SEX DEPENDENT ACTION OF AROCLOR 1254 ON BASAL AND sGnRHa-STIMULATED SECRETION OF LH FROM THE PITUITARY CELLS OF COMMON CARP, *CYPRINUS CARPIO* L.*

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Abstract

Polychlorinated biphenyls (PCBs) affect the hypothalamic-pituitary-gonadal axis in many vertebrates, changing the hormonal regulation of reproduction. To identify one of the possible sites of action of PCBs on gonadotropin release in common carp, the direct effects of Aroclor 1254 on luteinizing hormone (LH) secretion from dispersed pituitary cells were investigated. Pituitary cells were obtained from sexually mature male and female common carp (*Cyprinus carpio* L.) at the time of natural spawning. The cells were incubated with different concentrations of Aroclor 1254 (5, 10, 50 and 100 ng mL⁻¹ medium) and/or salmon gonadotropin-releasing hormone analogue (sGnRHa) at a concentration of 10⁻⁸ M. LH levels were measured in the cultured medium by the ELISA method after 10 hours of cell incubation. Incubation of male pituitary cells in the presence of tested concentrations of Aroclor did not change the basal LH secretion to the media. In the female pituitary cell incubations Aroclor (5, 10 and 100 ng mL⁻¹ medium) caused a significant increase in LH concentrations in comparison to control incubations. In the case of sGnRHa-stimulated LH secretion in incubations of cells of both sexes, all the concentrations of Aroclor significantly stimulated LH release and potentiated stimulatory effects of sGnRH analogue. These results indicate that endocrine disrupters, such as Aroclor 1254, may affect reproduction in fish, acting also directly on gonadotrophs at the level of the pituitary gland, changing LH secretion.

Key words: polychlorinated biphenyls, fish, cyprinids, gonadotropin releasing hormone, luteinizing hormone

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Gonadotropin secretion in fish is regulated by many endogenous factors, acting at all levels of the hypothalamo-pituitary-gonadal axis. Among them, the most extensively investigated are brain neurohormones: gonadoliberein (GnRH), dopamine and gonadal steroids, which regulate the production and release of gonadotropins in different phases of gonad maturation. All levels of the axis are subjected to the influence of environmental factors, some of which are considered to be endocrine-disrupting chemicals. Many of them, like polychlorinated biphenyls, present in an increasing amount in a polluted environment, may accumulate in organisms living in water (Van Geest et al., 2011). They cause reproductive dysfunctions in vertebrates (Gregoraszcuk et al., 2005; Andric et al., 2006) and in different fish species they cause permanent structural damage to the gonads (Garcia et al., 2016) or affect the secretion of endogenous hormones regulating reproduction (Khan and Thomas, 1997, 2001, 2006; Khan et al., 2001; Thomas and Doughty, 2004; Benninghoff and Thomas, 2005; Socha et al., 2013 a). The site of their action probably includes all the glands on the hypothalamo-pituitary-gonadal axis. Nevertheless, the results of *in vivo* experiments demonstrating the influence of PCBs on gonadotropin secretion in mammals and in fish do not allow it to be stated whether PCB action on gonadotropin secretion is caused mainly or exclusively by a disturbance to the hypothalamic neurohormones controlling gonadotropic cell secretion or additionally by direct action on gonadotropic cells.

Most of the data demonstrated that exposing fish to PCB lowers the maturation indexes of the GnRH-LH system during the gonadal recrudescence phase: Aroclor 1254-induced disruption of LH secretion in Atlantic croaker (Khan et al., 2001), and in Nile tilapia (*Oreochromis niloticus*) and Atlantic croaker (*Micropogonias undulatus*) it decreased the basal and GnRH-a-stimulated LH secretion (Khan and Thomas, 1997, 2001, 2006; Coimbra and Reis-Henriques, 2007). In contrast, Jansen et al. (1993) demonstrated that cultured anterior pituitary cells of female rats exposed to varying concentrations of Aroclor 1242 (1 to 50 ppm) increased basal and GnRH-stimulated gonadotropin secretion. These results were the first demonstration that PCBs can directly affect basal LH and FSH secretion from gonadotropic cells and also alter pituitary responsiveness to gonadoliberein. The potentiation of pituitary responsiveness to the PCB mixture (Aroclor 1242) observed in the latter study was similar to the E2 effect (the *in vivo* experiments in the study demonstrated the estrogenicity of Aroclor 1242 and several ortho-substituted PCB congeners). Despite the fact that the authors suggest that Aroclor 1242 may affect pituitary LH production and release via mechanisms unrelated to its estrogenic action, their conclusion is that Aroclor 1242 acts as an estrogen to affect the pituitary gonadotropin release.

Little information is available regarding a possible direct influence of PCBs on pituitary function. Brandt (1977) reported the accumulation of certain PCBs within the pituitary, which suggests that this may be a site of PCB action and this phenomenon could explain some of the disorders in reproductive functions. Raggi et al. (2016) showed that Aroclor 1254 induced the apoptosis of pituitary cells as well as the effector caspase-3 activity and expression.

The aim of this work was to examine the direct effects of Aroclor 1254 (PCB) on the basal and sGnRH-analogue-stimulated release of gonadotropin LH from common carp male and female pituitary dispersed cells in an *in vitro* culture.

Material and methods

The experiment was conducted on sexually mature common carp (*Cyprinus carpio* L.) in June. Pituitaries were obtained from 8 females (the mean body weight 2.20 ± 0.40 kg) and 10 males (the mean body weight 1.52 ± 0.20 kg) netted from outdoor ponds at the Fishery Station belonging to the Department of Ichthyobiology and Fisheries, University of Agriculture in Kraków, Poland. Gonad maturity was specified as a percentage of body weight (gonadosomatic index GSI), which was $21.3 \pm 1.84\%$ for female gonads and $4.3 \pm 0.87\%$ for male gonads. The enzymatic dispersion of the pituitary glands was performed according to the method described by Mikolajczyk et al. (1990). In brief, collected pituitary glands (separate pools of female and male pituitaries) were chopped into small pieces and subjected to dispersion for 8 hours at 20°C in a medium containing 0.1% (w/v) collagenase H (Boehringer Mannheim, Germany) and 1% BSA – bovine serum albumin (Sigma-Aldrich, USA). After enzymatic dispersion, the cells were harvested by 10 min centrifugation ($200 \times g$ at 20°C) and washed twice with a pre-incubation medium containing 2% Ultrosor SF (Sepracor S.A., France) and 1% antibiotic-antimycotic (Sigma-Aldrich, USA). A cell viability test was performed after enzymatic dispersion with the use of 20 μL of pituitary cell suspension treated for 5 minutes with 0.4% trypan blue. Unstained (viable) and stained (nonviable) cells were counted on a Thoma haemocytometer. The percentage of viable cells was 93 and 91 for female and male pituitaries, respectively.

Cells were resuspended in the pre-incubation medium and transferred into four 96-well microplates (Nunc A/S, Denmark) coated with Poly-L-lysine (Sigma-Aldrich, USA). Each well contained approximately 5×10^4 cells in 250 μL of medium. Then the plates were sealed and incubated for 48 hours at 22°C .

On the third day of incubation, the pre-incubation medium was replaced (after washing) with a medium containing Aroclor 1254 (at concentrations: 5, 10, 50 or 100 ng mL^{-1} medium) and/or 10^{-8} M of salmon gonadotropin-releasing hormone analogue (sGnRHa). The doses of Aroclor 1254 were selected on the basis of a publication (Lerner et al., 2007), as well as our own *in vitro* studies (Socha et al., 2012; 2013 b), in which similar concentrations (ng mL^{-1}) of PCBs were used. Control wells were filled up with the medium without any supplementation. Each treatment group consisted of five wells containing cells either from the pool of male or female pituitaries. For both sexes identical microplates were prepared. They were incubated for 10 hours at 22°C . At the end of the incubation period the plates were centrifuged ($200 \times g$ for 10 min at 20°C) and the media were collected and frozen at -20°C until LH determination by ELISA (Kah et al., 1989).

The obtained LH concentrations were analysed using GraphPad Prism statistical software (version 5, 2007, GraphPad Software, USA). All data are presented as means $\pm$ SEM. A nonparametric two-tailed Mann-Whitney U-test was performed. The differences between the means were determined as significant for $P \leq 0.05$.

Results

I. The effect of Aroclor 1254 on LH secretion from dispersed male pituitary cells:

a) basal secretion (Figure 1).

Figure 1. The effects of Aroclor 1254 at 5, 10, 50 or 100 ng mL⁻¹ on LH secretion from the dispersed pituitary cells of male common carp. Bars represent mean LH levels $\pm$ SEM, n=5

No statistically significant changes in LH levels under the influence of any of the concentrations of Aroclor were found.

b) sGnRHa-stimulated LH secretion (Figure 2).

Figure 2. The effects of Aroclor 1254 at 5, 10, 50 or 100 ng mL⁻¹ in combination with 10⁻⁸ M of salmon gonadotropin-releasing hormone analogue (sGnRHa) on LH secretion from the dispersed pituitary cells of male common carp. Bars represent mean LH levels $\pm$ SEM, n=5

sGnRHa alone did not significantly change the LH levels in the media when compared with control incubations. The combination of Aroclor (at all tested concentrations) with s-GnRH analogue resulted in significantly higher secretions of LH to the incubation media in comparison with control incubations. In the case of the two lowest concentrations of Aroclor (5 or 10 ng mL⁻¹) combined with sGnRHa, gonadotropin levels were significantly higher than in the media with sGnRHa only.

II. The effect of Aroclor 1254 on LH secretion from dispersed female pituitary cells:

a) basal secretion (Figure 3).

Figure 3. The effects of Aroclor 1254 at 5, 10, 50 or 100 ng mL⁻¹ on LH secretion from the dispersed pituitary cells of female common carp. Bars represent mean LH levels $\pm$ SEM, n=5

After 10 hours of cell incubation with Aroclor at concentrations of 5, 10 and 100 ng mL⁻¹, significant increases in LH concentrations compared to the control media were found. The strongest stimulation was observed in the case of the smallest concentration of Aroclor (5 ng mL⁻¹). Only 50 ng mL⁻¹ of Aroclor did not change LH levels in comparison with the control incubations.

b) sGnRHa-stimulated LH secretion (Figure 4).

Figure 4. The effects of Aroclor 1254 at 5, 10, 50 or 100 ng mL⁻¹ in combination with 10⁻⁸ M of salmon gonadotropin-releasing hormone analogue (sGnRHa) on LH secretion from the dispersed pituitary cells of female common carp. Bars represent mean LH levels $\pm$ SEM, n=5

sGnRH α alone significantly increased LH levels when compared with control incubations.

In the media of all Aroclor-tested concentrations combined with sGnRH α , gonadotropin levels were significantly higher than in the control incubations, as well as in the incubations with sGnRH α only.

Discussion

LH level analysis in the culture media demonstrated that in the case of male carp pituitary cells, Aroclor 1254 did not significantly change the concentration of this gonadotropin in comparison to control incubations, regardless of the concentration used in the experiment. In contrast to male cells, an increase in LH concentration was observed in the incubation media of female-derived pituitary cells under the influence of 5, 10 and 100 ng mL⁻¹ of Aroclor. These results suggest the direct action of PCBs on gonadotropic cells in mature carp. As was already mentioned in the Introduction, data on the direct influence of PCBs on pituitary gonadotropic cell secretion are very scarce. Jansen et al. (1993) demonstrated that Aroclor 1242 enhanced basal gonadotropin release from the pituitary cells in rats and potentiated pituitary responsiveness to GnRH, similarly to estradiol action. On the contrary, Khan and Thomas (1997) found that in Atlantic croaker (*Micropogonias undulatus*), Aroclor 1254 caused the loss of the *in vitro* pituitary gonadotropic response to LHRH analogue. Socha et al. (2013 b), incubating Prussian carp pituitary cells in the presence of Delor 106 (10, 50 or 100 ng mL⁻¹), showed a dose-dependent increase in LH concentrations in the incubation medium. These observations confirm the direct action of PCBs at the pituitary level. In the present experiments, the increase in LH concentrations in the incubations of female pituitary cells in the presence of Aroclor 1254 was not dose-dependent: the lowest tested concentration of Aroclor (5 ng mL⁻¹) increased LH levels threefold (by 212%), in comparison to the control incubations. Higher concentrations of Aroclor (10 and 100 ng mL⁻¹) were less effective and the increase in LH content reached 90%.

The difference in the response of pituitary cells derived from male or female common carp to Aroclor is not surprising, as Kautzky-Willer et al. (2016), Braun (2017), Heindel et al. (2017) or Wahlang et al. (2019) demonstrated that in mammals the effects of exposure to PCBs and other persistent organic pollutants are sex-specific and age-related. Such a sex-dependent phenomenon in the action of PCBs was also observed in fish: Li et al. (2019) demonstrated that low concentrations of Aroclor 1254 (0.2 and 2.0 μ g L⁻¹) exerted a sex-specific influence on lipid metabolism in zebrafish, where male fish were more sensitive to the metabolism-disturbing effects of PCB than female fish. The expression of vitellogenin (egg yolk precursor protein, a marker of feminization) released by the hepatocytes during vitellogenesis was up-regulated by increased concentrations of Aroclor 1254 in the liver and visceral mass of male fish but was not significantly changed in the female fish. Vos et al. (2000) confirm that the consequences of elevated vitellogenin levels in male fish

are serious for the population of males living in the natural environment, because such nonfunctional vitellogenin production is responsible for observed intersexuality (ovotestis) (Jobling et al., 1998), which can have an adverse effect at the population level. Thus, laboratory and field experiments confirmed that various xenobiotics are capable of inducing vitellogenin production and the development of ovary tissue in male fish, but that interspecies differences clearly exist. All these results indicate that in the water environment, the existence of persistent organic pollutants causes more harmful effects in males than in females.

Maisano et al. (2016) noted that male blue tuna were more sensitive to the influence of PCBs on fat deposition and the expression of lipid metabolism-related genes. In Atlantic cod (*Gadus morhua*) males, Bratberg et al. (2013) observed the higher detoxification and enhanced metabolism of PCB than in females. In zebrafish (*Danio rerio*) embryos (Berg et al., 2011), the earlier onset of puberty and a higher proportion of males than females was observed as a result of exposure to PCBs. Wahlang et al. (2019) concluded that the evidence of sex-dependent metabolic disruption with environmental toxicants supports the importance of sex differences when risks related to natural populations are considered.

Such sex differences in the action of PCBs on different aspects of human and animal physiology are dependent on many factors and in the context of our and others' results we can confirm that PCBs affect the process of gonadotropin release regulation in fish in a sex-dependent manner, also at the level of the pituitary gland.

PCBs are endocrine-disrupting chemicals interacting with endocrine receptors, which was demonstrated by Luthe et al. (2008). Most low molecular weight PCBs activate estrogen receptors (α and β) and antagonize androgen receptors, depending on the amount of chlorine in the molecule (Pěňčíková et al., 2018).

PCBs of higher molecular weight are considered anti-estrogenic and this fact is responsible for sex-specific effects. The differential responses of male and female carp pituitary cells to Aroclor 1254 – a mixture of chlorobiphenyls, which can have both estrogenic and/or anti-estrogenic properties (Jansen et al., 1993), may be the consequence of the well-known phenomenon that gonadotropin secretion from the pituitary gland is an estrogen-dependent process. There are examples that in rats the sensitivity of pituitary to GnRH stimulation is modulated by estrogens (Desaulniers et al., 1999; Desotelle et al., 2005) and pituitary sensitivity to exogenous GnRH is significantly altered by the presence of PCBs 126 and 52, where lower doses inhibit and higher doses facilitate GnRH-stimulated secretion of LH (Desotelle et al., 2005). Pituitary effects are the results of PCBs binding and activation of estrogen receptors in gonadotropes.

In our study, despite the lack of Aroclor 1254 effects on the basal LH release from male pituitary cells, GnRH-analogue-stimulated LH secretion was increased by all Aroclor-tested concentrations and in the case of the lower concentrations (5 and 10 ng mL⁻¹), the potentiation of GnRH analogue effects was observed. Aroclor alone at the concentrations of 5, 10 and 100 ng mL⁻¹ affected the basal secretion of LH from female pituitary cells, and all the concentrations of Aroclor also potentiated the stimulatory effects of the GnRH analogue. These results demonstrate that Aroclor directly affects gonadotropin-producing cells in the carp pituitary, probably

by activating estrogen receptors present in gonadotropic cells. In rainbow trout (*Oncorhynchus mykiss*), Kah et al. (1997) presented the estrogen receptors in the proximal *pars distalis* of the pituitary, where the gonadotrophs are located and also found that GnRH cells do not express estrogen receptors. Our findings therefore suggest that PCBs may increase the responsiveness of gonadotrophs to GnRH. Kraugerud et al. (2012) found similar phenomenon explaining the possible mechanism as upregulation of the GnRH receptor number in female lambs.

On the basis of our preliminary *in vitro* results, it is difficult to draw conclusions about the effects of Aroclor 1254 on *in vivo* LH release from the pituitary gonadotropic cells of male and female carp under natural conditions. However, it is clear that male pituitary cells were insensitive to all the doses of Aroclor we used, while a significant increase in LH levels to all doses of Aroclor was noted in female pituitary cells. This lack of an *in vitro* response of male pituitary cells to Aroclor may be related to their lower overall sensitivity at this stage of carp gonadal maturity (GSI = 4.3% – partially spawned fish), which is also evident in the lack of a response to the GnRH analogue alone caused by the low sensitivity of gonadotropic cells to stimulation by gonadoliberein (probably by small number of GnRH receptors). The combined action of Aroclor and sGnRH α changed this state and significant stimulation of LH secretion was noted in relation to the control and sGnRH α alone. The results of an *in vivo* experiment on Atlantic croaker (Khan et al., 2001) suggest that Aroclor 1254 disrupts LH secretion by alterations in the GnRH component of the GnRH-LH system. Its enhanced degradation causes the lower GnRH content (it impairs GnRH synthesis), acting on GnRH neurons directly and/or via other neurotransmitter system and also affects the number of pituitary GnRH receptors, which remained significantly lower in the PCB-exposed group. These results of the *in vivo* experiment cannot be directly compared with our *in vitro* study on different fish species, but confirm the phenomenon of impairment of the GnRH-LH system in fish.

In conclusion, the above results show that Aroclor 1254 – an endocrine disrupter – may affect reproduction in common carp (male/female) by acting at the level of the pituitary, directly on gonadotropic cells, changing basal and sGnRH-analogue-stimulated LH secretion. This action is sex-dependent: in male cells Aroclor did not affect basal LH secretion, whereas in female pituitary cells, incubations caused a significant increase in LH concentrations. In the case of cells of both sexes, all the concentrations of Aroclor potentiated stimulatory effects of the sGnRH analogue.

Further research is needed to confirm that this observation holds true for other fish species and that the direct action of PCBs at the level of pituitary cells could explain some of the disorders in reproductive functions in fish caused by the presence of xenobiotics in adversely changed water environments.

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