Int. J. Radiat. Biol., Vol. 86, No. 12, December 2010, pp. 1050–1060

informa
healthcare

Studies on the synergistic effects of extremely low-frequency magnetic fields and the endocrine-disrupting compound atrazine on the thyroid gland

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(Received 30 June 2009; Revised 28 May 2010; Accepted 13 June 2010)

Abstract

Purpose: The aim of this study was to examine the effect of extremely low-frequency magnetic fields (MF) and the endocrine-disrupting compound atrazine, each separately, on the thyroid gland of juvenile-peripubertal rats, and to investigate the possible synergistic effect of these two factors combined.

Materials and methods: The study was performed on male Wistar rats from postnatal day 23–53. Animals were divided into six groups: (1) 4 h/day exposure to MF (50 Hz, 100–300 μT, 54–160 V/m), (2) 20 mg/kg of body weight (bw) of atrazine, (3) 200 mg/kg bw of atrazine, (4) MF with 20 mg/kg bw of atrazine (5) MF with 200 mg/kg bw of atrazine, and (6) control.

Results: Light and electron microscopic studies demonstrated no significant alterations in the thyroid structure between the treated groups and the control. Significant outcomes were found regarding the volume density of thyroid follicles and the connective tissue between the MF-exposed group when compared to both atrazine treatments and the combined treatments. The high dose of atrazine significantly affected the number of mast cells compared to the control.

Conclusions: No synergistic effect of the MF and the endocrine-disrupting compound atrazine on the thyroid gland has been found. The specific histological alterations of the thyroid parenchyma observed in some treated groups require further investigation.

Keywords: *magnetic fields, atrazine, thyroid gland, mast cell, stereology*

Abbreviations: MF, magnetic fields; ELF, extremely low-frequency; TSH, thyroid stimulating hormone; T4, thyroxine; T3, triiodothyronine; bw, body weight; PND, postnatal day; EDC, endocrine-disrupting compound; HE, hematoxylin and eosin; Vv, volume density; Nv, numerical density; Ia, thyroid activation index; LD50, lethal dose, 50%; EGF, epidermal growth factor; TGF-β1, tranforming growth factor beta 1; NADPH, nicotinamide adenine dinucleotide phosphate; cAMP, cyclic adenosine monophosphate

Introduction

The possible biological effects of the electric and magnetic fields in the extremely low-frequency (ELF) range have been extensively studied in the last decades. Particular attention by the scientific community has been attached to the assessment of possible health outcomes from exposure to 50/60 Hz frequency fields due to their ubiquity in the living and working environment.

Previous studies have demonstrated that ELF electric and magnetic fields adversely affect the thyroid gland. The levels of the thyroid hormones were found decreased or increased in the peripheral blood of exposed rats (50 Hz, 20 mT), depending on the duration of exposure (Udintsev et al. 1978, Zagorskaya and Rodina 1990). After a nine-hour exposure to magnetic fields (MF) (50 Hz, 10 μT) during one night no alterations in the measured thyroid stimulating hormone (TSH), thyroxine (T4), triiodothyronine (T3), free T3, free T4 and thyroxin-binding globulin were recorded in 32 male volunteers aged between 20 and 30 years (Selmaoui et al. 1997). Investigations of acute exposure of linesmen to 400 kV power line with estimated mean exposure to electric and magnetic fields being 2.8 kV/m and

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ISSN 0955-3002 print/ISSN 1362-3095 online © 2010 Informa UK, Ltd.
DOI: 10.3109/09553002.2010.501837

23.3 μT demonstrated a linear decrease of serum TSH over the workday and consequently the significant differences in TSH concentrations between the three blood sampling occasions (before, during and after a workday) (Gamberale et al. 1989). Animal studies on the MF effects on the thyroid have shown moderate or no alterations in the level of the thyroid hormones (Lafreniere and Persinger 1979, Burchard et al. 2006).

Nevertheless, the overall issue of ELF electric and magnetic field effects still remains controversial and other factors, different than these fields, may also be involved. According to a number of authors, it is unlikely that ELF fields directly damage subcellular structures as their energy is far too low to break even the weakest chemical bonds. It is therefore assumed that these fields act as a promoting or co-promoting agent rather than an initiator. Most of the positive results reported in a number of studies could be ascribed to a synergistic action of the ELF fields with a certain chemical or physical agent(s). Among various chemical environmental pollutants atrazine, as a frequently used chlorotriazine herbicide, has been investigated due to its endocrine-disrupting potential. According to literature data, atrazine causes a number of imbalances in endocrine organs, primarily in the reproductive system. Atrazine was reported to decrease the weight of pituitary gland, adrenal glands and the ovary in the concentration of 200 mg/kg body weight (bw) (Laws et al. 2000). Both acute and chronic treatments with atrazine (50 mg/kg bw) reduced the levels of testosterone in serum and intratesticular tissue in immature male rats (Friedmann 2002). Measurements of TSH, T3 and T4 concentrations in peripheral blood showed no significant alterations after atrazine treatments from postnatal day (PND) 22–41 in male rats (12.5, 25, 50, 100 and 200 mg/kg bw) (Laws et al. 2000), while the treatment with 200 mg/kg bw from PND 23–53 significantly increased the serum T3 level, but not T4 and TSH (Stoker et al. 2000a).

In the present study we have performed a detailed morphometrical analysis of thyroid gland parenchyma and stroma in order to identify subtle changes in the glandular structure and a possible synergistic effect of MF and atrazine. In addition, a detailed analysis was attributed to thyroid mast cells, a multifunctional cell type of the thyroid connective tissue. Mast cells play a role in allergic reactions, acute and chronic inflammations, immune regulation, tissue repair, angiogenesis and extracellular matrix remodeling through the production of biomolecules, including amines as histamine and serotonin, proteoglycans, neutral proteases as tryptase and chymase, lipid-derived substances and cytokines (Metcalfe et al. 1997, Yong 1997).

Methods

Animals and experimental procedure

The experiment was performed on male Wistar rats under laboratory conditions with 20 $\pm$ 2°C temperature and a controlled photoperiod (14 h light/10 h dark). On PND 22 rats were weighted and randomly assigned to one of the six experimental groups consisting of 12 rats each: (1) Control, (2) MF, (3) low dose of atrazine, (4) high dose of atrazine, (5) MF and a low dose of atrazine, and (6) MF and the high dose of atrazine. All treatments were performed from PND 23–53.

The MF group was subjected to MF exposure (50 Hz, 100–300 μT, 54–160 V/m) 4 h daily from 10.00–14.00 h. The exposure system, by which MF was produced, was made of a single coil of solenoid type (Electronic Equipment Factory 'Novkabel', Novi Sad, Serbia) equipped with a cooling system and energised from 50 Hz, 220 V and 10 A via an autotransformer, which provided a 100 V output. Cages with animals were placed on both sides of the coil, perpendicular to the coil axis, at a 12 cm distance, and were covered with a plastic lid. The coil axis was parallel to the lines of force of the geomagnetic field (north-south direction). The MF produced by the coil was in the horizontal direction regarding the geomagnetic field; it was inhomogeneous and of decaying intensity along the animal cages, with values of 300 μT and 160 V/m on the side of the cage near the coil and 100 μT and 54 V/m on the opposite side. The value of the electric field at any other point in the room was less than 10 V/m. The magnetic field generated by the coil with the value of 100 μT was equal to the exposure limit for the general public recommended by the International Commission on Non-Ionizing Radiation Protection (ICNIRP) and the value of 300 μT was within the exposure limit for the working environment, while the intensity range of the produced electric field was below ICNIRP recommended exposure limits. The residential values of the magnetic (AC milligaussmeter, model 42B-1, Monitor Industries, Boulder, CO, USA) and electric fields (HI-3607 E.L.F. sensor, Holaday Industries, Eden Prairie, MN, USA) were measured to be 0.2 μT and 2.9 V/m, while the value of the geomagnetic field (Gauss/Tesla meter, model 4048, F.W. Bell, Orlando, FL, USA) was 40 μT.

Atrazine (98% purity, a gift from professor Sanja Lazic, Institute for Environmental and Plant Protection, Faculty of Agriculture, University of Novi Sad, Serbia) was dissolved in edible olive oil and administered per os in the concentration of 20 mg/kg bw (low dose) and 200 mg/kg bw (high dose) between 08.00 and 09.00 h daily. The animals were sacrificed on PND 53, after daily treatments. The particular

period of ontogeny (PND 23–53) and the doses used for atrazine treatments were selected based on previous studies on atrazine effects on premature rats (Stoker et al. 2000b). The investigation was made with the permission of the Committee of Ethics on Animal Experiments of the University of Novi Sad.

Light microscopy

After the decapitation, the thyroid gland from 10 animals of each experimental group was removed in a tissue block composed of trachea, esophagus and surrounding connective tissue. Samples were fixed in 4% paraformaldehyde (Merck, Darmstadt, Germany), embedded in paraffin and sectioned into 5 μm thick serial slices. For the histological analysis of thyroid structure and the morphometrical evaluation, paraffin sections were stained with hematoxylin and eosin (HE) (both stains by Merck). The histochemical staining method with toluidine blue (Carlo Erba, Milano, Italy) was used in order to demonstrate the population of mast cells in the thyroid.

Electron microscopy

The thyroid lobes from two animals of each experimental group were dissected after the sacrifice, fixed in 2.5% glutaraldehyde (Merck) in 0.1 M sodium cacodylate buffer (pH 7.4) (Fluka, Basel, Switzerland) at 4°C and postfixed in 1% osmium tetroxide (Fluka) for 1 h. Specimens were dehydrated through a graded series of acetone (J. T. Baker, Deventer, Holland) to propylene oxide (Merck) and embedded in epon resin (Merck). Sections of 0.5 μm and 1 μm thickness were obtained using an Leica ultracut UCT (Leica Microsystems, Nussloch, Germany), and they were stained with toluidine blue-azur (Merck and Sigma Chemicals Co., St Louis, MO, USA, respectively) to select areas for further sectioning. These areas were cut with a diamond knife into ultrathin sections, collected on copper grids (300 mesh) (Agar Scientific Ltd, Cambridge, UK) and contrasted with uranyl acetate (Merck) and lead citrate (Merck). The sections were examined and photographed using a JEOL JEM-100CX (Tokyo, Japan) transmission electron microscope.

Stereological analysis

The thyroid sections of 10 rats from each of the experimental groups were analysed using a multipurpose stereological grid M42 placed in the ocular of a Reichert light microscope (Reichert, Vienna, Austria) under a total magnification of $\times 400$. HE-stained sections were analysed on every fourth serial section (in total of three thyroid sections per each gland sample) and 60 fields of vision per thyroid sample. The point-counting was performed starting from the middle of the thyroid lobe (facing trachea) to the periphery. The volume density (Weibel 1979) of follicular epithelium (Vve) and colloid (Vvk) were determined and further used to calculate the volume density of follicles (Vvf) ($Vvf = Vve + Vvk$) and the thyroid activation index (Ia) ($Ia = Vve/Vvk$). The volume density of interfollicular tissue (Vvi) was also determined. Toluidine blue-stained sections were analysed on four thyroid sections per sample and 100 fields of vision per animal. The numerical (Nvm) (Weibel 1979) and volume (Vvm) density of the total mast cell population was determined as well as of the degranulated and of intact cells. In addition, the ratio of the degranulated to the intact mast cells was calculated.

The estimations were made by the same observer on blind-coded sections. The data were statistically analysed by analysis of variance (one-way ANOVA) and the Kruskal-Wallis test. When there were indications of statistical significance with the analysis of variance, the post-hoc Bonferroni test was used to compare the differences between the experimental groups, as well as the multiple comparison test for the significant outcomes of the Kruskal-Wallis test. The data were further analysed by a two- and a three-way analysis of variance (ANOVA). Two-way analysis of variance was performed with one between group levels (the six experimental groups) and one within stereological parameters (parameters for the thyroid gland vs. parameters for the mast cells). To discern quantitative interactions, a three-way analysis of variance was performed with two between the treatment levels (atrazine 20 mg/kg bw vs. atrazine 200 mg/kg bw and atrazine vs. combined treatments) and one within (parameters for the thyroid gland vs. parameters for the mast cells). *P* (probability)-values less than 0.05 were considered significant.

Results

Histological analysis

The results of histological analysis showed the predominance of microfollicles and columnar thyrocytes in the MF group, and a reduced interfollicular space compared to the control (Figure 1A, 1B). The thyroids of rats treated with a lower dose of atrazine (20 mg/kg bw) were composed mainly of macrofollicles with cuboidal epithelial cell lining and abundant colloid matter (Figure 1C). Ultrastructural analysis revealed the occasional presence of a rough endoplasmic reticulum with a massive dilated, irregularly shaped cisternae, containing heterogeneous electron-dense material and sparse ribosomes in the thyrocytes, compared to the typical

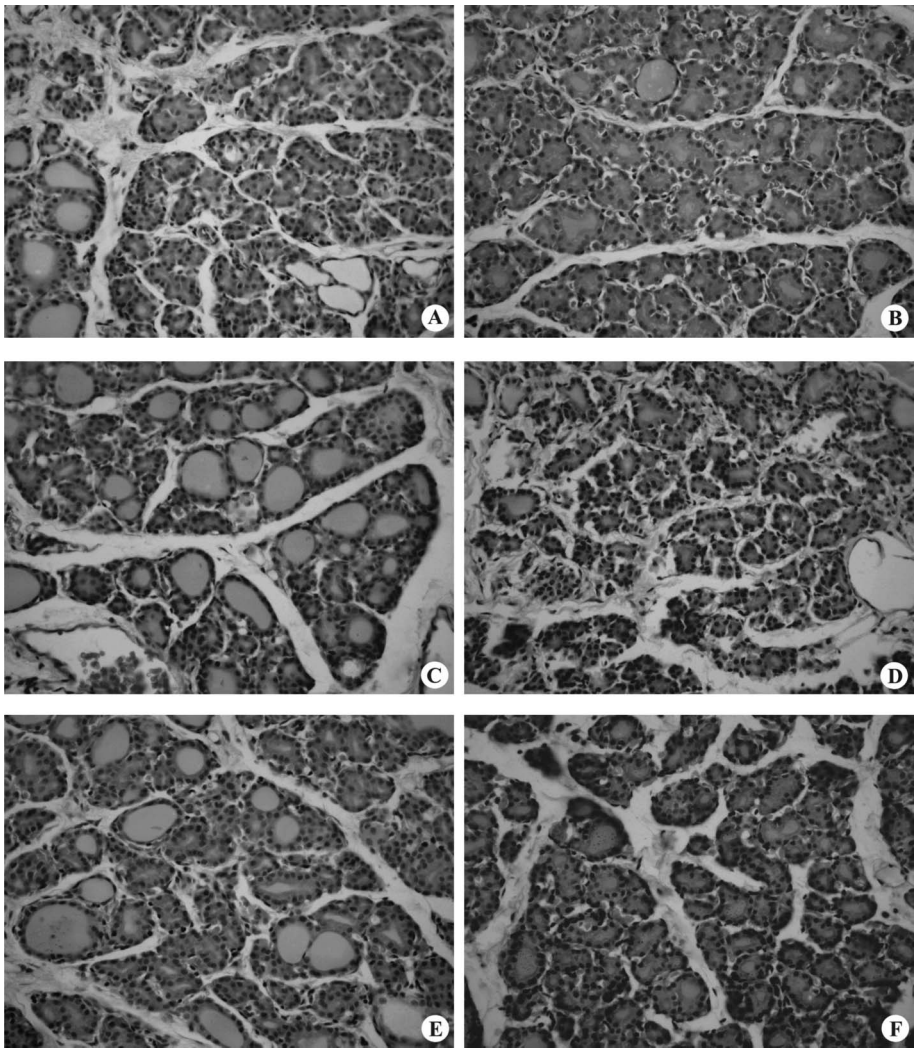

Figure 1. Photomicrographs of the thyroid gland stained with hematoxylin-eosin in experimental groups. (A) Control, (B) MF, (C) atrazine 20 mg/kg bw, (D) atrazine 200 mg/kg bw, (E) MF and atrazine 20 mg/kg bw, and (F) MF and 200 mg/kg bw. All photomicrographs are of the same magnification, $\times 400$.

ultrastructure of the control (Figure 2A, 2B). The experimental group treated with a higher dose of atrazine (200 mg/kg bw) was characterised by a number of microfollicles surrounded by the amassed connective tissue (Figure 1D). In the combined treatments of MF and atrazine (20 mg/kg bw or 200 mg/kg bw), both micro- and large follicles were observed with cuboidal epithelium and uniformly dense colloid (Figure 1E, 1F). Certain follicular cells in the group treated with MF and low dose of atrazine were characterised by a number of extended microvilli on the apical cell surface and by an abundance of electron-dense lysosomes varying in diameter in the apical cytoplasm (Figure 2C). Electron microscopic analysis of the thyroid follicular cells in the MF group, the high dose atrazine group and the combined group of MF with high dose of atrazine demonstrated typical subcellular characteristics (not shown).

The appearance of microfollicles with a rather narrow lumen without colloid, as the most prominent morphological change of the thyroid gland, were observed in half (five) of the MF-treated animals, in four of those treated with the high-dose of atrazine and in three animals exposed to combined treatments of MF and the high dose of atrazine (Figure 3A–E). In all cases, these microfollicles were observed only in one thyroid lobe while the other showed usual structural features. The contribution of follicles and of the connective tissue in these altered areas of the gland differed among animals of the same group and between the treatments too (Figure 3A–E).

The analysis of mast cells involved the total mast cell population within the thyroid and the functional heterogeneity of these cells. This analysis was based on morphological criteria which enabled the classification of mast cells into intact and degranulated,

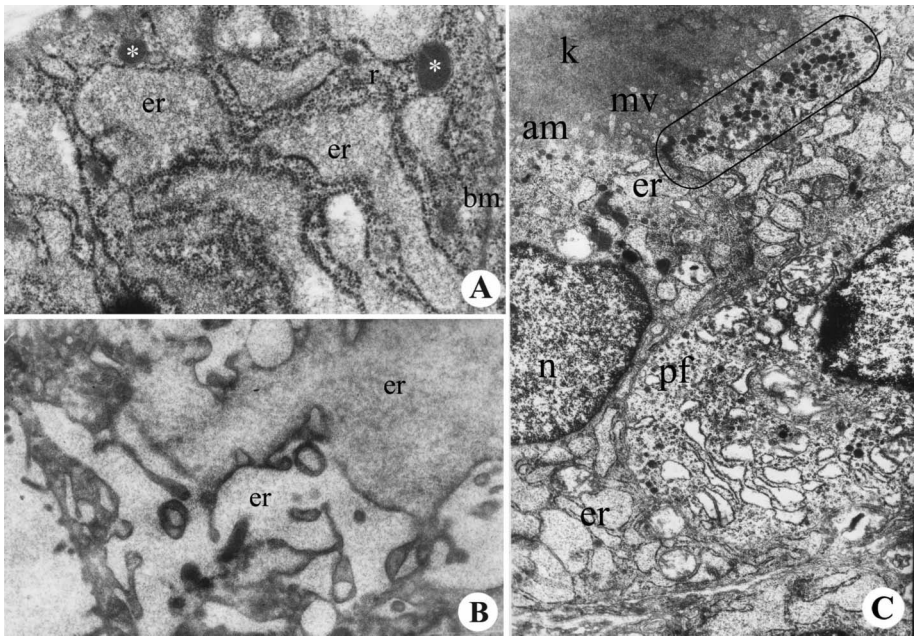

Figure 2. Electron micrographs of a part of the follicular cells in the thyroid gland. Control (A), atrazine 20 mg/kg bw (B) and MF and atrazine 20 mg/kg bw (C). Basal compartment of a thyrocyte in (A) with rough endoplasmic reticulum (er) and an abundance of ribosomes (r); lysosomes (asterisk), baso-lateral membrane (bm), magnification ×13,000. Rough endoplasmic reticulum (er) with dilated cisternae containing material of heterogeneous electron density in (B), magnification ×13,000. A number of: microvilli (mv) at the thyrocyte apical surface and lysosomes (frame) in the apical cytoplasm of the thyrocyte in (C); nucleus of the follicular cell (n), apical membrane (am), colloid (k), parafollicular cell (pf), magnification ×6,600.

the latter identified by the reduced cellular staining and granule content extrusion. Histological analysis revealed that mast cells commonly resided closely opposed to thyroid follicles or perivascularly. The occurrence rate of degranulated mast cells was lower in all the treated groups compared to the control (Figure 4A–F). No major differences in the distribution of mast cells among the groups were observed (Figure 4A–F).

Stereological analysis

According to the stereological analysis of the thyroid parenchyma and the connective tissue, the differences in the calculated values of all parameters of the treated groups compared to the control were not statistically significant at the $p < 0.05$ level (Table I). However, the volume density of the thyroid follicles was significantly higher in the MF group compared to both low (F test [F] = 4.75, $p = 0.0078$) and high (F = 4.75, $p = 0.0018$) dose of atrazine treatments as well as to the combined treatments with low (F = 4.75, $p = 0.014$) and high (F = 4.75, $p = 0.011$) dose of atrazine (Table I). The volume density of the connective tissue was significantly lower in the MF group compared to both low (F = 5.0, $p = 0.0014$) and high (F = 5.0 $p = 0.0028$) dose of atrazine treatments as well as to the combined treatments with low (F = 5.0, $p = 0.025$) and high (F = 5.0, $p = 0.016$) dose of atrazine (F = 5.0, $p = 0.016$) (Table I). The volume density of colloid in the group treated with low dose of atrazine was significantly higher (H = 14.7, $p = 0.0058$) compared to the group treated with the high dose of this compound (Table I). The numerical density of total mast cells was significantly lower (F = 3.42, $p = 0.014$) in the group treated with 200 mg/kg bw of atrazine as compared to the control group (Table II). The calculated ratio of the number of the degranulated to the intact mast cells points to its decrease in all the treated groups compared to the control (Table III).

Statistical testing of the data by a two-way analysis of variance revealed a significant interaction between the thyroid parameters and the parameters for mast cells (F = 5.88, $p = 0.0002$, partial eta-squared = 0.35). A three-way analysis of variance showed that the three factor interaction (thyroid and mast cell parameters, two atrazine doses, atrazine and combined treatments) was not statistically significant ($p > 0.05$). However, there was a statistically significant two-way interaction between thyroid and mast cell parameters (F = 13.72, $p = 0.0007$).

Discussion

The experiment was performed in order to investigate a possible synergistic effect of the MF and the

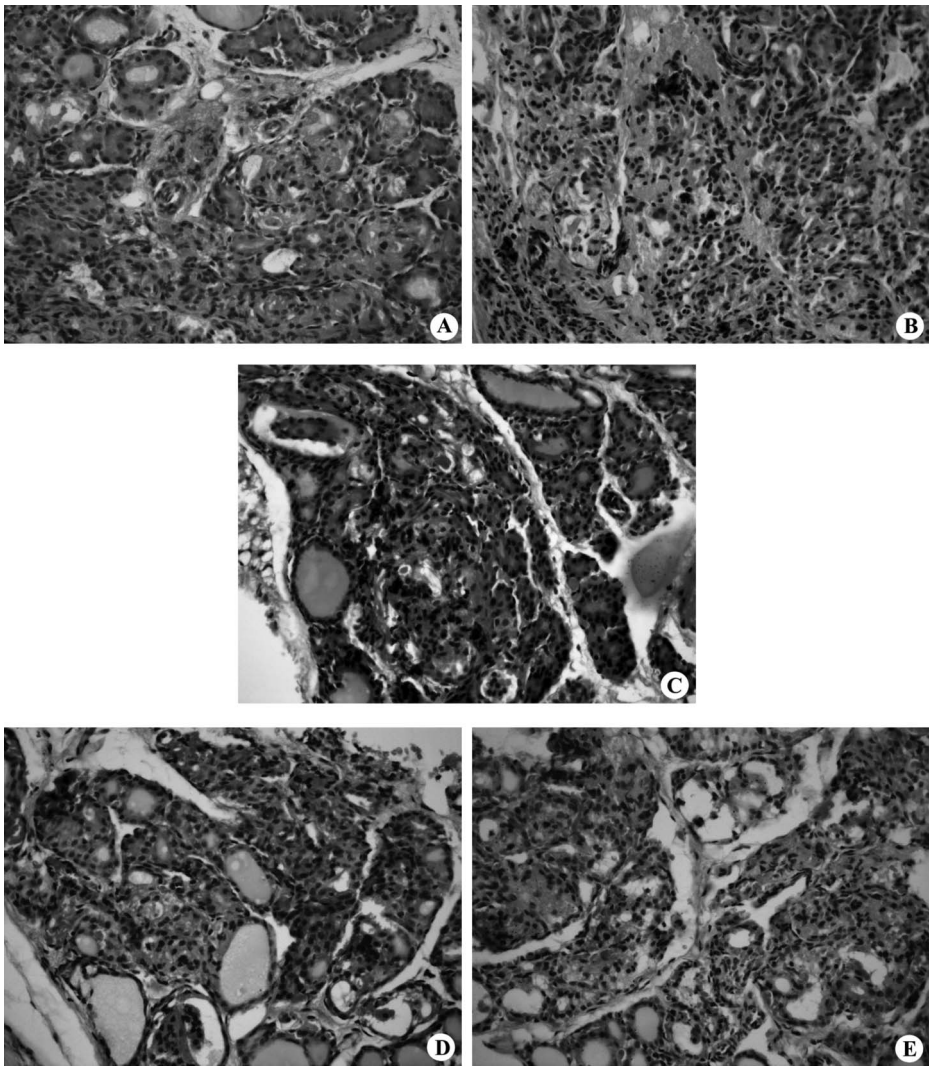

Figure 3. Photomicrographs of the thyroid gland stained with hematoxylin-eosin in the MF group (A, B), the group treated with a higher dose of atrazine (200 mg/kg bw) (C) and the group treated with MF and 200 mg/kg bw (D, E). Note the clusters of microfollicles with a very narrow lumen devoid of colloid or no lumen in (A–E) and the proliferation of the connective tissue in (A–E). All photomicrographs are of the same magnification, ×400.

endocrine-disrupting compound atrazine on the thyroid gland of juvenile/peripubertal male rats. The overall results of the investigated morphological parameters demonstrated no significant effect of the applied single treatments and their combinations, compared to the controls. However, the thyroids of certain treated groups were characterised by structural features somewhat different from those usually seen in the euthyroid states. The thyroid mast cells were significantly affected only by the treatment with a high concentration of atrazine.

MF treatments

The small diameter follicles with a low colloid content and columnar follicular cells point to a higher thyroid activity in the MF-exposed group, compared to the control. In the half animals of this group (five) the appearance of microfollicles with a rather narrow lumen devoid of colloid material was observed. These altered areas of the thyroid lobes were excluded during the stereological analysis, which might be the reason for the small differences in the numerical values between the control and the MF group, particularly for the volume density of the follicular epithelium.

The similar thyroid response to MF exposure was observed in our previous study in male rats aged 13 weeks at the beginning of the experiment (Rajkovic et al. 2006). During one month, the rats were exposed to MF of the same frequency and intensity, and with the same exposure dynamics as in the present study. The thyroid histology in both studies demonstrated similar structural characteristics after the MF exposure. However, there was a statistically significant difference between the control and the

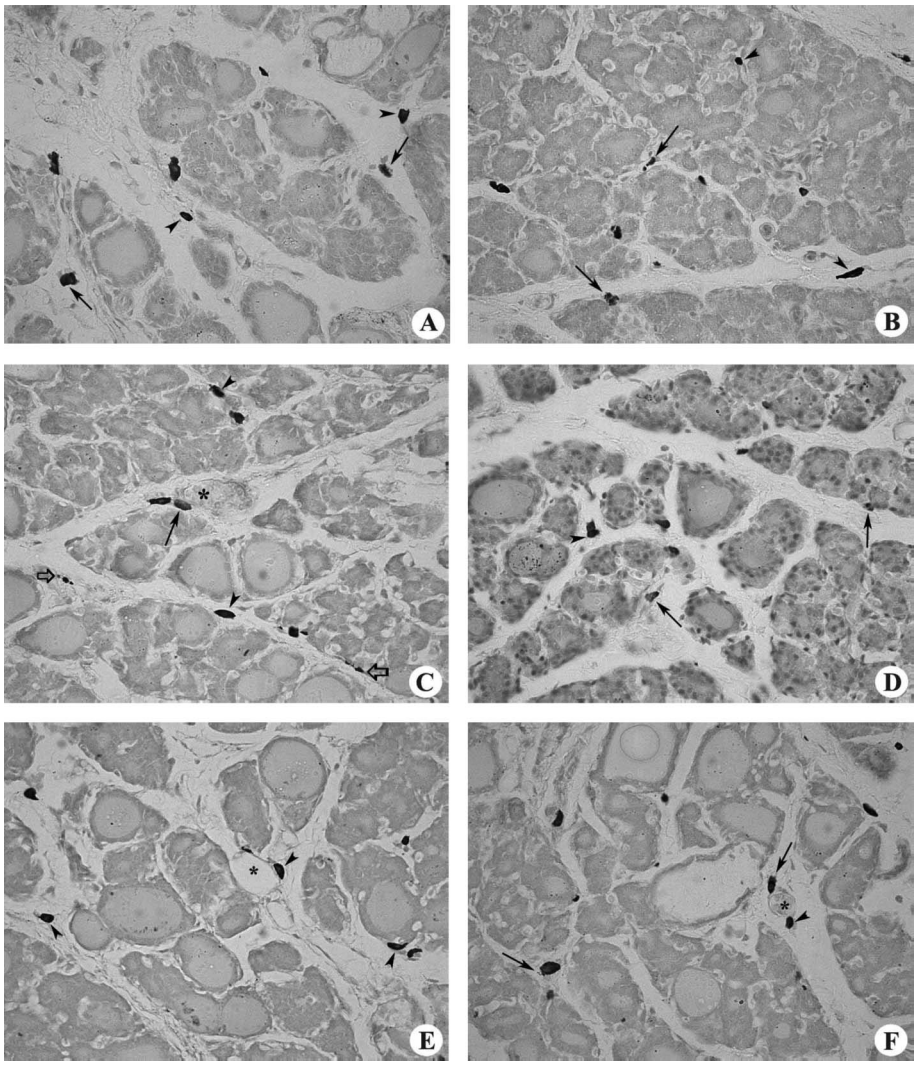

Figure 4. Mast cells in the thyroid gland of the experimental groups. (A) Control, (B) MF, (C) atrazine 20 mg/kg bw, (D) atrazine 200 mg/kg bw, (E) MF and atrazine 20 mg/kg bw, and (F) MF and 200 mg/kg bw. Intact cells (arrow heads), degranulated cells (arrows), extruded mast cell granules (open arrow) and blood vessels (asterisk). All photomicrographs are of the same magnification. Toluidine blue, ×400.

Table I. Mean values with the standard deviation (SD) of all investigated stereological parameters in the thyroid gland of control and exposed animals are given. Vvf, Vve, Vvk and Vvi represent, respectively, the volume density of: follicles, follicular epithelium, colloid and interfollicular connective tissue. Ia presents the thyroid activation index. ATR represents the atrazine treatment.

Stereological parameter	Control	MF	ATR 20 mg/kg bw	ATR 200 mg/kg bw	MF + ATR 20 mg/kg bw	MF + ATR 200mg/kg bw
Vvf (%)	59.0 ± 3.3	63.4 ± 5.4	56.1[b]** ± 3.2	55.2** ± 2.7	56.5* ± 4.6	56.3* ± 6.2
Vve (%)	48.6 ± 4.4	50.8 ± 7.7	43.2 ± 1.8	45.8 ± 3.9	43.8 ± 2.9	46.6 ± 5.3
Vvk (%)	10.4 ± 3.2	12.6 ± 5.1	13.6 ± 2.7	8.3** ± 2.5	12.8 ± 5.8	9.8 ± 2.1
Ia	5.2 ± 1.9	4.7 ± 1.9	3.3 ± 0.6	5.4 ± 1.8	4.1 ± 1.7	4.9 ± 1.0
Vvi (%)	40.7 ± 3.4	36.6 ± 5.5	44.9** ± 2.9	44.5** ± 2.7	43.1* ± 4.5	43.4* ± 6.2

Analysis of variance: Vvf, Vvi; Kruskal-Wallis: Vve, Vvk, Ia; Vvf: Significantly different from the MF group at $p < 0.05$ (*) or at $p < 0.01$ (**). Vvk: Significantly different from the ATR 20 mg/kg bw group at $p < 0.01$ (**). Vvi: Significantly different from the MF group at $p < 0.05$ (*) or at $p < 0.01$ (**).

exposed group in the previous investigation regarding the measured stereological parameters related to the epithelium, colloid and the connective tissue. Despite the similarities in the thyroid response to MF exposure in juvenile/peripubertal and pubertal rats, the inner-group variability was probably, due to the age, too high to lead to any statistically significant difference in the younger animals. This leads to the

Table II. Mean values with the standard deviation (SD) of all investigated stereological parameters for mast cells in the thyroid gland of control and exposed animals are given. Vv represent the volume density and Nv the numerical density of: total mast cell population (Vvm, Nvm), the intact cells (Vvm_{INT}, Nvm_{INT}) and degranulated cells (Vvm_{DEG}, Nvm_{DEG}). ATR represents the atrazine treatment.

Stereological parameter	Control	MF	ATR 20mg/kg bw	ATR 200mg/kg bw	MF + ATR 20mg/kg bw	MF + ATR 200mg/kg bw
Vvm (%)	0.223 ± 0.089	0.272 ± 0.116	0.249 ± 0.068	0.308 ± 0.087	0.268 ± 0.08	0.269 ± 0.078
Vvm_{INT} (%)	0.073 ± 0.065	0.09 ± 0.039	0.127 ± 0.066	0.086 ± 0.032	0.095 ± 0.048	0.066 ± 0.045
Vvm_{DEG} (%)	0.152 ± 0.065	0.342 ± 0.521	0.135 ± 0.046	0.22 ± 0.099	0.173 ± 0.07	0.202 ± 0.089
Nvm (mm^{-3})	18843 ± 5026	17214 ± 3154	15563 ± 3372	13608* ± 2214	15212 ± 2667	18001 ± 2888
Nvm_{INT} (mm^{-3})	5258 ± 4033	5767 ± 2839	6659 ± 1586	5694 ± 2688	7274 ± 1845	7795 ± 4430
Nvm_{DEG} (mm^{-3})	13090 ± 5635	11759 ± 2722	8717 ± 3732	8186 ± 2160	8603 ± 2078	11708 ± 1971

Analysis of variance: Vvm, Vvm_{INT}, Vvm_{DEG}, Nvm; Kruskal-Wallis: Nvm_{INT}, Nvm_{DEG}.
Nvm: Significantly different from the control at $p < 0.05$ (*).

Table III. Ratio between the degranulated and intact mast cells in the thyroid gland of control and exposed animals are given. Nvm represent the numerical density of mast cells. ATR represents the atrazine treatment.

Ratio	Control	MF	ATR 20mg/kg bw	ATR 200mg/kg bw	MF + ATR 20mg/kg bw	MF + ATR 200mg/kg bw
Nvm	2.5	2.04	1.31	1.44	1.18	1.5

conclusion that the thyroid gland of male rats exposed to the MF prior to adulthood responds to the treatment by increasing its activity which is less prominent in the prepubertal period than during the puberty. Results from the available literature on MF effects on the thyroid gland, involving the investigation of structural features of the thyroid parenchyma, showed no changes in the morphology of thyroid follicles after the exposure of adult rats to 0.5 Hz MF (1, 10 or 100 μT) (Lafreniere and Persinger 1979) and mice to 50 Hz MF (14 μT) (Svedenstal and Johanson 1998).

Atrazine treatments

Similarly to the MF group, the thyroids of the high dose atrazine group demonstrated morphological signs of an increased activity. Conversely, the treatment with ten times lower concentration of this substance caused the decrease in glandular activity, as indicated by the decreased thyroid activation index, compared to the control. This descrepancy in thyroid response to two administered concentrations of atrazine led to a significant difference in the content of colloid in the thyroid follicles between the two treated groups, but not when compared to the control. Therefore, the histological and stereological analyses point to an opposite, weak dose-response effect of atrazine upon the thyroid gland.

Our results regarding the treatment with 20 mg/kg bw of atrazine are in accordance with the data published by Kornilovskaya et al. (1996). The authors studied the effect of atrazine (0.2 LD50 [lethal dose 50%]) on rats during 6 and 12 days and demonstrated an increase in the thyroid follicular volume and the number of thyrocytes, as well as a decrease in the serum T3 concentration. However, investigating the effect of atrazine of various concentrations (12.5, 25, 50, 100 and 200 mg/kg bw) on the rat thyroid gland from PND 22–41, Laws et al. (2000) found no alterations in the amount of colloid material in the thyroid follicles, thickness of the follicular epithelium and no changes in the shape and diameter of the follicles either.

Combined treatments

The histological and stereological analyses demonstrated a decreased glandular activity in rats exposed to both combined treatments, compared to the control group. Similar to atrazine treatments, the differences in numerical values of stereological parameters between the control and the treated groups were more pronounced in the animals treated with MF and a lower concentration of atrazine. The morphometrical data revealed significant differences in the volume density of thyroid follicles and of connective tissue between the MF group and both atrazine treatments as well as between the MF group and both of the combined treatments. The discrepancies between these two stereological parameters, the first in glandular paranchyma and the second in the interstitium, indicate a difference in the action of the MF and atrazine on the thyroid tissue. The MF promoted the proliferation of glandular parenchyma while atrazine contributed to the proliferation of the connective tissue stroma, all in comparison to the referent thyroid structure of the controls.

In the light of the evidence that our study was performed on juvenile-peripubertal rats, the thyroid status in this period of ontogeny should not be considered apart from reproductive events. This is substantiated with the data regarding the initial increase in plasma testosterone between PND 25 and 35 and the more significant increase between PND 35 and 55 (reviewed in Stoker et al. 2000b). It is also important to note that the postnatal increase of TSH occur at about the time of puberty (Klug and Adelman 1979). Literature data suggest the testosterone effects on the thyroid physiology in means of its modulatory action on TSH-binding in the thyroid (Christianson et al. 1981, Watanobe and Takebe 1987). The orchidectomy significantly decreases serum TSH concentration in immature rats and decreases TSH binding to receptors on thyrocytes under in vitro conditions (Banu et al. 2001). Results of the experimental studies have demonstrated that MF, as well as atrazine, have a potency to alter serum testosterone levels leading to either an increase of this hormone by the MF (Forgacs et al. 1998) or a decrease by the atrazine treatments (100 or 200 mg/kg bw) (Trentacoste et al. 2001). Regarding the MF exposure in our study, the morphological features of the 53-day old male rats correspond to the reproductive parameters, e.g., to a possible facilitation of TSH binding in the thyroid by testosterone. Furthermore, the experimental data also suggest the testosterone effect on the proliferation of the thyroid follicular cells in prepubertal rats (Banu et al. 2002), which might explain the alterations in thyroid morphology identified in some animals of the MF group, characterised by a predominately microfollicular pattern. This characteristic structural feature of the thyroid in certain MF-exposed animals could not be solely attributed to testosterone, but potentially to other factors involved in the regulation of follicular organisation of the thyroid tissue. In addition to TSH, a primary thyroid morphological and physiological regulator, the assembly of thyrocytes into small diameter follicles is known to be regulated by the epidermal growth factor (EGF) and the tranforming growth factor beta 1 (TGF-ß1) (Westermark et al. 1991, Nilsson et al. 1995). Recent studies have provided valuable information about the importance of the latter in the regulation of the thyroid follicular cell physiology including the inhibition of thyroid peroxidase expression, protein iodination, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and the decrease in basal and TSH-stimulated cyclic adenosine monophosphate (cAMP) production and TSH receptor expression (Delorme et al. 2002). In addition, a positive correlation exists between the epithelial cell proliferation, the expression of growth and vascular factors and the extent of vascularisation indicating that the thyroid microcirculation is also involved in the control of the thyroid economy (Gérard et al. 2002). Taken together, reproductive and/or non-reproductive factors could participate in formation of microfollicles devoid of colloid not only in the MF, but also in the high-dose atrazine group and the group treated with the combination of these two factors (MF and atrazine).

Mast cells

The stereological analysis demonstrated a significant reduction in the number of thyroid mast cells, expressed through their numerical density, by the high dose of atrazine alone. The calculated data further revealed no significant alterations in the number of intact and degranulated mast cells in any of the applied treatments compared to the control. However, a trend of a dose-dependent decrease in the atrazine treatments compared to the control was found for the number of degranulated mast cells. The reduced degranulation of thyroid mast cells after the atrazine treatments was demonstrated previously for the perifollicular mast cells, while similar changes in the stromal mast cells were not found (Kornilovskaya et al. 1996). Investigating the response of cutaneous mast cells in our recently published study, we found a statistically significant increase in the total number of mast cells and in the number of degranulated cells in the low dose of atrazine treatment (20 mg/kg bw) and in both of the combined treatments (MF with 20 mg/kg bw atrazine and MF with 200 mg/kg bw) compared to the control (Rajkovic et al. 2010). The data point to a synergistic effect of MF and atrazine upon cutaneous mast cells, but also indicate that these cells are more sensitive to single (atrazine) and combined (MF with atrazine) treatments than the mast cells in the thyroid gland.

Although recent studies have demonstrated a rapid degranulation of rat basophilic leukemia mast cell line (RBL-2H3) and peritoneal mast cells by atrazine under in vitro conditions (Mizota and Ueda 2006), it seems that mast cells moderately add to the thyroid general response to the atrazine administered at low dose in our study. However, the number of the total mast cell population was significantly lower in the rats treated with the high dose of atrazine compared to the control, which could be attributed to a direct atrazine effect on mast cells or the effect of TSH. Although the number of intrathyroidal mast cells is positively correlated to plasma TSH and a release of metachromatic material is promoted by this hormone (Melander et al. 1971), the calculated stereological parameters for mast cells as well as for the thyroid parenchyma, seem to point clearly to a direct effect of atrazine. Regarding the MF influence on the mast cells, it should be mentioned that a theoretical model was proposed regarding the mast cell sensitivity to MF exposure according to which MF causes mast cell

degranulation and release of biologically active substances including histamine (Gangi and Johansson 2000). As mentioned above, our results have demonstrated that the thyroid mast cells of juvenile-peripubertal rats were not susceptible to our MF treatment. It is, however, conceivable that the use of immune markers against the mast cell mediators would more accurately reveal a possible MF- or atrazine-related involvement of these cells within the thyroid.

The calculations of changes in the ratio of the number of the degranulated to the intact mast cells demonstrated a high ratio in the MF group, but similarly low in other treatments compared to the control. This provided an important implication of atrazine having the greater power to affect the thyroid mast cells than the MF. In both atrazine and both combined treatments, the intact cells had a tendency to increase, as opposed to the degranulated cells. Consequently, the ratio was lower in comparison to the control, with the lowest value found in the combined treatment of MF with the low dose of atrazine. The overall data indicated a modification in the ratio of the two functional stages of the mast cells in all experimental groups that correlated well with the histological appearance of the thyroid under the applied treatments. This is further supported by the significant interaction between the thyroid and the mast cell parameters, which is of particular importance since mast cells may play an important role in folliculogenesis, hormogenesis and angiogenesis in the thyroid gland and are also involved in the control of thyroid blood flow and the secretion of thyroid hormones.

Conclusion

According to our results, the combined exposure to MF and atrazine had no severe impact on the investigated morphological parameters of the thyroid gland. Although there is a lack of statistical significance between the control and the treated groups, the histological and morphometrical results indicate that exposure to MF resulted in opposite effects on the thyroid gland structure from the treatment with a low dose of atrazine and from both of the combined treatments, respectively. The number of thyroid mast cells was significantly affected only by the high dose of the atrazine treatment. Furthermore, the data presented here demonstrated no synergistic effect of the MF and the endocrine-disrupting compound atrazine on the thyroid gland. However, the specific histological alterations of the thyroid parenchyma observed in some treated groups require further investigation.

Acknowledgements

The work was supported by the Serbian Ministry of Science and Technological Development grant No. 153001B (Belgrade, Serbia) and, partly, by the Cancer-och Allergifonden (Stockholm, Sweden), the Hjälpfonden (Stockholm, Sweden), Stiftelsen Tornspiran (Stockholm, Sweden) and the Karolinska Institute (Stockholm, Sweden). The authors thank Professor Tomas Hökfelt at the Department of Neuroscience, Karolinska Institute, Stockholm, Sweden, for general support. The authors also thank Ms Milena Stosic for the skilful technical assistance and Ms Margareta Krook-Brandt (at the Department of Learning, Informatics, Management and Ethics, The Medical Statistics Service Group, Karolinska Institute, Stockholm, Sweden) for expert support with the statistical evaluation.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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