data regarding the combined effects of TCDD and genistein on apoptosis of ovarian cells. However, genistein diminished apoptotic effects of TCDD on a lymphoblastic T cell line (L-MAT) via inhibition of tyrosine kinases pathways [53]. In addition, genistein inhibited glutamate-induced apoptosis of primary neuronal cell cultures and both estrogen and aryl hydrocarbon receptors were involved in the interaction [54]. It appears that the effects of TCDD and/or genistein on steroid hormone secretion and receptor expression in porcine

Fig. 8. Exemplary dot plots of Annexin V-FITC and propidium iodide double stained granulosa cells isolated from medium porcine follicles and cultured for 24 h. A) control cells; B) cells treated with TCDD; C) cells treated with genistein; and D) cells treated with TCDD and genistein. Following pre-culture (40 h), the cells (2×10^6 cells/ 3 ml Eagle's medium, 5% calf serum) were cultured with TCDD (100 pM) and/or genistein (500 nM) for 24 h. R24: live cells (no fluorescence; $FITC^-/PI^-$); $FITC^+/PI^-$: early apoptotic cells; $FITC^+/PI^+$: late apoptotic cells; $FITC^-/PI^+$: necrotic cells.

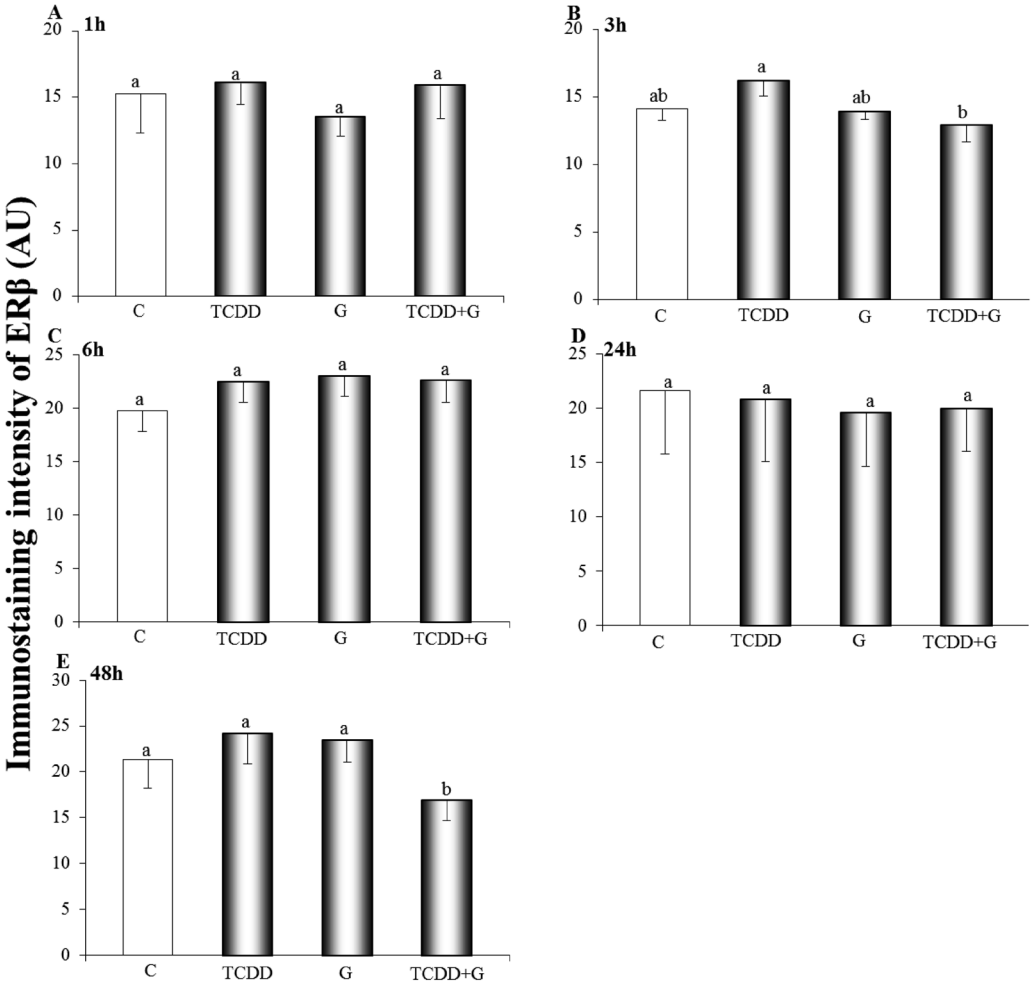

Fig. 6. The effects of TCDD and/or genistein on the intensity of ERβ immunostaining (mean ± SEM) in cultured granulosa cells isolated from porcine medium follicles (n = 4 independent experiments). Following pre-culture (40 h), the cells (1×10^5 cells/ 0.3 ml Eagle's medium, 5% calf serum) were cultured with TCDD (100 pM) and/or genistein (500 nM) for A) one; B) three; C) six; D) 24 and E) 48 h. One-way ANOVA for repeated measurements was followed by the LSD post-hoc test. The same letters over the bars indicate a lack of significant differences between the groups ($P < 0.05$). AU: arbitrary units; ERβ: estrogen receptor β; C: control; G: genistein; TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

granulosa cells are not related to apoptosis.

In summary, we found that a low, environmentally relevant dose of TCDD potentiated the inhibitory effect of similarly relevant dose of genistein on *in vitro* P_4 production by porcine granulosa cells. TCDD and genistein administered together decreased ERβ (48 h) and AhR (24 h) protein expression in the cells in contrast to the individual treatments. Moreover, the inhibitory effect of TCDD on AhR mRNA expression was abolished by genistein (48 h). It should be emphasized that these differences could not be detected if the receptor expression was examined only in one time point, as it is a common practice. It is likely that differences in steroid hormone secretion and receptor protein expression in cells treated with both TCDD and genistein resulted from AhR and ERβ 'cross talk'. Future studies should explore the nature of such cross talk. The results presented in the current paper clearly indicate that effects exerted by low doses of EDCs applied in combination must be taken into consideration when studying potential risk effects of EDCs on biological processes. This is especially important for compounds persistent in the environment such as genistein and TCDD, since the former substance is present in animal diets and the latter accumulates in fat tissue.

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