Toxicology and Applied Pharmacology 314 (2017) 63–71

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

Secretory function of ovarian cells and myometrial contractions in cow are affected by chlorinated insecticides (chlordane, heptachlor, mirex) *in vitro*

Wrobel Michael Hubert *, Mlynarczuk Jaroslaw

Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Tuwima Street 10, 10-748 Olsztyn, Poland

ARTICLE INFO

Article history:
Received 17 June 2016
Revised 20 October 2016
Accepted 20 November 2016
Available online 22 November 2016

Keywords:
Chlordane
Heptachlor
Mirex
Ovary
Uterus
Cow

ABSTRACT

The aim of the study was to investigate the effect of chlordane, heptachlor and mirex, on hormonal regulation of the force of myometrial contractions.
Myometrial, endometrial, granulosa and luteal cells as well as strips of myometrium from non-pregnant cows were incubated with three insecticides at environmentally relevant doses (0.1, 1 or 10 ng/ml).
None of the insecticides affected the viability of studied cells. Chlordane stimulated, while heptachlor and mirex inhibited, secretion of testosterone and estradiol from granulosa cells as well as secretion of progesterone from luteal cells, respectively. Secretion of oxytocin (OT) from granulosa cells was increased after incubation with all studied insecticides. Only mirex stimulated OT secretion from luteal cells, while heptachlor inhibited this effect. None of them affected synthesis of OT in luteal cells and prostaglandins (PGF2 and PGE2) secretion from uterine cells, except PGE2 secretion from endometrial cells was decreased when the cells were incubated with 0.1 ng/ml of chlordane. Basal and OT-stimulated myometrial contractions were increased by mirex and decreased by heptachlor.
The data show that the insecticides altered secretory function of ovarian cells. Heptachlor and mirex affected also myometrial contractions *in vitro*, but uterine secretion of prostaglandins were not involved in the mechanism of that adverse effect of insecticides. The data indicate on potential of these insecticides to disturb fertilisation, blastocyst implantation or even the length of gestation.

1. Introduction

Insects, because of their amount and variety of species, tend to be the most common and numerous group among pests. Therefore, the intensive use of insecticides tends to improve quantity of crops in modern agriculture. However, they enter to food chain and therefore, insecticides can disturb ecosystem. Organochlorine insecticides, like derivatives of cyclopentadiens (chlordane, heptachlor and mirex) went into production shortly after the end of World War II. Already in the end of the last century, these insecticides were mentioned as carcinogen for human (Jennings and Li, 2015). Therefore, they were included to the group of persistent organic pollutants listed in the Stockholm convention, which have to be eliminated and their use have been restricted (UNEP, 2001). However, due to their low degradation, concentration of chlordane and heptachlor in soil (Mahugija et al., 2014; Wang et al., 2016) or atmosphere (Shunthirasingham et al., 2016) and heptachlor in water (Wu et al., 2014) still remain measurable. They easy penetrate from environment to animals bodies and, because of their high lipophilicity, chlordane, heptachlor and mirex are still detect in human (Byrne et al., 2015; Freire et al., 2014; Guo et al., 2014; Zhu et al., 2015) and animals tissue (Byrne et al., 2015; Savinov et al., 2011; Wang et al., 2015) as well as in bovine milk (Avancini et al., 2013). Into organism these pesticides, interfere with the synthesis, secretion or transport of endogenous hormones, and act as endocrine disrupting factors. It can be followed by disturbances in many reproductive processes (Stefansdottir et al., 2014). Admittedly, estrogenic properties of many organochlorine compounds have been well documented (Grünfeld and Bonefeld-Jorgensen, 2004; Kuiper et al., 1998; Inadera, 2006; McLachlan, 2001). while chlordane, heptachlor and mirex have not shown an estrogenic activity (Gellert, 1978; Kim et al., 2011; Soto et al., 1995). It was also shown that heptachlor and mirex have exerted even anti-estrogenic properties (Okoumassoun et al., 2002; Uslu et al., 2013).

Abbreviations: AA, arachidonic acid; Act D, actinomycin D; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane; E2, estradiol; KRS, Krebs-Ringer's solution; MTT, tetrazolium salt; NP-I/OT, neurophysin-I/OT; OT, oxytocin; P4, progesterone; PCBs, polychlorinated biphenyls; PGA, peptidyl-glycine-α-amidating monooxygenase; PG(s), prostaglandin(s); PGFM, 13,14-dihydro-15-keto-PGF2α; T, testosterone; TBP, TATA box binding protein.

* Corresponding author.
E-mail address: m.wrobel@pan.olsztyn.pl (M.H. Wrobel).

http://dx.doi.org/10.1016/j.taap.2016.11.011
0041-008X/

Estradiol (E2), as a result of testosterone (T) conversion in the bovine granulosa cells (Henderson and Swanston, 1978) increases the number of oxytocin (OT) receptors in endometrium (Fuchs et al., 1992) and myometrium (Richter et al., 2004), and it stimulates mRNA and protein expression of prostaglandin (PG) F2α receptor in oviductal smooth muscle (Huang et al., 2015). Admittedly, E2 can augment uterine contraction *in vivo* (Hawk, 1983) and myometrial strips *in vitro* (Wrobel et al., 2005) in cows, while ovarian OT and uterine PGF2α are the most potent uterotonic agents (Fuchs et al., 1992; Olson, 2003; Ruckebusch and Bayard, 1975). In contrast, the stimulation of PGE2 receptors promotes uterine relaxation (Olson, 2003). The positive feedback loop between PGF2α and OT is well documented in cows (Kotwica et al., 1999; Skarzynski et al., 1999). Moreover, progesterone (P4) abolishes the effect of OT on the motor activity of uterus, and P4 is considered to be essential factor to the maintenance of the uterine quiescence during gestation (Lye and Porter, 1978). Therefore, the unaffected regulation of the uterine motility is crucial for the course of estrous cycle as well as fertilisation, implantation of blastocyst, the maintenance of pregnancy and initiation of labor at term (Bulletti et al., 2004). Abnormal contractility might underlie ectopic pregnancy, spontaneous miscarriage or preterm birth (Bulletti et al., 2004; Aguilar and Mitchell, 2010).

Thus, we wanted to check whether the insecticides can directly or indirectly affect the hormonal regulation of uterine activity. Therefore, the aim of this study was to investigate whether chlordane, heptachlor and mirex interfere with: 1) the secretion of ovarian steroids (T, E2 and P4), which are involved in regulation of myometrial receptivity for OT; 2) the ovarian and uterine secretion of primary regulators of the uterine contractions (OT and PGs); and 3) the basal and OT-stimulated myometrial contractions.

2. Material and methods

2.1. Preparation of material

Bovine ovaries and uteri (8–12th days of estrous cycle), were collected in a commercial slaughterhouse. All materials used in these studies were purchased from Sigma-Aldrich (PL) unless otherwise stated. Each medium was supplemented with gentamycin (20 μg/ml) and amphotericin (2 μg/ml) and they did not contain phenol red. For incubations exceeding 24 h, media were supplemented with antioxidants: ascorbic acid (20 μg/ml; Merck, USA), sodium selenite (5 ng/ml; ICN, USA) and transferrin (5 μg/ml). Granulosa cells were obtained by vigorous aspiration with follicular fluid from follicles >1 cm in diameter (10–15 follicles for each experiment). Luteal cells were obtained by perfusion with collagenase according to Okuda et al. (1992). Myometrial and endometrial cells were obtained by enzymatic dispersion according to Wrobel et al., 2015. Only cells showing viability above 80% were used for further studies. The cells suspensions were transferred into 48-well plates (2×10^5/ml of cells, Nunclon Δ-Surface, NUNC, NL). For the measurement of mRNA expression only, the 6-well plates (5×10^5/ml, Nunclon Δ-Surface, NUNC, NL) were used. The cells were precultured (95% air and 5% CO_2, 100% humidity, 38 °C; Memmert INCO 180, D) for 24 h (granulosa and luteal cells), 72 h (endometrium) or 96 h (myometrium). Next, they were washed twice with M199 and the medium was replaced with DMEM/HAM-12 supplemented with 0.1% BSA. Four strips (6–7 mm long and 3–4 mm wide) of longitudinal smooth muscle were dissected from each myometrium. The strips were incubated (24 h, 95% air and 5% CO_2, 4 °C) with treatments according to Wrobel et al. (2005, 2015), and the force of myometrial strips contractions was measured.

2.2. Treatments

Chlordan, heptachlor and mirex were dissolved in DMSO (HPLC purity grade). Final concentration of the DMSO in the culture medium did not exceed 0.1%. Therefore, 0.1% of DMSO was added to the control samples.

2.3. Evaluation of cell viability

The cell viability was determined after incubation (72 h, 38 °C) of granulosa (n = 5), luteal (n = 6), myometrial (n = 5) and endometrial (n = 4) cells with chlordan, heptachlor or mirex, each at dose of 10 ng/ml. Actinomycin D (Act D; 500 ng/ml), an inhibitor of RNA synthesis, was used as a positive control. Each treatment was performed in quadruplicate.

2.4. Hormone secretion from ovarian and uterine cells

The granulosa, luteal and endometrial cells were incubated (72 h, 38 °C) separately with chlordane, heptachlor or mirex (each at dose of 0.1, 1 or 10 ng/ml; n = 5). FSH (AFP-5679C, 100 ng/ml), LH (AFP-11743B, 100 ng/ml) and AA (20 μg/ml) were used as positive controls, respectively. Each treatment was performed in duplicate. The myometrial cells (n = 4 cows) were incubated (24 h) with all insecticides (separately, each at dose of 10 ng/ml) or arachidonic acid (AA; 20 μg/ml). After incubation, the medium was collected into tubes containing 10 μl of 0.3 M EDTA in 1% acetylsalicylic acid (Meyer et al., 1989) and stored at −20 °C for subsequent determination of T, E2 and OT in the granulosa cell cultures, P4 and OT in the luteal cell cultures and PGFM (the main metabolite of PGF2α) and PGE2 concentrations in the myometrial and endometrial cell cultures.

2.5. OT synthesis in the luteal cells

The luteal cells (n = 4) were incubated (8 h) with chlordane, heptachlor or mirex (10 ng/ml) in duplicate. Next, the cells were covered with Phenozol (300 μl for each well; A&A Biotechnology, PL) and analysis of mRNA expression for neurophysin-I/OT (NP-I/OT) and peptidylglycine-α-amidating monooxygenase (PGA) was done.

2.6. Myometrial contractions

Myometrial strips (4 from each cow) were incubated (24 h) with chlordane (n = 5 cows), heptachlor (n = 4 cows) or mirex (n = 4 cows), each at the dose of 0.1, 1 and 10 ng/ml, according to Wrobel et al. (2015). Next, the track of spontaneous and OT-stimulated (10^{-7} M) contractions was recorded.

2.7. Determination of cell viability

The viability of cells was measured by a TOX-1 test (*in vitro* toxicology assay kit, MTT-based) according to the manufacturer's protocol. The granulosa and myometrial cells were incubated with MTT (20 μl/well) for 4 h, whereas the endometrial and luteal cells were incubated with MTT for 6 and 2 h, respectively, The absorbance of the reaction product (formazan) was measured at λ = 570 nm, using ELISA reader (Multiscan EX, Labsystem, FI).

Table 1
Primer sequences used to analysis of gene expression in bovine luteal cells *in vitro*.

Gene	Accession no.	Sequence (5′–3′)	Product size (bp)
NP-I/OT	NM_176855.1	Forward: GTCTGCACCATGGCAGGTT Reverse: ACGGCAGGTAGTTCTCCTCTTG	255
PGA	NM_173948.2	Forward: GCGTTTGCCAATGGATGAG Reverse: TGGCATATTGCATCCAAACAG	99
TBP	NM_001075742.1	Forward: CAGAGAGCTCCGGGATCGT Reverse: ACACCATCTTCCCAGAACTGAATAT	194