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Combined Exposure of Peripubertal Male Rats to the Endocrine-Disrupting Compound Atrazine and Power–Frequency Electromagnetic Fields Causes Degranulation of Cutaneous Mast Cells: A New Toxic Environmental Hazard?

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Abstract The effects of single and combined treatments of the endocrine-disrupting compound atrazine and the power–frequency electromagnetic fields (EMFs) were investigated on cutaneous mast cells in juvenile/peripubertal male Wistar rats. Animals were divided into six groups: (1) 4 h/day exposure to EMFs (50 Hz), (2) 20 mg/kg of body weight (bw) of atrazine, (3) 200 mg/kg bw of atrazine, (4) EMFs with 20 mg/kg bw of atrazine, (5) EMFs with 200 mg/kg bw of atrazine, and (6) control. Both the atrazine and the combined treatments, but not the single EMF exposure, increased the number of degranulated mast cells. Statistically significant differences were demonstrated between the control and both of the combined treatments ($p < 0.01$ and $p < 0.001$, respectively). Additionally, low and high doses of atrazine combined with the EMFs were found significantly different when compared to the EMF group alone (both at $p < 0.001$). Considering the biological importance of mast cells in cutaneous immune reactions, future studies should reveal whether combined exposures to chemical and physical environmental agents pose a serious health risk.

Mast cells are resident in many tissues, including the connective tissue of the skin. They are found associated with blood vessels and nerves in cutaneous dermis and under the epithelial lining of the skin facing the external environment. Cytoplasmic granules of cutaneous mast cells contain preformed mediators, such as histamine and serotonin, two potent vasoactive amines involved in inflammatory and allergic reactions. In addition, mast cells are able to produce mediators *de novo*, typically during IgE-mediated activation, such as leukotrienes, prostaglandins, and cytokines, which are usually absent in resting cells (reviewed in Metcalfe et al. 1997). The effects of mast cells are related to the extracellular degranulation. In addition the immunologic stimuli, the release of mast cell mediators can be triggered by various chemical substances, including pesticides, as well as exposure to ionizing and nonionizing radiation (Albrecht et al. 2007; Malcotti et al. 2002; Rajkovic et al. 2005b; Rodgers and Xiong 1997b).

The chlorotriazine herbicide atrazine (IUPAC: 6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine) is excessively used on crop fields to control broadleaf weeds in the production of corn, sugar cane, and sorghum (US EPA 2001). Although banned in the European Union (EU), atrazine is continuously used in non-EU countries as well as in the United States. Due to its heavy use, the toxicological profile of atrazine has been investigated over the years. To date, the toxicity of atrazine was found in both wildlife and laboratory conditions primarily related to morphological and physiological endocrine and reproductive end points (Friedmann 2002; Jooste et al. 2005; Solomon et al. 2008; Stoker et al. 2000a). Reports on the immunotoxicity of atrazine particularly relating to its effect on mast cells are scarce. A study using 1-month-old mice demonstrated a significant dose-dependent decrease in spleen and thymus cell numbers after a 14-day oral application of atrazine at doses of 5, 25, 125, or 250 mg/kg body weight (bw) (Filipov et al. 2005). Seven weeks after the last applied atrazine dose of 25 mg/kg bw and greater, the cell numbers in the spleen were still decreased (Filipov et al. 2005). Maternal exposure to 35 mg/kg bw of

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atrazine decreased the primary (IgM) T-dependent antibody response and delayed-type hypersensitivity responses in 8-week-old male offspring (Rooney et al. 2003). In a similar study assessing the potential developmental toxicity of atrazine, the number of CD8+ T-cells, CD4+ T-cells, or B220+ B-cells were not altered in 3-month-old mice after gestational–lactational exposure to subcutaneously applied atrazine (700 μg/day), but an increase in T-cell proliferation and cytolytic activity were detected after in vitro stimulation (Rowe et al. 2006). In vitro studies demonstrated the dose-dependent degranulation of mast cells after atrazine treatment (Mizota and Ueda 2006).

Along with the chemical contamination of the environment in industrialized societies, a potential toxic environmental "pollution" originating from the use of electricity emerges in both residential and occupational environments. The number of artificial sources producing electromagnetic fields (EMFs), particularly power–frequency fields (50/60 Hz), constantly increased from the beginning of the 20th century. Current literature data regarding the biological effects of these fields point to their influence on all organ systems, including the skin (Johansson 2009). The effects of EMFs (50 Hz and 2, 16, or 32 kA/m) on mast cells were found in the skin, lymph nodes, and intestine in experimental rodents after exposure for 4 h during 5 days (Iurina et al. 1997) and in rat brain after exposure to EMFs (7 or 40 Hz and 50 or 500 nT) for 15 nights (Cook et al. 2000). However, peritoneal mast cells were unaffected by acute exposure to EMFs (60 Hz, 5 mT) in in vitro conditions (Price and Strattan, 1998). In skin biopsies of healthy volunteers exposed to TV or PC screens for 2 or 4 h, the number of mast cells increased in the papillary dermis, and migration of these cells toward the epidermis and their degranulation was also observed (Johansson et al. 2001).

Investigating the interaction between the occupational exposure to EMFs and cancerogenic chemicals in male industrial workers in Sweden, Navas-Acien and associates (2002) found a significantly increased relative risk of glioma associated with exposures to 0.13–0.20-μT EMFs and arsenic or peak pesticide exposures. For the workers exposed to somewhat higher EMFs ranging from 0.20 to 0.30 μT, a significant relative risk of glioma was found with exposure to lead and elevated risks for the chromium/nickel, metallic compounds, or petroleum products. The data analysis for the combined exposures to EMFs and different chemical substances for meningiomas demonstrated an increased risk only for lead (Navas-Acien et al. 2002). The experimental study on Jurkat cells designed to investigate the combined effects of EMFs (1 mT) and the carcinogenic substance benzene or benzene-hydroxylated metabolites (catechol, hydroquinone, and 1,2,4-benzenetriol) demonstrated a significant increase in DNA damage for the joint exposures to EMFs and hydroquinone or 1,2,4-benzenetriol (Moretti et al. 2005). The single exposure to EMFs or to hydroquinone was unable to cause genotoxic effects in cultured cells, whereas 1,2,4-benzenetriol was demonstrated to be a potent genotoxic agent (Moretti et al. 2005).

In the present study, we aimed at investigating the effect of atrazine and power–frequency EMFs on cutaneous mast cells separately and in combination based on the following grounds: (1) Atrazine is an environmental contaminant commonly detected in groundwater and surface water, including drinking water (Barbash et al. 2001; Rice et al. 2004; WHO 2003), (2) EMFs generated by power lines and electric appliances currently represent an unavoidable physical factor in the human living and working environment, and (3) it is likely that humans are exposed simultaneously to various combinations of chemical and physical environmental agents, which raises concern about their possible health effects on the human population.

Materials and Methods

Animals

The study was performed on male rats of the Wistar strain subjected to treatments from postnatal day (PND) 23 to PND 53. Animals were housed in plastic cages under laboratory conditions at 20 ± 2°C and were subjected to a controlled photoperiod (14 h light, 10 h dark). Before the beginning of the experimental procedure (PND 22), all males were weighted and randomly assigned to one of the experimental groups. Animals were sacrificed on PND 53, after daily treatments. The investigation was made with the permission of the Ethical Committee on Animal Experiments of the University of Novi Sad.

Considering the lack of consensus on animal experiments with EMFs, the particular period of ontogeny (PND 23 to PND 53) was chosen according to the EPA (Environmental Protection Agency, USA) protocol for the assessment of the impact of endocrine-disrupting compounds (EDCs) on male rats (Stoker et al. 2000b).

Experimental Procedure

Animals were divided into six groups. Each group consisted of ten rats with similar initial body weights. The first group (EMF group) was subjected to daily 4-h exposure to EMFs (50 Hz, 100–300 μT, 54–160 V/m), the second and third groups were receiving atrazine *per os* at a concentration of 20 and 200 mg/kg bw, respectively, two groups were subjected to the combined exposures to EMFs and either 20 or 200 mg/kg bw of atrazine, and the sixth was the control group.

For the EMF treatments, the exposure system was used as previously described (Rajkovic et al. 2005a). Animals were exposed to EMFs from 10.00 to 14.00 h. Atrazine (98% purity, a gift from Sanja Lazic, Institute for Environmental and Plant Protection, Faculty of Agriculture, University of Novi Sad, Serbia) dissolved in edible olive oil was administered daily between 08.00 and 09.00 h. Dose groups were selected based on previous studies on atrazine effects on premature rats (Stoker et al. 2000b).

Light Microscopy

After decapitation, samples of skin (10 per group) from the interscapular region were taken, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned into 5-μm-thick sections. For the histological analysis and quantitative estimation of mast cells, the histochemical staining method with toluidine blue (Carlo Erba, Milano, Italy) was used.

Samples of skin (two per group) designated for Epon embedding were dissected, fixed in 2.5% glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 M sodium cacodylate buffer (pH 7.4) (Fluka, Basel, Switzerland) at 4°C, and postfixed in 1% osmium tetroxide (Fluka, Basel, Switzerland) for 1 h. Specimens were dehydrated through a graded series of acetone (J. T. Baker, Deventer, Holland) to propylene oxide (Merck, Darmstadt, Germany) and embedded in Epon resin (Merck, Darmstadt, Germany). Semithin sections of 0.5 and 1 μm were obtained using an Leica Ultracut UCT (Leica Microsystems, Nussloch, Germany) and stained with toluidine blue–azur (Sigma Chemicals Co., St. Louis, USA).

Stereological Analysis

Skin sections from ten rats of each of the experimental groups were analyzed using a multipurpose stereological grid M42 placed in the ocular of a Reichert light microscope under a total magnification of ×400. Point-counting was performed starting from the epidermal–dermal junction on 4 skin sections per sample and 50 fields of vision per sample. Skin sections were randomly selected but avoiding analysis of subsequent sections and, consequently, duplicate counts of the same cells. Consecutive fields of vision were counted in order to provide a comprehensive analysis and to enable the screening of mast cells populating the upper dermis of the entire section analyzed. The numerical (Nvm) and volume (Vvm) densities (Weibel 1979) of the total mast cell population of degranulated cells and of intact cells in skin dermis were determined.

Estimations were made by the same observer on blind-coded sections. Each of the variables was analyzed with the Kruskal–Wallis test among the six groups. When there were indications of an overall statistical significance, the multiple comparison test was used with adjusted p-values in order to discern differences between the experimental groups. Additionally, a principal component analysis was performed on the data followed by a one-way analysis of variance for the first component, which was approximately normally distributed, with the six groups as the factor. p-Values less than 0.05 were considered significant.

Results

Histological Analysis

Toluidine blue-positive mast cells were identified in the upper, middle, and lower dermis. Deeper parts of the dermis were occupied with a number of large-diameter intact and degranulated cells, and the middle dermis was occupied with only scarce cells, mostly intact with an intense metachromatic reaction. Mast cells of the upper dermis were characterized by a decreased metachromatic reaction, reduction of the cell volume, and a predominance of degranulated over intact cells (Figs. 1a–f and 2a–c). Semithin sections revealed a number of degranulated cells in both of the combined groups and were frequently found in close proximity to small blood vessels situated in the upper dermis (Fig. 2a–c). Further analysis using stereological methods enabled a detailed screening of the differences in the number and volume of the total population of mast cells as well as of intact and degranulated subtypes.

Stereological Analysis

Results of stereological measurements demonstrated an increase in the total volume density of mast cells in all treatments compared to the control, whereas the differences between the control and the low dose of atrazine and between the control with the combined treatment of EMFs with the low dose of atrazine was statistically significant ($p < 0.01$ and $p < 0.001$, respectively) (Table 1). A similar trend of increased numerical data in the treated groups compared to the control was obtained for the volume density of degranulated mast cells with the statistically significant difference found between the control and the low dose of atrazine ($p < 0.01$) and between the control and the low-dose atrazine treatment combined with the EMF exposure ($p < 0.001$) (Table 1).

The numerical density of total mast cells and of degranulated cells in all treated groups was increased compared to the control, except for the EMF treatment alone. The outcomes of the statistical analysis of these two parameters were the same regarding statistical significance between particular experimental groups. Therefore, the

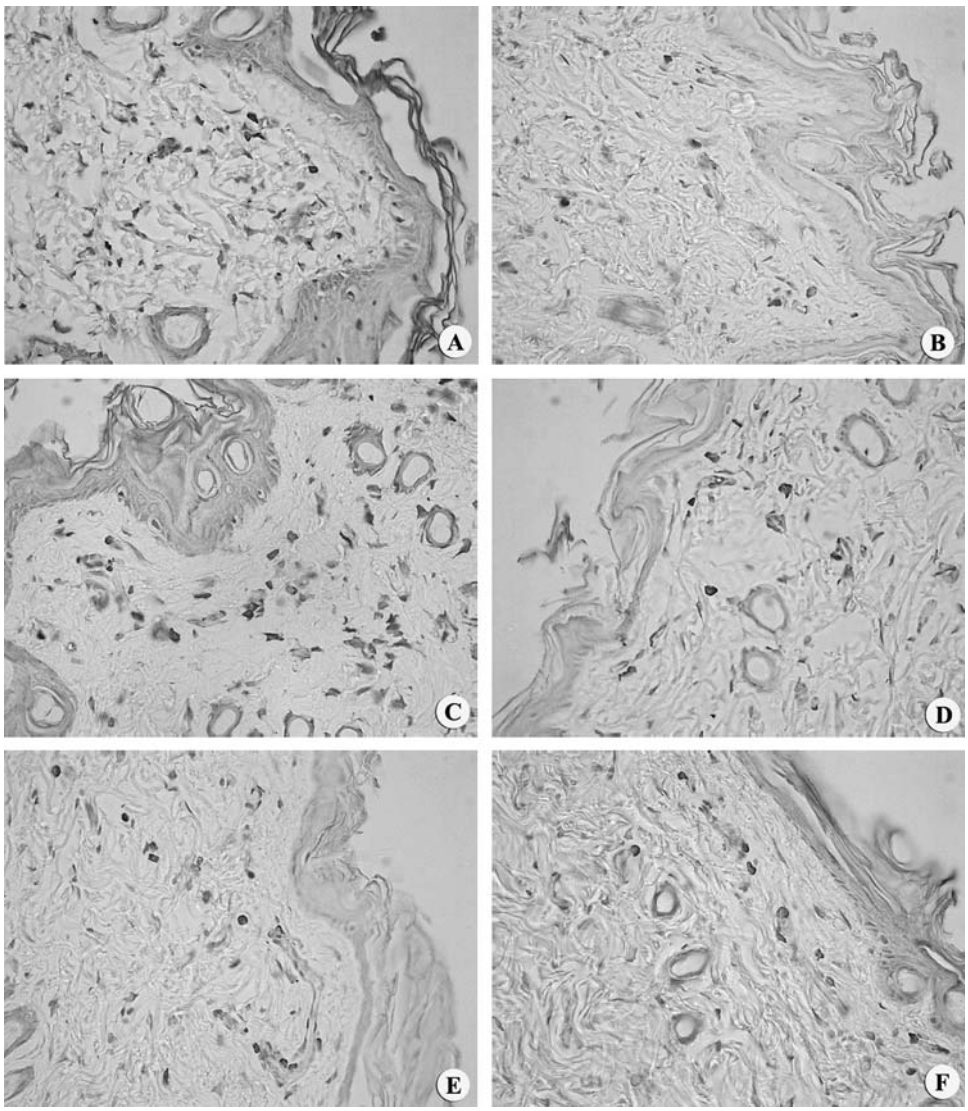

Fig. 1 Photomicrographs of mast cells in the upper dermis of paraffin sections stained with toluidine blue: **a** control, **b** EMF, **c** atrazine 20 mg/kg bw, **d** atrazine 200 mg/kg bw, **e** EMF with atrazine 20 mg/kg bw, and **f** EMF with 200 mg/kg bw. Note the difference in the mast cell number among the groups and the difference in the intensity of the metachromatic staining. All photomicrographs are of the same magnification, ×400

results for the total and degranulated cells are presented together in the following section. Statistically significant differences were demonstrated between the control and the low dose of atrazine ($p < 0.05$) and between the control and both of the combined treatments of EMFs and the low and high doses of atrazine ($p < 0.01$ and $p < 0.001$, respectively) (Table 1). Additionally, low and high doses of atrazine combined with the EMFs were found significantly different when compared to the EMF group alone (both at $p < 0.001$) (Table 1). The differences between the high dose of atrazine treatment and the combined treatment of EMFs with the high dose of atrazine were also found to be significantly different ($p < 0.05$ and $p < 0.01$, respectively, for the total number of mast cells and both at $p < 0.01$ for the degranulated cells) (Table 1).

Analysis of the intact mast cells demonstrated no significant alterations for the volume density of these cells, whereas a significant difference was found for the numerical density between the control and the low dose of atrazine ($p < 0.01$) and between the control and the group exposed to EMFs and the low dose of atrazine ($p < 0.001$) (Table 1). The statistically significant differences ($p < 0.05$) between the different treatments (low dose of atrazine, high dose of atrazine, EMF) are demonstrated in Table 1.

Our findings demonstrate that, compared to the control, the low concentration of atrazine had influenced all investigated stereological parameters for mast cells in a greater extent than the high dose of atrazine (e.g., numerical data show no dose-response effect of atrazine, except for the numerical density of intact mast cells) (Table 1). Consequently, the same trend was found for the combined treatments of a low or high dose of atrazine with EMFs compared to the control (Table 1).

One-way analysis of variance with factor group revealed an overall statistical significance ($p < 0.05$),

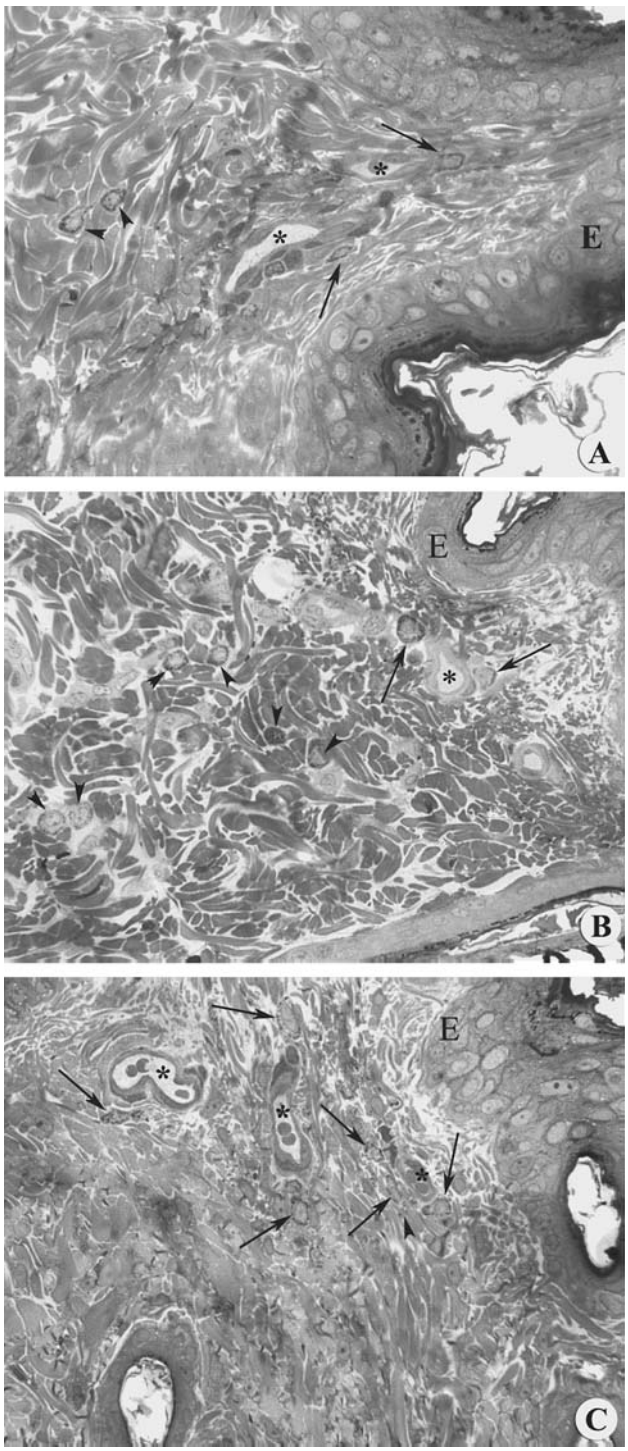

Fig. 2 Photomicrographs of mast cells in the upper dermis of semithin sections stained with toluidine blue–azur: **a** control, **b** EMF with atrazine 20 mg/kg bw, and **c** EMF with 200 mg/kg bw. Skin epidermis (*E*), mast cells (*arrow head*) in the cutaneous connective tissue, and degranulated mast cells (*arrow*) closely aposed to blood vessels (*asterisk*). All photomicrographs are of the same magnification, ×630

however, the post hoc Tukey test demonstrated no statistically significant differences between the experimental groups.

Discussion

According to our findings, single low- and high-dose atrazine treatments and both of the combined treatments (atrazine/EMFs) caused degranulation of cutaneous mast cells in juvenile/peripubertal male rats. The most pronounced responses were detected in the treatment with a low dose of atrazine and both of the combined treatments. Atrazine at both low and high doses in combination with EMFs had a synergistic effect. Significant differences, found between the treatments and the control, related to degranulated cells point to the importance of mast cells in the biological response of skin to the applied treatments.

Literature data regarding atrazine effects on mast cells provide substantial data from both in vitro and in vivo studies about the sensitivity of these cells to atrazine treatments and the mechanism involved in the process of mast cell degranulation. An experimental study, aimed to test whether EDCs affect the mast cell line RBL-2H3, demonstrated degranulation of mast cells by atrazine as measured through the release of granule-associated β-hexosaminidase, but no degranulation was detected by the other investigated substances (amitrol, benzophenol, bisphenol A, pentachlorophenol, tetrabromophenol A) (Mizota and Ueda 2006). The atrazine-induced degranulation was dose dependent between 10 nM and 1 μM and was mediated through putative membrane receptors as assumed by blockage of degranulation with the membrane-impermeable BSA-conjugate PROG-BSA (Mizota and Ueda 2006). Further characterization of mast cell degranulation showed that this process is mediated through the neurosteroid receptor $G_{q/11}$, phospholipase C, and calcium mobilization from intracellular stores (Mizota et al. 2005, Mizota and Ueda 2006). In light of the well-known vascular effects of mast cell-derived mediators, it is important to note that an in vivo study with the Evans blue extravasation test demonstrated an increased vascular permeability in the skin after intraplantar administration of atrazine (10 fmol) to mice (Mizota and Ueda 2006).

Contrary to atrazine, another triazine herbicide, simazine (1, 10, or 100 μM), had no potency to increase either spontaneous or antigen-stimulated histamine release from peritoneal mast cells in in vitro conditions (Sato et al. 1998). However, the organophosphate pesticide malathion at the concentration of 1 mg/kg bw led to degranulation of cutaneous mast cells in mice after oral administration for 90 days (Rodgers and Xiong 1997b). A 10-fold higher concentration of this substance increased the level of serum histamine in mice 4 h after *per os* application (Rodgers and Xiong 1997a).

Current data based on experimental animal studies and studies involving human subjects point to the ability of EMFs to affect mast cells under certain conditions. Our

Table 1 Median values with the lower and upper quartiles (in parentheses) of all investigated stereological parameters for mast cells in the skin of control and exposed animals

Stereological parameter	Control ($n = 10$)	EMF ($n = 10$)	ATR 20 mg/kg bw ($n = 10$)	ATR 200 mg/kg bw ($n = 10$)	ATR 20 mg/kg bw and EMF ($n = 10$)	ATR 200 mg/kg bw and EMF ($n = 10$)
Vvm (%)	0.19[a]	0.26[a,b]	0.38[b,c]	0.24[a,b]	0.35[c]	0.26[a,b,c]
	(0.19)	(0.24)	(0.24)	(0.19)	(0.28)	(0.19)
	(0.24)	(0.28)	(0.52)	(0.28)	(0.48)	(0.28)
Vvm_{INT} (%)	0.048[a]	0.048[a]	0.048[a]	0.048[a]	0.048[a]	0.048[a]
	(0.048)	(0.048)	(0.048)	(0.048)	(0.048)	(0.048)
	(0.048)	(0.048)	(0.048)	(0.048)	(0.048)	(0.095)
Vvm_{DEG} (%)	0.14[a]	0.19[a,b]	0.33[b,c]	0.19[a,b]	0.30[c]	0.19[a,b]
	(0.095)	(0.14)	(0.19)	(0.14)	(0.24)	(0.14)
	(0.19)	(0.24)	(0.48)	(0.24)	(0.43)	(0.24)
Nvm (mm^{-3})	18797[a]	16163[a,b]	24744[c,d]	20551[a,b,c]	30262[d]	30180[d]
	(15941)	(14379)	(19618)	(18190)	(23978)	(27184)
	(19653)	(19402)	(32250)	(24345)	(37140)	(50316)
Nvm_{INT} (mm^{-3})	2458[a]	954[b,c]	1195[a,b,c]	2591[a,b]	609[c]	2755[a,b]
	(1563)	(609)	(726)	(981)	(609)	(1261)
	(4420)	(1563)	(2054)	(3362)	(996)	(4688)
Nvm_{DEG} (mm^{-3})	17012[a]	14358[a,b]	23550[c,d]	17988[a,b,c]	30610[d]	29423[d]
	(14607)	(13501)	(17416)	(16092)	(23161)	(25203)
	(19593)	(19248)	(30642)	(22278)	(36391)	(53463)

Note: *Vv* represent the volume density and *Nv* represents the numerical density of: total mast cells (*Vvm*, *Nvm*), intact cells (Vvm_{INT}, Nvm_{INT}), and degranulated cells (Vvm_{DEG}, Nvm_{DEG}). All median values designated with the same letter indicate homogenous groups (Kruskal–Wallis, $p > 0.05$)

previous studies have demonstrated an increase in toluidine blue-positive mast cells and a significant increase in serotonin-positive mast cells in the skin of 3-month-old male rats exposed to power–frequency EMFs (50 Hz, 100–300 μT) 4 h/day for 30 days (Rajkovic et al. 2005a, 2005b). Analysis of histamine-positive mast cells revealed a majority of cells to be degranulated, but the overall number was lower compared to the control (Rajkovic et al. 2005b). In addition, a moderate increase in the eosinophil cationic protein (ECP)-positive cells was recorded indicating inflammatory response of the skin to EMF exposure (Rajkovic et al. 2005b). An increased number of mast cells as well as alterations in the content of calcitonin-gene-related peptide (CGRP), somatostatin, and protein S-100 in skin cells and nerve fibers were found in skin biopsies of human subjects expressing subjective and/or objective skin symptoms of erythema, papules, pustules, and sensations of heat, itch, pain, and smarting related to video display terminal exposure (Johansson et al. 1994, 1996).

In the present study, we demonstrated the ability of atrazine applied at a dose of 20 mg/kg bw to degranulate mast cells and a trend for the high dose of 200 mg/kg bw. Our findings demonstrate a nonlinear dose-response of cutaneous mast cells to atrazine exposure for all of the investigated parameters. An explanation for such findings is made difficult by the application of atrazine in two concentrations that are 10-fold apart. Substantial further work is needed in order to test the observation of these adverse effects involving the use of immune markers against mediators derived from mast cells, a series of different atrazine doses, and different durations of exposure. At this point, it could be hypothesized that a high dose of atrazine effectively affected mast cells in juvenile/peripubertal rats only for a short duration, thus disabling the assessment of its full activation potential after a subchronic application. The lack of dose-response to atrazine treatments could also be attributed to the adaptation of mast cell activity in the skin to the high dose of atrazine. Nevertheless, findings from this study support the need not only to further study atrazine effects on cutaneous mast cells in order to clarify the mast cell response to atrazine exposure but also to investigate its nonlinearity in young rats and to test the existence of a nonlinear dose-response in the adults. In addition, a different stereological approach might be used aimed to yield better numerical scores for the volume density parameters, thus providing fewer discrepancies between the outcomes for the volume and numerical densities.

Morphological and quantification-based analysis demonstrated that the potential of atrazine to cause cutaneous

mast cell degranulation was further emphasized by the synergistic action of EMFs. The toluidine blue, to which mast cells in the skin were exposed during the histochemical staining procedure, yields a metachromatic staining of the proteoglycan molecules stored in the cytoplasmic secretory granules. It has been recently demonstrated that histamine and serotonin are costored with proteoglycans in mast cell secretory granules and released upon cellular activation (Ringvall et al. 2008). Based on these data, it can be assumed that these two amines were released into the dermal connective tissue in the atrazine and combined groups in our study, as evidenced by a weak metachromatic staining of a number of mast cells. Because it is known that the number of mast cells increases at sites of inflammation and allergy and that histamine and serotonin are potent vasoactive mediators exerting increased vascular permeability and plasma extravasation (Yong 1997), further studies are needed to reveal the type of reaction involved in the skin.

In summary, it can be concluded that the EDC atrazine at both investigated doses produced a significant effect on cutaneous mast cells when combined with EMFs under the present experimental conditions. Considering the biological importance of mast cells in cutaneous physiology, future studies are needed to identify the exact mast cell mediators involved. Additionally, research involving prospective epidemiological studies should reveal the importance of combined environmental chemical and physical exposures and whether they pose a serious risk to human health.

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