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Original article

The protein expression disorders of connexins (Cx26, Cx32 and Cx43) and keratin 8 in bovine placenta under the influence of DDT, DDE and PCBs

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Abstract

Polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT) and its metabolite, dichlorodiphenyldichloroethylene (DDE) can disturb the secretory function of the ovary and both contractions and secretory function of the uterus during the estrus cycle and pregnancy. Additionally, PCBs can pass through the placental barrier into allantoic and amniotic fluid. The presence of PCBs in these fluids is associated with higher frequency of spontaneous abortions and premature births in humans and animals. Therefore, the effect of PCBs, DDT and DDE on the connexins (Cx26, Cx32 and Cx43) and keratin 8 (KRT8) expression in bovine placentomes was investigated. The placentome slices from the second trimester of pregnancy were incubated with PCB153, 126, 77, DDT and DDE (each at doses of 1, 10 or 100 ng/ml) for 48 h. Then, the slices were stained using immunohistochemistry. The density of Cxs staining was measured with AxioVision Rel. 4.8 software in fetal-maternal connections and binuclear cells (BNC). None of the tested xenobiotics (XBs) affected the localization of Cxs and KRT8 in the fetal-maternal connection area, but the XBs affected the density of Cxs in fetal-maternal connections and binuclear cells (BNCs). Depend on the doses, in fetal-maternal connections all used PCBs changed the protein expression of different Cxs, while in BNCs, all tested XBs except DDT increased the expression of Cxs. None of investigated XBs affected on KRT8 expression. In summary, used XBs affect the expression of Cxs and change the quantitative relationships between them. Therefore, XBs can unfavorably influence function of the utero-placental barrier in cows.

Key words: connexins expression, KRT8, chlorinated xenobiotics, cotyledons, cow

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Introduction

Polychlorinated biphenyls (PCBs) and pesticide DDT as well as its metabolite DDE are resistant to biodegradation (Borja et al. 2005), and due to their lipophilic properties, they can accumulate in tissues of living organisms (Glynn et al. 2000). These environmental pollutants can act as agonists or antagonists of gonadal steroid hormones (Goldman and Yawetz 1992, Safe 1994, Schrader et al. 2003), influence oxytocin (OT) secretion (Mlynarczuk et al. 2009, Wrobel et al. 2014), and decrease the sensitivity of granulosa and luteal cells to gonadotropins (Mlynarczuk and Kowalik 2013). We observed that these xenobiotics (XBs) in concentrations detected in the reproductive tract (Kamarianos et al. 2003, Mlynarczuk et al. 2005) disturb the secretory function of the ovaries and both contractions and secretory functions of the uterus and oviducts during the estrus cycle and pregnancy (Mlynarczuk et al. 2009, Wrobel et al. 2009, Wrobel et al. 2010). Additionally, the presence of xenobiotics (XBs) in maternal and fetal blood as well as amniotic and allantoic fluid (Hirako et al. 2005) is associated with spontaneous abortions and premature births in humans and animals (Macklin and Ribelin 1971, Korrick et al. 2001, Venners et al. 2005, Pollack et al. 2011). However, other studies demonstrated that chloroorganic XBs can influence gene expression for connexins (Cx26, 32, and 43) in the endometrium and chorion (Heikaus et al. 2003, Wojciechowska et al. 2017ab) which form a utero-placental barrier. Therefore, it is possible that disadvantageous effects of these compounds on pregnancy and fetal development can occur through adverse changes in the expression of proteins to form structures that ensure the proper function of the utero-placental barrier.

Placentomes in ruminants consist of embryo-originated trophectodermal cotyledons and uterine mother-originated endometrial caruncles. In the last trimester of pregnancy, trophoblast binuclear cells (BNCs) begin to migrate and fuse with endometrial cells (Pfarrer et al. 2003). The placenta transforms from a chorionic-epithelial form into a syndesmochorial form (Bjorkman 1954), which can be considered a limited invasion type of placenta. The placental barrier is formed between both parts of the placenta, and its integrity depends on the expression of connexins (Cxs), which contribute to the formation of gap junction connections (Risek and Gilula 1991, Pfarrer et al. 2006). Connexin 26 (Cx26) takes part in the migration of binuclear cells (BNCs) across the fetal-maternal border as well as fusion with maternal cells and in the trophoblast invasion process (Wooding and Whates 1980, Winterhager et al. 1993, Pfarrer et al. 2006). Connexin 32 (Cx32) is responsible for the differentiation of syncytial villi and placental growth (Zhou and Jiang 2014), whereas connexin 43 (Cx43) regulates cell growth (Moorby and Patel 2001) in the maternal part of the placenta (Pfarrer et al. 2006) and is involved in trophoblast invasion during the fusion of BNCs with endometrial cells (Winterhager et al. 1993, Frendo et al. 2003, Pfarrer et al. 2006). Keratin 8 (KRT8) is also responsible for the integrity of the maternal-fetal connections and can be considered a marker of placental barrier function (Jaguemar et al. 2003, Watson 2007).

Therefore, the aim of this study was to test the effects of PCBs, DDT and DDE on the connexins (Cx26, Cx32 and Cx43) and KRT8 protein expression to maintain the placental barrier structure and integrity.

Materials and Methods

Preparation and incubation of placentome sections

Uteri from four cows in the second trimester of pregnancy were collected 15-20 min after slaughter. The period of pregnancy was classified based on morphological observations of the reproductive tract and fetus (Jainudeen and Hafez 1980) and the Keller formula calculation: x(x+2)=length of fetus, where "x" is the gestational age. The uteri were transported to the laboratory within 1 h in ice-cold 0.9% NaCl supplemented with 10 IU/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml amphotericin and 100 μg/ml L-glutamine. The placentomes were cut using a razor blade into 1–2 mm thick slices, and which next were divided into 60–80 mg sections with complete fetal-maternal connections, as described earlier (Wojciechowska et al. 2015). The placentome sections (in three replicates) were incubated in M-199 medium that was supplemented with 2% fetal calf serum (FCS) and 10% amniotic fluid (AF) under a controlled atmosphere (95% O_2 + 5% CO_2) at 37.5°C as described in a previous study (Wojciechowska et al. 2015). The medium without phenol red was enriched with gentamycin (40 mg/ml; KRKA, Novo Mesto, Slovenia) and amphotericin (20 μg/ml; ICN Pharmaceuticals, New York, NY, USA).

After 2 hrs, the preincubation medium was supplemented with PCB 153, 126, 77, DDT and DDE at doses of 1, 10 or 100 ng/ml, respectively. After 48 h of incubation, the placentome sections were placed in 4% paraformaldehyde (PFA) immediately after preparation. We found earlier that placentome sections can maintain the integrity of the placenta after 48 h of incubation in the conditions described above (Wojciechowska et al., 2015). All materials used in this study were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated.

Immunohistochemistry (IHC)

Sections of placentomes preserved in PFA were washed several times in deionized water, dried and placed in paraffin blocks. Next, they were cut into 5-µm thick sections and then incubated at 56-60°C in a humidity chamber for 20-30 min. Subsequently, the sections were dewaxed two times in xylene for 10 min and rinsed for 5 min in alcohol (100%, 96% and 70%). To expose the antigens, the sections were boiled three times for 5 min in citrate buffer (pH=6) and then cooled for 20 min. Next, the slices were rinsed three times for 5 min in TBS buffer (0.05 M Tris-HCl, pH=7.4) and incubated in 10% hydrogen peroxide for 30 min at room temperature, and washed in TBS again. To prevent non-specific binding, the sections were incubated for 60 min with PAV (0.1M phosphate-buffered saline, 0.1% bovine serum albumin and 0.05% thimerosal) containing 10% serum (Abcam, Cambridge, UK), and they were incubated overnight with specific antibodies at 4°C in a dark, humid chamber. The following antibodies for Cxs and KRT8 purchased from Abcam were applied: anti-Cx26 rabbit polyclonal antibody at a 1:500 dilution (ab65969), anti-Cx32 rabbit polyclonal antibody at a 1:40 dilution (ab66631), anti-Cx 43 mouse monoclonal antibody at a 1:300 dilution (ab11369) and an anti-KRT8 mouse monoclonal antibody in a 1:1800 dilution (ab28650). After the sections reached room temperature, they were washed four times in TBS buffer for 5 min and then incubated with secondary antibodies (1:400 dilution, all supplied by Vector Laboratories, Burlingane, CA) for 30 min at 4°C. After washing with TBS buffer, the sections were incubated with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and washed again in TBS. Next, the sections were dried and incubated in DAB solution, tinted with Mayer's hematoxylin, and dehydrated in alcohol and xylene, and closed in DPX mounting medium (Sigma-Aldrich, Poznan, Poland). All materials and reagents for TBS preparation were purchased from Sigma-Aldrich (Poznan, Poland). The sections were observed under 100x and 400x magnification using an Axio Imager Z1 light microscope (Zeiss, Jena, Germany). The relative optical density of Cxs and KRT8 staining was measured in the fetal-maternal connections area and BNCs using AxioVision Rel.4.8 software (Zeiss, Jena, Germany). Briefly, 4 pregnant uteri sections for each of the XBs treatments were prepared. The optical density was calculated based on 5-6 images taken from each of two sections chosen for fixation. In addition, 3-6 stained parts of the section and 3 parts of the background were marked on each of analyzed images, and the results were saved as individual mean values.

Statistical analysis

Statistical analysis of the mean values (± SEM) was performed using one-way ANOVA followed by a Newman-Keuls post-test. Differences ($p<0.05$) between staining intensity of each connexin expression compared to its control value were marked by asterisks, whereas differences ($p<0.05$) of the staining intensity between values in the dose treatment groups were marked by means letters. GraphPad PRISM 6.0 software (Graph Pad Software, San Diego, CA, USA) was used for analysis.

Results

The expression of Cx26 was found mainly in the maternal part of placenta on the contact surface of the placenta portions and in BNCs (Fig. 1A). Intense staining for Cx32 was demonstrated in BNCs and much weaker staining was observed in the stromal layer of the maternal part of the placenta (Fig. 1B). Cx43 protein expression was present only in the stromal layer of the maternal part of the placenta (Fig. 1C), whereas the intense staining for KRT8 occurred mainly at the border of fetal-maternal connections area and in the cytoplasmic membranes of BNCs (Fig. 1D).

The quantitative relationships between Cxs in the control sections of the placentome were similar to each other. The expression of Cx26 and Cx43 protein did not differ significantly ($p>0.05$), whereas there was markedly lower expression ($p<0.05$) of Cx32 (Fig. 2).

PCB 153 at 1 ng/ml increased ($p<0.05$) the expression of Cx26 protein in the fetal-maternal connections area, while the 1 and 100 ng/ml doses increased ($p<0.05$) Cx32 and Cx43 protein expression (Fig. 2A). In BNCs, each of the PCB153 doses stimulated ($p<0.05$) Cx26 and Cx32 protein expression, whereas this XB did not affect ($p>0.05$) Cx43 protein expression (Fig. 3A). Moreover, PCB153 disturbed ($p<0.05$) the Cxs quantitative relationships after treatment with 1 or 100 ng/ml (Fig. 2A) in fetal-maternal connections and after treatment with 100 ng/ml in BNCs (Fig. 3A).

Around maternal-fetal connection area, PCB126 (1 ng/ml) led to increased expression ($p<0.05$) of both Cx32 and, at the 10 ng/ml dose, Cx43. Each of the PCB126 doses changed the ratio ($p<0.05$) of Cxs compared to the control (Fig. 2B). In BNCs, PCB 126 (1 ng/ml) increased ($p<0.05$) only Cx32 protein expression and only in this case, the quantitative relationships of individual Cxs were disturbed ($p<0.05$) compared to the control sections (Fig. 3B).

All doses of PCB 77 stimulated ($p<0.05$) Cx32 expression in the fetal-maternal connections area, but their ratio did not ($P>0.05$) change (Fig. 2C). All PCB

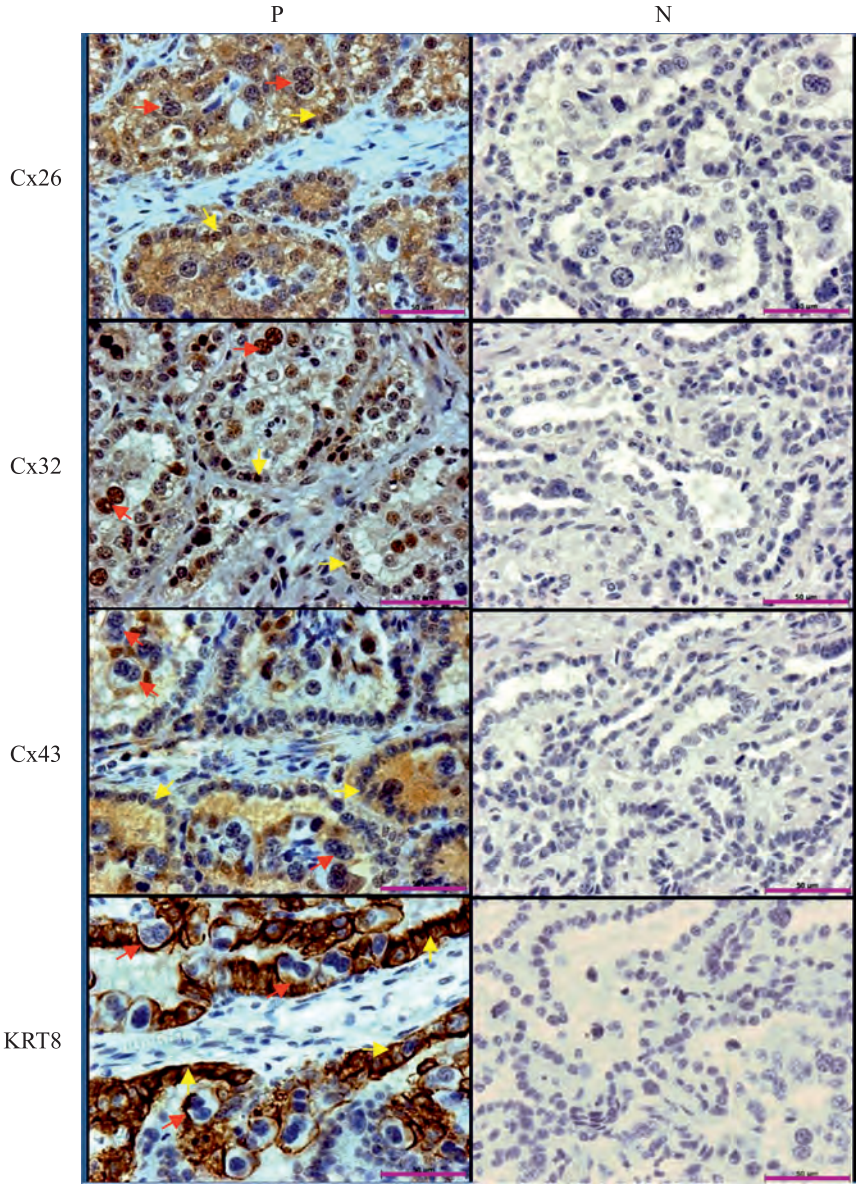

Fig. 1. The immunolocalization of connexin 26 (Cx 26), 32 (Cx 32), 43 (Cx 43) and keratin 8 (KRT8) in non-incubated bovine placentome slices from the second trimester of pregnancy, positive (P) and negative (N) staining. Scale bar = 50 μm. The yellow arrows point fetal-maternal connections area and the red arrows point to BNCs.

77 treatments increased ($p<0.05$) the expression of Cx32 protein in BNCs, and the 10 and 100 ng/ml doses also stimulated Cx26 expression. Despite changes in the expression of individual connexins, their quantitative relationships were not changed by PCB77 (Fig. 3C).

Effects of DDT (1 ng/ml) around maternal-fetal connections resulted in an increase ($p<0.05$) in Cx43 expression and a decrease ($p<0.05$) in Cx26 expression after treatment with 100 ng/ml. These changes simultaneously influenced the ratio ($p<0.05$) of the Cxs in this site (Fig. 2D). In BNCs, this pesticide changes only quantitative relationships ($p<0.05$) of Cxs (Fig. 3D).

Each dose of DDE increased ($p<0.05$) Cx32 and Cx43 protein expression in the maternal-fetal connection area. However, changes in the quantitative relationships of individual Cxs ($p<0.05$) were recorded for doses of 1 and 100 ng/ml (Fig. 2E). In BNCs, this XB increased Cx32 expression ($p<0.05$) at the 1 or 100 ng/ml dose, yet only the lowest dose affected ($p<0.05$) the quantitative relationships for Cxs (Fig. 3E).

The expression of KRT8 was not altered ($p>0.05$) by any XBs used in these experiments (Fig. 4).

Discussion

The XBs investigated in this study not affect the location of individual Cxs in the placental barrier or

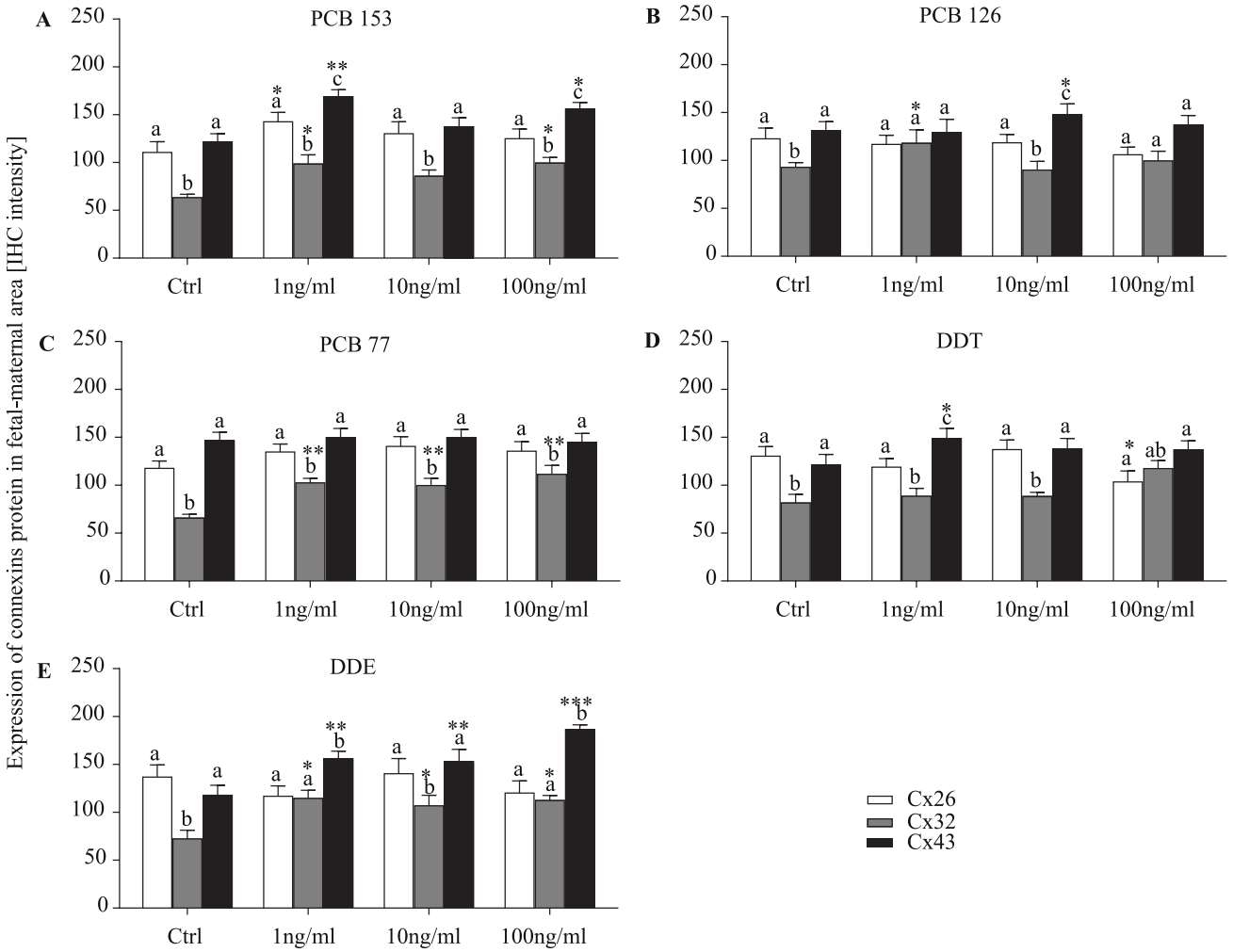

Fig. 2. The expression of Cxs protein in fetal-maternal connections area (±SEM; n=12) after treatment with PCB153 (A), PCB126 (B), PCB77(C), DDT (D) and DDE (E) at doses of 1, 10, 100 ng/ml. The letter above the bars indicates the differences ($p<0.05$) between values in the dose treatment groups while the asterisk (*$p<0.05$, **$p<0.01$, ***$p<0.001$) points to the differences of each connexin expression compared to its control value.

BNCs. In addition, it has been shown that the mutual relationship between different types of Cxs in BNCs and in placental barrier cells is similar to the relationship in control slices. However, changes in the staining intensity in the XBs-treated sections indicate that there is different expression of these proteins in the fetal--maternal connection area. Some of the XBs can mimic or block the effects of E2 and P4, which are involved in Cxs expression (Grümmer et al. 1994, Johnson et al. 2016). Therefore, increased expression of Cx 32 and Cx43 under the influence of PCBs 153 and 77 may be associated with the estrogen-like effects of both congeners (Danse et al. 1997, DeLisle et al. 2001). However, this outcome does not explain the effects of DDT and DDE, which have estrogen-like properties that are less evident. Previous studies (Grümmer et al. 1999) have shown that the ratio of E2 to P4 is an important factor affecting the expression of *Cx26* and *Cx43* genes in the area of fetal-maternal connections. DDT and DDE may stimulate P4 secretion without a simultaneous effect on E2 secretion from the chorion (Wojciechowska et al. 2017), which could change E2:P4 ratio in favor of P4. This effect would explain the increase of Cx43 expression and decline of Cx26 in chorion slices treated with the highest doses of DDT and DDE.

The expression of Cxs is also affected by prostaglandin PGF2α (Xu et al. 2015), and both endometrial and chorionic cells have receptors for this prostaglandin (Slama et al. 1994, Ulbrich et al. 2009). It has also been shown that all XBs used in this study may increase the secretion of PGF2α from endometrial cells (Wrobel et al. 2009, Wrobel et al. 2010). Therefore, PGF2α could affect the expression of Cxs in fetal-maternal connections area in either an autocrine or paracrine manner. It indicates that even XBs that do not have estradiol-like activity like PCB126, DDT or DDE, may disturb the mutual relationship of Cxs. It is important to note that chorionic villi have a significantly lower

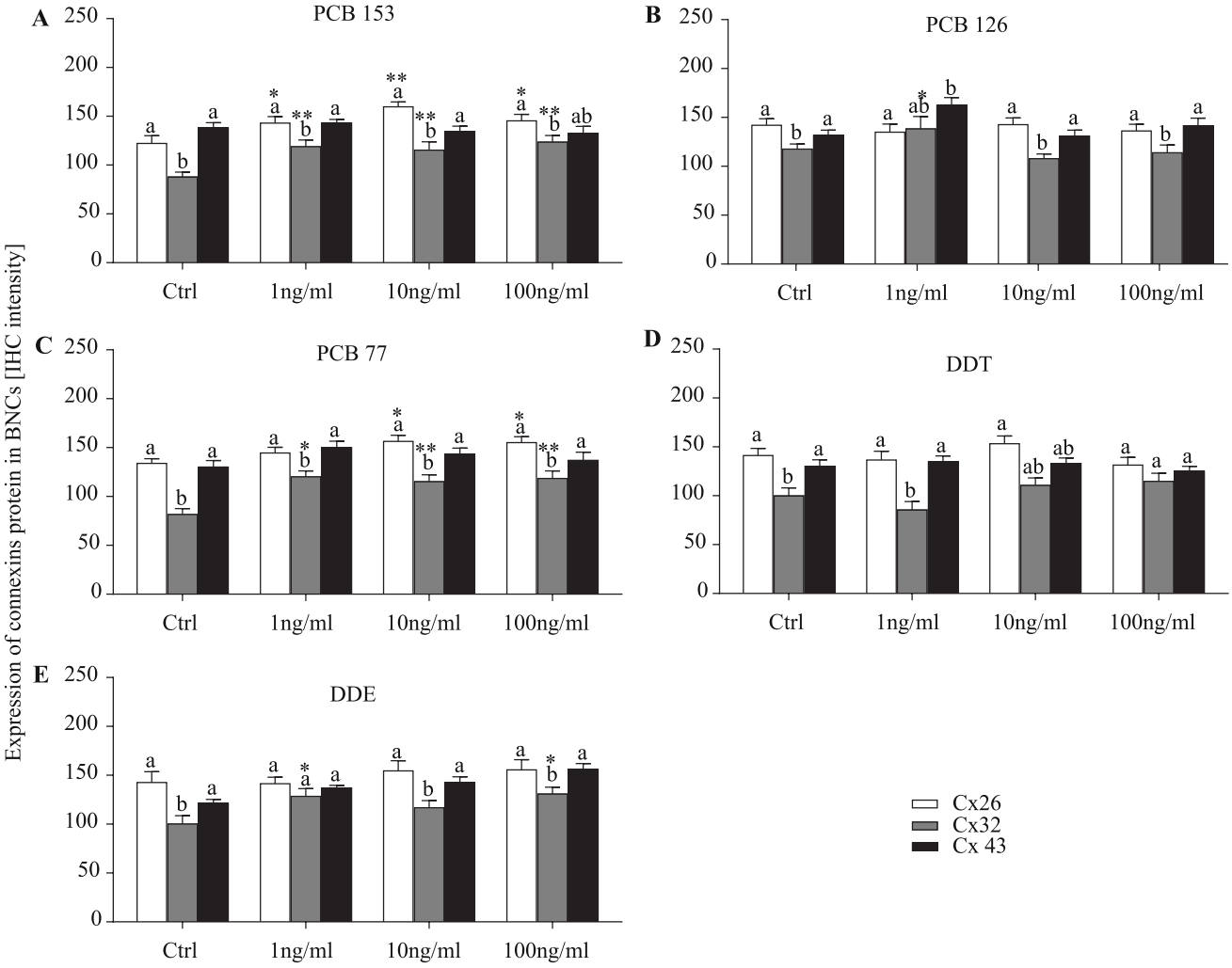

Fig. 3. The expression of Cxs protein in BNC cells (±SEM; n=12) after treatment with PCB153 (A), PCB126 (B), PCB77(C), DDT (D) and DDE (E) at doses of 1, 10, 100 ng/ml. The letter above the bars points the differences ($p<0.05$) between values in the dose treatment group while the asterisk (*$p<0.05$, **$p<0.01$, ***$p<0.001$) points to the differences of each connexin expression compared to its control value.

ability to metabolize prostaglandins compared to that of uterine tissues. This reduced ability increases the susceptibility of villi on to PGF2α influence. Unfortunately, changes in Cxs expression in BNCs are difficult to explain because there are no data that indicate these cells can secrete prostaglandins or have specific receptors for them. Present study has revealed that BNCs show lower expression of Cxs after XB treatment compared to other trophoblast cells. These differences may be an effect of the lower expression of steroid hormone receptors in BNCs (Schuler et al. 2008) compared to that in the endometrium or chorion cells.

We assume that the changes in Cxs expression induced by XBs are the result of their influence on the mRNA expression of *Cx* genes. Discrepancies in mRNA expression and protein levels for individual Cxs observed earlier (Wojciechowska et al. 2017a, Wojciechowska et al. 2017b) and found in the present study can be evoked by a different rate of posttranslational and posttranscriptional processes of Cxs in the fetal-maternal connections area (Grümmer et al. 1999, Salat-Canela et al. 2015).

There was no effect of the XBs on the expression of the KRT8 protein, which confirms that the XBs investigated did not significantly influence both gene expression and post-translational changes of this protein, as found previously (Wojciechowska et al. 2017b).

Disorders in Cxs expression may adversely affect direct communication between cells through gap junctions in fetal-maternal connections area, which significantly disrupts the regulation of physiological functions in placenta cells (Meşe et al. 2007, Johnson et al. 2016). Furthermore, disturbances in Cxs expression may affect a migration and fusion of BNCs to endometrial cells. Thus, the barrier function of the placenta and its development, especially the transformation of the placenta

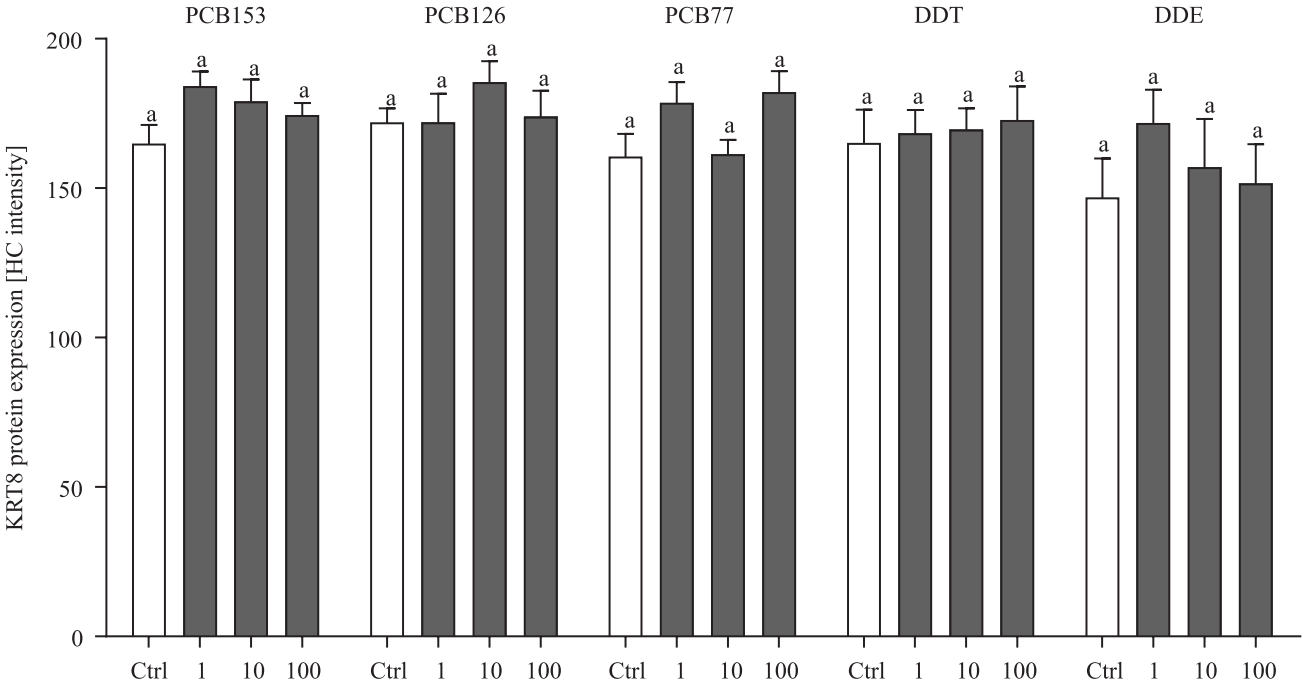

Fig. 4. The mean (±SEM; n=12) protein expression of keratin 8 (KRT8) in the fetal-maternal connection area of bovine placentome sections after incubation with XBs (1, 10 or 100 ng/ml).

from epitheliochorial to syndesmochorial form, during third trimester of bovine pregnancy (Winterhager et al. 1996), may be impaired. By this way, changes in Cx 26 and 43 expression may disturb nutrient and metabolite exchange processes in placentomes (Gabriel et al. 1998, Liao et al. 2001), which impairs fetal development (Zhang et al. 2014). The presence of these xenobiotics in tissues of pregnant females correlated with the neonatal low birth weight (Casas et al. 2017, Bloom et al. 2017) supports this view.

The results obtained in this study show that chloroorganic XBs affect the expression of Cxs in fetal-maternal connections area of the placenta and may impair its barrier-function.

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