Co-expression of the aryl hydrocarbon receptor and estrogen receptor in the developing teeth of rat offspring after rat mothers' exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and the protective action of α -tocopherol and acetylsalicylic acid

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Conflict of interest

None declared

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Abstract

Background. Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can cause adverse effects in many organs. Toxic effects are caused due to the formation of a TCDD complex with the cytoplasmatic aryl hydrocarbon receptor (AhR), whose mechanism of action is similar to that of the estrogen receptor (ER). Some substances, including α -tocopherol (E) and acetylsalicylic acid (ASA), can reduce the toxic effects of TCDD in offspring.

Objectives. The objective of this study was to evaluate the co-expression of AhR and ER in the incisors of rat offspring whose mothers were exposed to TCDD, using immunohistochemical and histological techniques. Moreover, the possible protective role of E and ASA was investigated.

Material and methods. Four groups of 2-day-old rat offspring, whose mothers were intoxicated by TCDD before mating, were established: control group (C), TCDD group, TCDD+E group and TCDD+ASA group.

Results. In the TCDD group, there was an increase in ER expression and a decrease in AhR expression in comparison with the C group. In the TCDD+E and TCDD+ASA groups, there was a weak or negative ER expression and slightly stronger expression of AhR than in the TCDD group.

Conclusions. The co-expression of AhR and ER during tooth development suggests the role of AhR and ER in the control of this process. Both receptors are also involved in the process of detoxification of TCDD. The increase in AhR in TCDD+E and TCDD+ASA groups indicate a preventive action of antioxidant and anti-inflammatory pharmaceuticals, which may limit negative effects of TCDD.

Key words: rat, estrogen receptor, tooth, dioxin, aryl hydrocarbon receptor

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Introduction

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the strongest dioxins, which is why exposure to it may induce severe malfunctions of many organs.^{1,2} Our study and those of other authors showed that TCDD induces inflammatory processes and activates the processes of enzymatic digestion of connective tissue elements.^{3,4} On the other hand, dioxins inhibit alkaline phosphatase in osteoblasts, thus disturbing the mineralization process.^{5,6} Moreover, TCDD inhibits collagen I synthesis by altering gene expression and induces oxidative stress, leading to the activation of interleukins, which further activate osteoclastogenesis.^{5,7} Disturbed fiber synthesis of type I collagen may result in a defective structure of dentin and cementum as well as the bone. The dioxin may influence the structure of teeth and the bone, especially during their development. In our previous studies, we found that there is a transmission of toxic effects of dioxins to the offspring of mothers who were exposed to TCDD.⁴ Other authors reported that dioxins may alter calcium deposition in mineralized tissues by influencing the activity of some hormones (estrogens, corticosterone, T3).^{8–11}

The aryl hydrocarbon receptor (AhR) plays a key role in the pathomechanism of TCDD-induced intoxication.^{12–19} Apart from the classical response to xenobiotic exposure, this receptor is also involved in diverse endogenous processes, such as cell proliferation, T cell differentiation, hematopoietic stem cell expansion/differentiation, lung response to polycyclic aromatic hydrocarbons, and glucose tolerance via mechanisms that are not clearly defined for the most part.^{18,19}

To prevent the expansion of the inflammation process caused by biotransformation of TCDD, numerous pharmaceuticals have been used, i.e., α -tocopherol (E), a powerful antioxidant antagonist of AhR,^{7,12,17,20–22} and acetylsalicylic acid (ASA).^{8,9,21}

During development, estrogen controls numerous processes, including cell proliferation and differentiation via estrogen receptors (ER). Subsequently to hormone binding and transformation, receptor-ligand complexes interact with specific hormone response elements on target genes, which results in the regulation of transcription.²³

Two classes of ER exist: nuclear estrogen receptors (ER α and ER β), which are members of the nuclear receptor family of intracellular receptors, and membrane ERs, which are mostly G protein-coupled receptors. Estrogen receptors are regarded to be cytoplasmic receptors in their unliganded state; however, immunocytochemical research has shown that only a small fraction of ERs reside in the cytoplasm, while most ERs are found constitutively in the nucleus. Estrogen receptors have been reported to occur in fish, lizards and several mammals, including humans.²³

Both ERs are widely expressed in different tissue types; however, there are several differences in their expression patterns. A wide distribution of ER α immunoreaction has been reported in fibroblasts, endothelial cells, mammary gland cells, ameloblasts, odontoblasts, chondrocytes, osteoblasts, and osteoclasts in addition to female reproductive tissues, which suggests that there is a diverse effect of estrogen on development, growth, and homeostasis.^{23,24}

Estrogens play an important role during tooth development and its survivability in the surrounding tissues.^{25,26} Alhodhodi et al. suggest that estrogens stimulate osteogenic differentiation and that this action is mediated mainly through the ER β isoform. These findings may have profound consequences in terms of the investigation and treatment of oral pathologies, which are associated with imbalances in estrogen concentrations.^{24,26} Nebel proved that estrogen affects chemokine expression in periodontal cells, which shows a complex pattern involving downregulation as well as upregulation of chemokines. Estrogen exerts both anti-inflammatory and proinflammatory effects through these mechanisms.^{22,27} Therefore, the loss of the ER β function during development may have an impact on periodontal diseases and tooth decay.²⁸

Enamel formation and its subsequent maturation to become the hardest tissue in mammals are closely related to the differentiation of ameloblasts and morphological changes in them.^{23,25} From the apical to the incisal end, rat incisor ameloblasts are classified regionally into presecretory, secretory, transitional, maturation, pigmentation, and reduced ameloblasts according to their maturation level.^{23,25} Immunohistochemistry of the rat enamel organ revealed ER α expression as nuclear localization in ameloblasts as well as in mature and immature odontoblasts. The ER α pattern exhibited a periodic change at the maturation stage coherent with constant higher labeling in ruffle-ended ameloblasts than in smooth-ended ameloblasts. These findings suggest a possible role of ER α in ameloblast proliferation and differentiation.²³ In addition, Jukic et al. reported that several odontoblasts in humans exhibited ER α immunoreaction (1.0–1.3%), which suggests that tooth-forming cells are considered to be a target for estrogen action. To our knowledge, there has been no report on the expression of ER α in the cytoplasm in rat ameloblasts and associated structures.²⁹ There are no reports concerning TCDD-influence on tooth development and the co-expression of AhR and ER, and the protective role of chosen anti-inflammatory pharmaceuticals.

The aim of this study was to identify AhR and ER co-expression in the developing incisors of rats, whose mothers were exposed to TCDD and treated with ASA or E. Additionally, it was evaluated whether there are possibilities of reducing potential post-dioxin defects in the structure of enamel and dentin in the intoxicated mothers' offspring by simultaneous application of α -tocopherol or ASA.

Material and methods

To conduct the experiment, 40 offspring from 24 female Buffalo rats (with body mass 130–150 g, at the age of 9–11 weeks) were used. The study was approved by the Local Ethical Committee on Animal Testing (permission No. 38/2009).

All female rats were kept in polystyrene cages (60 cm × 40 cm × 40 cm) with metal lids (6 animals per cage). The experiments were carried out under standard conditions. The rats were fed with standard Labofeed H feed and received water ad libitum.

The experimental animals were divided into 4 groups of 6 females, from which infants for investigation were obtained:

- control group (C)
- TCDD group – females, 3 weeks before mating, into which 5 µg/kg b.w. of TCDD (Greyhound Chromatography and Allied Chemicals, Birkenhead, UK) was injected intramuscularly.
- TCDD+E group – females, 3 weeks before mating, to which TCDD was administered as in the TCDD group and into which, additionally, α-tocopherol acetate (Hasco-Lek SA, Wrocław, Poland) at a dose of 30 mg/kg b.w. s.c. was injected every day for 3 weeks
- TCDD+ASA group – females, also 3 weeks before mating, in the case of which the TCDD injection pattern was the same as in the TCDD+E group and to which ASA (Bayer Polska, Warszawa, Poland) at a dose of 50 mg/kg b.w. p.o. was administered instead of α-tocopherol.

The females from all groups were mated with randomly chosen males from the same strain that were not exposed to any chemical substances. After birth, 2-day old infants were euthanatized with Phenobarbital (Morbital®; Bio-wet, Puławy, Poland) and mandibles from 10 infant rats from each of the 4 groups were taken for analysis. Samples of incisors and the periodontal tissue were placed in a 4% buffered formalin solution for 48 h. Next, the material was rinsed in tap water and decalcified in ethylenediaminetetraacetic acid (EDTA), dehydrated in alcohol series, cleared in methyl benzoate, and embedded in paraffin.

For immunocytochemical analysis, serial sections 7–9 µm thick were cut, deparaffinized in xylene and rehydrated in alcohol series. For each of the antibodies, a minimum of 3 slides were used. Negative and positive probe was performed. Endogenous peroxidase activity was blocked with Peroxidase Blocking Reagent (DAKO, Gdynia, Poland). Then, the sections were rinsed twice for 5 min in distilled water. Afterwards, the sections were digested with proteinase K (DAKO) and rinsed again twice for 5 min in distilled water. The sections were incubated with rabbit Anti Human AhR primary antibody (Serotec, Kidlington, UK) or Anti-Estrogen Receptor antibody, rabbit monoclonal antibody (Sigma-Aldrich, Dorset, UK) for 1 h in a dilution of 1:80; then, the sections were rinsed twice in phosphate-buffered

saline solution (PBS) solution (pH 7.3) for 5 min. Later, the Novolink MinPolymer DS Novocastra (Leica Biosystems, Wetzlar, Germany) kit was used. The next stage involved staining with DAB+ substrate buffer and DAB+ chromagen (DAKO) visualization system; Mayer's hematoxylin (Merck, Darmstadt, Germany) was used to stain cell nuclei. The material was analyzed with a Nikon Eclipse 80i microscope (Nikon Corp., Minato, Tokyo, Japan).

In all subjects from each study group, the degree of expression of a given receptor was analyzed in 100 cells in at least 3 fields of view. Each individual cell received a numerical value 0–3 corresponding to the intensity of the reaction. Next, the average value for each of the studied groups was calculated. The statistical analysis of the obtained data of individual groups was performed with STATISTICA v. 12.0 (StatSoft Inc., Tulsa, USA) using the Student's test.

Results

In the C group, a differentiation process of the enamel organ was observed at the apex of the incisor. The cells are arranged in a columnar epithelium, the cells of which proliferate, differentiate and secrete enamel and dentin. At this stage, both enamel and dentin are not mineralized. The amount of the tooth matrix is slowly reduced towards the neck and root as well as the number of cells, which becomes undifferentiated (Fig. 1). The expression of the ER in the cytoplasm in odontoblasts, ameloblasts, cementoblasts, and osteoblasts is very weak or completely negative in the enamel organ (Fig. 1). A positive reaction for AhR was noted in the majority of cells of the developing teeth (Fig. 2). A clearly positive immunohistochemical reaction to AhR was noted in the inner enamel epithelium, whereas this reaction is weaker on the outer surface. A weaker reaction was noted in odontoblasts compared to ameloblasts. Aryl hydrocarbon receptor was expressed at different levels in the pulp, alveolar bone and connective tissue surrounding the teeth (Fig. 3).

In the TCDD group, the development of enamel and dentin was retarded. Ameloblasts were mostly cuboidal, rarely cylindrical. Odontoblasts were in close relation with mesenchymal cells of the pulp. The amount of synthesized dentin and enamel was twice as small as in the C group. An increased expression of the ER was mainly observed in odontoblasts and ameloblasts, but also in mesenchymal cells. The main localization of the ER was in the cytoplasm. This expression was noted in ameloblasts in the odontoblastic layer of the papilla (Fig. 1). In this group, a much weaker immunohistochemical reaction to AhR has been observed in ameloblasts (Fig. 2). In less differentiated ameloblasts, AhR is rarely present. The immunohistochemical reaction in the mesenchymal cells area is also weaker. The immunohistochemical reaction to AhR is primarily seen in cells of epithelial origin in the cytoplasm (Fig. 3).

Fig. 1. Expression of ER in rat incisors in respective groups

A – control group; the apical portion of rat incisor; weak positive reaction to estrogen receptor (ER) (arrow) in odontoblasts (Od); note the lack of ameloblasts on enamel (letter "E") (×600 magnification); B – TCDD group; the lateral portion of the tooth; intensive synthesis of enamel by cylindrical, ER-positive in nucleus (arrow) ameloblasts (Am); dental pulp (DP) contains numerous capillary vessels (×600 magnification); C – TCDD+E group; very weak positive reaction to ER in cuboidal Am and cylindrical Od in area adjacent to the apical portion of the tooth; between layers of Am and Od cells thin layer on enamel (arrow) is visible (×600 magnification); D – TCDD+ASA group; numerous Am on the apical part of the tooth are ER-negative in cytoplasm; single cells in this region are ER-positive in nucleus (arrow); DP and surrounding mesenchyme are less developed than the C group (×600 magnification).

In the TCDD+E group, the histological image of developing incisors is similar to the image seen in the TCDD group. However, ameloblasts become mostly cylindrical. There are fewer cells at the differentiation stage (cuboidal) (Fig. 1). A weak or negative reaction to AhR in the enamel organ was observed (Fig. 2). In the apical and lateral area, the immunohistochemical reaction to the ER was very weak. No ER presence has been observed in the area of dental sac and alveolar bone in the cytoplasm. There was a weak positive reaction in the enamel organ; however, it was a little stronger than in the TCDD group (Fig. 1 and 3).

In the TCDD+ASA group, the effects of developmental disorders in comparison to the TCDD group could be observed. Ameloblasts are mostly cylindrical, but an increased proliferation of odontoblasts is observed. Moreover, numerous cells were polarized, which means that they were more mature than the incisors from C

and TCDD groups (Fig. 1). In the enamel organ, the immunohistochemical reaction to AhR and ER was similar to that of the TCDD+E group and was noted in both cytoplasm and the nucleus. A weak immunohistochemical reaction to the ER was observed in odontoblasts and mesenchymal cells adjacent to the pulp (Fig. 1). A positive immunohistochemical reaction to AhR was observed in odontoblasts and mesenchymal cells in the cytoplasm and nucleus (Fig. 2,3).

Discussion

Expression of AhR at different tooth development stages in mice has been precisely described by Sahlberg et al. and Partanen et al.^{15,30} In 14-day-old embryos, AhR expression in incisors, epithelium and osteoblasts was observed,

Fig. 2. AhR expression in rat incisors in respective groups

A – control group; the apical portion of rat incisor; strong positive reaction to aryl hydrocarbon receptor (AhR) in nucleus and cytoplasm of odontoblasts (Od) and ameloblasts (Am) ($\times 600$ magnification); B – TCDD group; the lateral portion of the tooth next to the apical part of the rat incisor; intensive proliferation of enamel by cylindrical, AhR negative (arrow) Am; Od show weak positive reaction to AhR ($\times 600$ magnification); C – TCDD+E group; positive reaction (arrow) to AhR in Am and Od in the apical portion of the tooth ($\times 600$ magnification); D – TCDD+ASA group; numerous AhR-positive in cytoplasm Am on the lateral part of the tooth; single cells in this region are AhR-positive in nucleus (arrow); enamel (letter "E") is already mineralized ($\times 600$ magnification).

and no AhR expression was observed in the buds of molar teeth. At the bell stage, on the 17th day of embryonic life, the expression of this receptor was visible in the inner enamel epithelium, whereas between the 19th day of embryonic life and the 12th day of ontogenesis – in line with odontoblastic differentiation and dentin formation – AhR expression was observed in preameloblasts and odontoblasts (only after terminating differentiation from mesenchymal tissue). Moreover, it has been found that AhR expression is more intense in ameloblasts and odontoblasts than in the intermediate stratum of the enamel organ or osteoblasts. This changeable expression of AhR in ameloblasts and odontoblasts, which depends on the stage of tooth development, was observed in healthy animals.⁹

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin stimulates inflammation and, thus, reduces the content of collagen fibers in tissues and as a result influences tooth development.

Consequently, inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) are released; therefore, fibroblast activity is inhibited and osteoclasts are activated.^{3,6,31}

During our own research, the inhibition of a negative impact of TCDD on the dental organ in offspring from the TCDD+E group have correlated with an increased AhR expression after administration of E, especially in the layer of odontoblastic and amelogenic cells compared to the offspring of mothers that were exposed to the dioxin only.^{6,30} In rat newborns whose mothers were exposed to TCDD, a positive reaction to AhR was present mainly in epithelial cells but it was rare and of low intensity. This means that AhR, which is present in other areas of the dental organ, is blocked by trace amounts of TCDD in newborn organisms. On the basis of studies on mice genetically deprived of AhR, it has been proved that the lack of this

receptor significantly decreases the effects of dioxins, since the TCDD main pathway is through AhR.²² According to Gao et al., the administration of TCDD to developing rat newborns reduced the reaction to AhR in ameloblasts to a larger extent than in differentiating dental papilla cells.¹⁰ The observed range of AhR expression in ameloblasts and odontoblasts, demonstrated in our own and cited studies, may result from the difference in offspring age (2-day-old newborns – in case of our own studies, and 9- and 22-day-old in the study by Gao et al.¹⁰), as well as from the applied TCDD dose (5 µg/kg. b.w. – in case of our own studies and 50 µg/kg. b.w. and 1000 µg/kg b.w. – in the study by Gao et al.¹⁰). Moreover, it is possible that TCDD attached to AhR was transported into the nucleus. As a result, products of AhR-dependent genes suppress the expression of AhR.

A 3-week administration of E to the dams, from the moment of TCDD exposure to their pregnancy, was supposed to reduce the toxic activity of free radicals produced by the dioxin by inhibiting AhR in a direct and indirect

way.^{12,17,22,31} Other authors also demonstrated a positive influence of E on animals exposed to TCDD.²² The administration of E to the dams decreases the concentration of TCDD in the serum.^{31,32} Therefore, a smaller amount of this compound or end products is transferred to the fetus via the placenta and results in a higher expression of AhR in offspring in the TCDD+E group. The administration of ASA and E to the dams exposed to TCDD in our studies led to the observed immunohistochemical reaction to AhR present in TCDD+E and TCDD+ASA groups, which was stronger than in the TCDD group. Therefore, the supplementation with E or ASA administered to mothers intoxicated with dioxins before pregnancy could limit the number of defects in developing teeth in newborns.

Several developmental and experimental studies have shown the involvement of a steroid hormone including estrogen in dental tissues.^{23,29,33} Osteoblastic activity has been reported to affect hard tissue formation via the activation of its specific ERs.²⁹ This evidence strongly

Fig. 3. Average value of aryl hydrocarbon receptor (AhR) and estrogen receptor (ER) expression level in odontoblasts and ameloblasts

* – statistically significant difference at $p < 0.05$ to control group; + – statistically significant difference at $p < 0.05$ to TCDD group.

suggests that hard tissue-forming cells are under the control of estrogen.²³

Ohtake et al. and Martin et al. studied a dioxin-estrogen-related action in cultured breast and uterine cancer cells.^{34,35} The activated ERs dock to control regions of target genes and trigger their transcription, thus initiating a cascade of molecular and cellular events instead of AhR. The same estrogenic actions of dioxins were observed when researchers injected TCDD into ovariectomized mice, which had no circulating estrogens. The AhR-ligand complex was also found to repress the ER function when estrogens are present and bound to the receptors, thus providing an explanation for previous reports of anti-estrogenic activities of dioxins.^{29,36,37}

In offspring, at the moment of birth, the level of estrogen is minimal regardless of the sex of individuals. Along with the age, the level of estrogen increases to approx. 25 pg/mL in the 8th week in females, whereas it shows the same pattern between birth and puberty in males as it does in female littermates.^{29,36,37}

Therefore, in the absence of estrogens, an increased expression of ER within the cytoplasm in TCDD-treated animals may be explained by a permanent activation of ER synthesis by TCDD degradation end products or inhibition of its transfer to the nucleus.^{36,37}

We have also found that the increased expression of ER was noted in the TCDD group, whereas the expression of AhR decreased. An increased level of ER, as an alternative pathway in the anti-inflammatory process, may be a part of the cellular self-defense against TCDD biotransformation products.³⁸

Our findings demonstrate the localization and pattern of distribution of ER immunoreactions in rat ameloblasts and odontoblasts. Estrogen receptors and AhR were differently expressed in the C group and in the TCDD group. When considered together, these findings suggest a possible overlapping role of AhR and ER in the proliferation and differentiation of ameloblasts. However, a detailed mechanism of the involvement of estrogen in enamel formation including synthesis and secretion of an enamel protein remains unclear. Further research is required to clarify this issue.²³

Conclusions

The observed co-expression of AhR and ER in the cell cytoplasm during development suggests the role of AhR and ER in the control of cell proliferation, differentiation and apoptosis. Both receptors are also involved in the process of detoxification of TCDD. The increase in AhR in TCDD+E and TCDD+ASA groups indicates a preventive action of antioxidant and anti-inflammatory pharmaceuticals, which may limit negative effects caused by TCDD.

The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin delays embryonic development as a consequence of suppressing the AhR

synthesis and through the modulation of expression of estrogen-dependent genes. Moreover, in the TCDD group, ameloblasts and odontoblasts were at an early proliferative stage; therefore, ER was still present in the cytoplasm. The cells from TCDD+E and TCDD+ASA groups did not express ER.

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