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Scientific Paper

COMBINED EFFECTS OF CADMIUM AND DECABROMINATED DIPHENYL ETHER ON THYROID HORMONES IN RATS

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The aim of this study was to see how a mixture of cadmium (Cd) and decabrominated diphenyl ether (BDE209) affect thyroid function, namely thyroid-stimulating hormone (TSH), thyroxin (T4), free thyroxin (FT4), triiodothyronin (T3), and free triiodothyronin (FT3) in Wistar rats (eight per group) receiving either a single substance or their combination by gavage for 28 days. Three groups were receiving Cd alone in the doses of 2.5 mg kg^{-1}, 7.5 mg kg^{-1}, or 15 mg kg^{-1} b. w. a day, three groups were receiving BDE209 in the doses of 1000 mg kg^{-1}, 2000 mg kg^{-1}, or 4000 mg kg^{-1} b. w. a day, while nine groups were receiving different mixtures of Cd and BDE209 in these doses (3x3 design). The results have indicated that the Cd+BDE209 mixtures more potently disrupt thyroid hormone homeostasis than would be expected from these chemicals alone.

KEY TERMS: *BDE209, Cd, thyroid-stimulating hormone, thyroxin, triiodothyronin*

Chemical risk assessment procedures commonly rely on determining the effects of a single substance. Over the last decade, however, a number of well-designed studies have investigated the effects of multi-component mixtures (1-4). High-level exposure to cadmium (Cd) is usually a result of environmental contamination due to various anthropogenic activities (5, 6). Acute or chronic exposure to Cd can affect the liver, kidney, bone, and testes in humans and experimental animals (7-13). In addition, there is evidence that Cd can alter thyroid function (6, 14-17).

Flame retardants have also become very important environmental and occupational pollutants. Through history, BDE209 was thought to be released minimally into the environment during all phases of its use and not available biologically due to its large molecular size and low aqueous solubility. However, today it is a widespread environmental contaminant with evidence demonstrating its bioaccumulation and toxic potential (18-23). Recent studies with polybrominated diphenyl ethers (PBDEs) have shown that they disturb liver enzyme activity and thyroid hormone function (24-29), reduce epididymal sperm function (30), and may affect the nervous system and newborns (31, 32).

Cadmium and BDE209 are jointly present in the environmental media, food, biota, and human tissues

through several pathways as a result of emission from various sources. Combined exposure to Cd and organic pollutants may result in complex toxicity. In view of the importance of *in vivo* behaviour of contaminants concomitantly present in an organism, this study was aimed at determining the effects of mixtures of Cd and BDE209 on thyroid-stimulating hormone (TSH), thyroxin (T4), free thyroxin (FT4), triiodothyronin (T3), and free triiodothyronin (FT3) in rats.

MATERIALS AND METHODS

Experimental animals

Male albino Wistar rats weighing 200 g to 240 g were obtained from a disease-free stock bred at the Military Medical Academy in Belgrade, Serbia. The animals were housed in plastic cages with a plastic bottom and wire mesh top, in a climate-controlled facility with a constant 12-hour day and night cycle at 20 °C to 24 °C and relative humidity between 40 % and 60 %. The animals had free access to food and tap water throughout the study and were treated according to the guidelines for animal studies (no. 9667-1/2011) issued by the Academy's ethics committee.

After a quarantine period of 14 days, groups of eight animals each were receiving either a single substance or their combinations by gavage in a volume of 0.5 mL kg^{-1} b.w. per day for 28 days. Three groups were receiving Cd alone in the form of $CdCl_2$ x H_2O (Merck, Darmastadt, Germany) in the doses of 2.5 mg kg^{-1}, 7.5 mg kg^{-1}, or 15 mg kg^{-1} b. w. per day ($Cd_{2.5}$, $Cd_{7.5}$, and Cd_{15}, respectively) and three groups BDE 209 alone as a suspension in dimethyl sulphoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) in the doses of 1000 mg kg^{-1}, 2000 mg kg^{-1}, or 4000 mg kg^{-1} b. w. per day ($BDE209_{1000}$, $BDE209_{2000}$, and $BDE209_{4000}$, respectively). Cadmium doses were selected to reflect environmental to occupational exposure (7, 9, 16). The choice of BDE209 doses was based on literature data on its very low absorption rate of 0.3 % to 2 % (18, 19). The remaining nine groups were receiving combinations of Cd and BDE209 doses according to the 3x3 design.

Before gavage Cd salt and BDE209 were dissolved in DMSO.

Rats in the vehicle control group were receiving DMSO alone (DMSO group), while control animals were receiving saline (control group).

We did our best to minimise the suffering and the number of animals used. Throughout the study, the rats were continuously monitored for body weight, clinical signs such as changes in the skin, fur, eyes, mucous membranes, secretions and excretions, autonomic activity (lacrimation, piloerection, pupil size, unusual respiratory patterns), behaviour, and food and water intake. Signs of toxicity were monitored on a daily basis, while water and food intake were recorded weekly. After decapitation, blood was collected from the carotid arteries in glass tubes and then centrifuged at 3000×*g* for 30 min. The supernatant (serum) was transferred to polypropylene test tubes and stored at −70 °C until thyroid hormone analysis.

Hormone analysis

Serum samples were analysed for thyroid-stimulating hormone, thyroxin, triiodothyronin, and free triiodothyronin with commercial tests using a Roche Elecsys 2010 analyser (Roche Diagnostics, Mannheim, Germany). Values for T3 and T4 are expressed as nmol L^{-1}, for FT3 and FT4 as pmol L^{-1} while for TSH as mU L^{-1}. The analytical ranges were 0.005 mU L^{-1} to 100 mU L^{-1} for TSH, 0.30 nmol L^{-1} to 10 nmol L^{-1} for T3, 5.40 nmol L^{-1} to 320 nmol L^{-1} for T4, 0.40 pmol L^{-1} to 50 pmol L^{-1} for FT3, and 0.30 pmol L^{-1} to 100 pmol L^{-1} for FT4. Total precisions over these ranges (expressed as coefficients of variance) were 5.4 % for TSH, 4.8 % for T3, 3.3 % for T4, 2.2 % for FT3, and 2.7 % for FT4.

Statistical analysis

To establish significance of differences between the groups, we used one-way analysis of variance (ANOVA) and subsequently Tukey's paired comparisons or Fisher's least significant difference (LSD) as *post hoc* tests. The level of statistical significance for all tests was set at $p<0.05$. All data were analysed using the statistical package Statistica 7.0.

RESULTS

Slower body weight gain was the most common in groups treated with the combination of compounds (in eight out of nine groups), while Cd or BDE209 alone did not cause significant changes compared to controls (Table 1). However, water and food consumption did not differ significantly between animals receiving single compounds (Cd or BDE209) and animals receiving their combinations.

Table 1 *Body weight and food and water intake in Wistar rats orally exposed to BDE209, Cd, or their combinations for 28 days.*

	Body weight / g		Daily consumption	
Group	**Final**	**Gain**	**Food / g**	**Water / mL**
Control	312.50±5.01	115.7±9.04	40.75±1.50	73.25±5.32
DMSO	300.04±14.14	85.00±19.15	30.00±8.89	53.00±14.61
$Cd_{2.5}$	331.25±24.16	107.50±26.05	24.51±2.33a	45.66±2.37a
$Cd_{7.5}$	290.00±52.15	80.00±31.62	20.03±3.15a	36.35±3.08a,b
Cd_{15}	304.29±20.70	82.86±24.98a	17.51±3.38a,b	37.53±5.66a
$BDE209_{1000}$	317.50±19.08	113.80±16.85b	22.92±4.02a	44.58±12.58a
$BDE209_{2000}$	305.00±18.71	88.00±10.95a	25.00±5.47a	41.68±16.01a
$BDE209_{4000}$	291.67±48.75	84.00±25.10a	27.08±1.58a	43.77±4.15a,b
$Cd_{2.5}$+$BDE209_{1000}$	286.25±27.74	81.30±26.96a,c,d	21.56±4.72a	34.37±11.38a
$Cd_{7.5}$+$BDE209_{1000}$	258.33±41.19	20.00±1.41a,b,c,d	18.30±11.03a	30.45±15.28a,b
Cd_{15}+$BDE209_{1000}$	261.43±48.11	66.67±30.77a,d	23.84±5.55a	32.41±10.53a,b
$Cd_{2.5}$+$BDE209_{2000}$	243.33±30.11	26.00±8.94a,b,c,d	18.75±12.58a	26.25±18.33a
$Cd_{7.5}$+$BDE209_{2000}$	270.00±24.49	71.67±20.41a	22.08±5.49a	50.42±22.14
Cd_{15}+$BDE209_{2000}$	215.00±8.37	20.00±7.07a,b,c,d	16.48±7.69a	29.15±4.79a,b,d
$Cd_{2.5}$+$BDE209_{4000}$	250.01±38.47	22.50±8.25a,b,c,d	20.82±5.19a	45.85±4.79a
$Cd_{7.5}$+$BDE209_{4000}$	228.00±10.95	30.00±10.95a,b,c,d	19.57±4.17a	41.28±0.85a,b
Cd_{15}+$BDE209_{4000}$	232.00±21.68	57.50±23.63a,d	20.82±3.21a	33.75±11.15a

$Cd_{2.5}$, $Cd_{7.5}$, and Cd_{15}- receiving Cd alone in a daily dose of 2.5 mg kg^{-1}, 7.5 mg kg^{-1} or 15 mg kg^{-1} b. w., respectively
$BDE209_{1000}$, $BDE209_{2000}$, and $BDE209_{4000}$ - receiving BDE209 alone in a daily dose of 1000 mg kg^{-1}, 2000 mg kg^{-1}, or 4000 mg kg^{-1} b. w., respectively
a - significantly different from control; b - significantly different from the DMSO group; c - significantly different from the same Cd dose; d – significantly different from the same BDE209 dose (one way ANOVA and post-hoc *Fisher's Least Significant Difference (LSD) test, $p<0.05$).*

Food consumption was lower in all groups compared to control, and the Cd_{15} group also showed a significant decrease compared to the DMSO group (Table 1). Groups treated with combinations did not differ in food consumption from groups treated with Cd or BDE209 alone. Water consumption decreased in all groups compared to control, with the exception of the $Cd_{7.5}$+$BDE209_{2000}$ group. Compared to the DMSO group, only the $Cd_{7.5}$ and the $BDE209_{4000}$ groups showed lower water consumption, and the same effect was observed in the following four combination groups: $Cd_{7.5}$ or Cd_{15}+$BDE209_{1000}$, Cd_{15}+$BDE209_{2000}$ and $Cd_{7.5}$+$BDE209_{4000}$. The only statistically significant difference between the combination groups and corresponding single-compound groups was noticed for the Cd_{15}+$BDE209_{2000}$ *vs.* $BDE209_{2000}$ group; the first drank about 30 % less water than the $BDE209_{2000}$ group. Decreases in food and water intake of almost 50 % in some groups are in accordance with lower body weight gain. Except for food and water intake and body weight gain, there were no other clinical signs of poisoning.

All three Cd doses induced a significant decrease in T4 and FT4 levels *vs.* control, whereas 2.5 mg kg^{-1} Cd led to a decrease in FT3 and 15 mg kg^{-1} Cd to decrease in T3 and FT3 levels (Table 2). Significant differences from the DMSO group were seen in the $Cd_{2.5}$ (FT4), $Cd_{7.5}$ (FT3, T4), and Cd_{15} (FT3) groups. Similar to Cd alone, all three doses of BDE209 induced a significant decrease in T4 and FT4 levels *vs.* control. Compared to the DMSO group, a significant decrease in hormone levels, given in brackets, was observed in the $BDE209_{1000}$ (FT3, T4), $BDE209_{2000}$ (FT3, T4, FT4), and $BDE209_{4000}$ (T3) groups.

Combinations added to these effects, particularly in the groups receiving BDE209 (1000 mg kg^{-1} or 2000 mg kg^{-1}) plus Cd (all three doses). Cd+BDE209 (1000 mg kg^{-1}) significantly decreased T4 and FT4 levels compared to the groups given the corresponding doses of either Cd or BDE209. BDE209 (2000 mg kg^{-1}) plus Cd also significantly decreased T4 and FT4, and additionally T3 levels. We measured TSH levels as well, but they were all below the limit of detection (0.005 mU L^{-1}).

Statistical analysis of the ratio between T3 and T4 pointed out that it was significantly higher in almost all groups compared to control. In the $Cd_{7.5}$ group and in the groups receiving combinations $Cd_{2.5}$+$BDE209_{1000}$ and $Cd_{2.5}$+$BDE209_{2000}$ this ratio was also significantly higher than in the DMSO group. All Cd combinations with the highest dose of BDE209 also showed a significant increase in respect to treatment with corresponding dose of BDE209 alone. Expressed in percentages, the ratio between T3 and T4 varied from 5 % to 27 %, while in groups Cd+$BDE209_{4000}$ it varied from 10 % to 18 %, compared to the DMSO group.

DISCUSSION

Both pollutants, alone or in combination lowered thyroid hormone levels compared to control, but the combined effect was more pronounced. The combined effect on the levels of T3, FT3, T4, and FT4 may be viewed as additive. A number of studies have already shown that Cd alters thyroid function in experimental

Table 2 *Serum thyroid hormone levels in Wistar rats orally exposed to Cd, BDE209, or their combinations for 28 days*

	Thyroid hormone levels				Ratio
Group	**T3 / nmol L^{-1}**	**FT3 / pmol L^{-1}**	**T4 / nmol L^{-1}**	**FT4 / pmol L^{-1}**	**T3/T4**
Control	1.52±0.06	4.57±0.19	82.66±3.36	42.51±2.22	0.0183
DMSO	1.46±0.05	4.37±0.33	62.93±4.45a	32.85±4.18a	0.0232
$Cd_{2.5}$	1.40±0.13	3.29±0.24a	54.66±4.11a	27.88±3.15a,b	0.0256a
$Cd_{7.5}$	1.40±0.15	3.69±0.45b	47.43±9.04a,b	23.84±3.60a	0.0295a,b
Cd_{15}	1.34±0.10a	3.56±0.25a,b	54.90±4.40a	29.44±2.65a	0.0244a
$BDE209_{1000}$	1.39±0.06a	2.98±0.87a,b	53.84±5.51a,b	31.02±2.88a	0.0258a
$BDE209_{2000}$	1.44±0.09	4.72±0.73b	49.61±6.77a,b	25.31±3.31a,b	0.0290a
$BDE209_{4000}$	1.30±0.12a,b	4.38±0.79	61.85±8.02a	29.82±3.82a	0.0210
$Cd_{2.5}$+$BDE209_{1000}$	1.34±0.05a	3.33±0.17a	47.48±4.04a,b,c	24.15±3.00a,b,c,d	0.0282a,b
$Cd_{7.5}$+$BDE209_{1000}$	1.38±0.06a	4.01±0.20	64.48±4.12a,c,d	31.00±3.36a,b,c	0.0214
Cd_{15}+$BDE209_{1000}$	1.30±0.05a,b	3.22±0.31a,b	47.78±7.74a,b,c	24.32±2.28a,b,c,d	0.0272a
$Cd_{2.5}$+$BDE209_{2000}$	1.28±0.14a,b,c,d	4.09±0.50c	43.46±5.42a,b,c	22.31±2.49a,b,c	0.0295a,b
$Cd_{7.5}$+$BDE209_{2000}$	1.27±0.12a,b,c,d	3.81±0.53d	47.43±7.58a,c,d	25.32±3.62a,b	0.0268a
Cd_{15}+$BDE209_{2000}$	1.22±0.17a,b,c,d	3.83±0.33d	47.13±8.58a,b,c	24.29±3.27a,b,c	0.0259a
$Cd_{2.5}$+$BDE209_{4000}$	1.31±0.15a,b	3.94±0.49c	51.41±14.21a,b,d	28.11±5.22a,b	0.0255a,d
$Cd_{7.5}$+$BDE209_{4000}$	1.32±0.14a,b	4.09±0.26	48.11±4.60a,b	23.90±2.22a,d	0.0274a,d
Cd_{15}+$BDE209_{4000}$	1.43±0.10	4.52±0.53c	54.15±7.65a,b	29.03±4.15a	0.0264 a,d

$Cd_{2.5}$, $Cd_{7.5}$, and Cd_{15}- receiving Cd alone in a daily dose of 2.5 mg kg^{-1}, 7.5 mg kg^{-1} or 15 mg kg^{-1} b. w., respectively
$BDE209_{1000}$, $BDE209_{2000}$, and $BDE209_{4000}$ - receiving BDE209 alone in a daily dose of 1000 mg kg^{-1}, 2000 mg kg^{-1}, or 4000 mg kg^{-1} b. w., respectively
a - significantly different from control; b - significantly different from the DMSO group; c - significantly different from the same Cd dose; d – significantly different from the same BDE209 dose (one way ANOVA and post-hoc *Fisher's Least Significant Difference (LSD) test, $p<0.05$).*

animals (6, 7, 9, 14, 15-17, 33) and humans (34). Yoshizuka et al. (17) suggested that Cd accumulated in the mitochondria of thyroid follicular epithelial cells might disturb the oxidative phosphorylation of this organelle, lower energy supply, and therefore inhibit the synthesis and release of thyroid hormones. Similarly, Pilat-Marcinkiewicz et al. (16) observed a dose-dependent effect on the structure and function of thyroid follicular cells in rats. Apart from the studies suggesting that Cd interferes with the thyroid function at the glandular level, there are findings supporting the effects at the peripheral level by inhibiting the conversion of T4 to T3 (14, 35-38). Thyroid hormones are metabolised in peripheral tissue by deiodination, conjugation, deamination, and decarboxylation enzyme reactions, and any change in these metabolic pathways may significantly affect thyroid function at the cellular level (35-37). As deiodination of T4 to T3, which occurs mainly in the liver, depends on 5′-monodeiodinase activity (35, 37), hepatic dysfunction is likely to affect thyroid hormone levels (36), T3 in particular. However, thyroid hormone levels are mainly regulated by the hypothalamic-pituitary-thyroid axis and if this axis is affected by Cd, so will hormone release (39, 40).

Several reports have shown that a broad range of chemicals to which humans are routinely and inadvertently exposed can bind to thyroid receptors (TRs) and may produce complex effects on thyroid hormone signalling (41-43). Polybrominated diphenylethers, structurally related to BDE209, have been also recognised to bind to TRs and perhaps mimic thyroid hormones (42, 43). We may therefore assume that BDE209 binds to thyroid receptors rather than acts through the hypothalamic-pituitary axis. However, Zhou et al. (28) found that decaBDE caused no changes in any of the thyroid hormone levels (28). In contrast, Kim et al. (44) found significant increases in thyroid weight of pregnant rats exposed to high doses of BDE209 and significantly lower concentrations of T4 in F1 female offspring exposed to BDE209 on postnatal day 42. In a recent study, Lee et al. (22), found significantly lower total serum T3 concentrations, degenerated follicular epithelium, and hepatocyte hypertrophy and vacuolisation in newborn rats exposed to BDE209. These findings confirm that BDE209 might affect thyroid hormone synthesis and metabolism.

The combination of Cd and BDE209 toxicity has even more pronounced effects on T3 and T4, which can be explained by different mechanisms of their action. Although, we assumed additive response, future studies should look into the type of interactions between Cd and BDE209 at dose levels relevant for human exposure.

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Sažetak

KOMBINIRANO DJELOVANJE KADMIJA I DEKABROMIRANOG DIFENIL ETERA NA HORMONE ŠTITNJAČE U ŠTAKORA

Cilj ovoga istraživanja bio je utvrditi na koji način smjesa kadmija (Cd) i dekabromiranog difenil etera (BDE209) djeluje na aktivnost štitnjače. Određivane su aktivnosti stimulirajućega hormona štitnjače (TSH), tiroksina (T4), slobodnog tiroksina (FT4), trijodtironina (T3) te slobodnog trijodtironina (FT3) kao parametara koji upućuju na funkcionalnost štitnjače. Kao eksperimentalni testni sustav korišteni su Wistar štakori (n=8 po grupi), kojima je tijekom 28 dana dozirana pojedinačna tvar ili smjesa kadmija i dekabromiranog difeniletera. Životinje su bile podijeljene u tri grupe koje su primale tri različite doze kadmija: 2,5 mg kg^{-1}, 7,5 mg kg^{-1} i 15 mg kg^{-1} tjelesne težine po danu. Tri grupe životinja primale su tri različite doze BDE209: 1000 mg kg^{-1}, 2000 mg kg^{-1}, odnosno 4000 mg kg^{-1} tjelesne težine po danu. Preostale životinje bile su podijeljene u devet grupa kojima su bile dozirane različite koncentracije kadmija i dekabromiranog difenil etera (3x3 dizajn). Rezultati pokazuju da Cd+BDE209 smjesa u većoj mjeri remeti homeostazu hormona štitnjače u odnosu na pojedinačne spojeve.

KLJUČNE RIJEČI: *BDE209, Cd, stimulirajući hormon štitnjače, tiroksin, trijodtironin*

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