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Long term effect of extremely low frequency electromagnetic field on islet of pancreas structure

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Summary. According to epidemiological studies, living and working in the presence of extremely low frequency electromagnetic fields (ELF EMFs) may contribute to several modern human diseases, and may explain the large increase in the incidence of diabetes and pancreatic cancer in urban areas. However, the influence of ELF EMFs on the pancreas remains unclear. The present study tests the effects of 5 months exposure to non-homogenous extremely low frequency electromagnetic fields (50 Hz) of two different intensity (500-50 μT and 1000-100 μT) on endocrine pancreatic structure in Wistar rats. Results show that animals exposed to ELF EMFs of low and high intensity display a reduced number of islets per section of pancreas: an effect particularly pronounced in animals exposed to stronger fields. The mean islet diameter was significantly higher in both groups versus control ($p<0.01$, respectively). In contrast, exposure to ELF EMFs had no significant influence on the total volume density of pancreatic islets. However, ELF EMF exposure did induce an increase in the volume density of connective tissue and blood capillaries in Langerhans islets, especially upon treatment with ELF EMFs of higher intensity. ELF EMFs of lower intensity increased the percentage of alpha and beta cells while reducing the percentage of D and PP cells. In addition, low intensity ELF EMF fields increased the volume density of alpha cells, decreased the volume density of D and PP cells, and had no influence on the volume density of beta cells. ELF EMFs of higher intensity decreased the percentage of alpha and D cells, while increasing the percentage of beta and PP cells. ELF EMFs of higher intensity induced an increase in the volume density of alpha cells, decreased D cell volume density, and had no influence on the volume density of beta and PP cells. Thus, according to our results, alpha cells displayed the greatest sensitivity to changes in ELF EMF intensity, while all other non-beta cells, as well as the beta cells do not alter their response profile depending on the intensity of the ELF EMFs. However, further investigations are required to elucidate intra-islet interactions between different endocrine islet cells types during ELF EMF exposure.

Key words: beta cells, extremely low frequency electromagnetic field (ELF EMF), islet of Langerhans, non-beta cells, pancreas, stereological analysis.

INTRODUCION

Extremely low frequency electromagnetic fields (ELF EMFs) are defined as those having a frequency of up to 300 Hz; and are a non-ionizing form of radiation, with photon energy that is too weak to brake atomic bonds (Lee et al. 2004). Numerous field and laboratory studies indicate that ELF EMFs originating from residentially proximate power lines, household electrical wiring, medical devices and other sources from our working and living environment, have negative influences on the behavior and physiology of living organisms (Thomas et al. 1999; Yousefi and Nasiri 2006; Paniagua et al. 2007). According to epidemiological studies, living and working under the constant influence of ELF EMFs may contribute to many modern human diseases (Floderus et al. 1993; Savitz and Loomis 1995; Thomas et al. 1999; Johansson 2009), including the large increase in the incidence of diabetes and pancreatic cancer observed in high-urban areas (Laitl-Kobierska et al. 2002; Sakurai et al. 2008). In particular, the influence of EMFs on diabetes is currently a new focus for scientists studying the causes of diabetes. In fact, a hypo-

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glycemic effect of ELF EMFs accompanied by an increase in insulin secretion has been confirmed in experimental (Bonner-Weir 2000; Bonhomme-Faivre et al. 2003; Gholampour et al. 2011) and clinical studies involving healthy volunteers and diabetics (Bonner-Weir 2001). Because these issues have been poorly studied to date, further investigations are required. Thus, the present study was designed to test the effects of long term (5 months) exposure to non-homogenous extremely low frequency electromagnetic fields (50 Hz) of two different intensity (500-50 μT and 1000-100 μT) on the structure of the endocrine pancreas in Wistar rats.

MATERIAL AND METHODS

Animals

The present study was performed on male Wistar rats subjected to treatments from post natal day (PND) 23 to 53. Animals were housed in plastic cages under laboratory conditions with 20 ± 2 °C temperature and 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 conducted with the permission of the Committee of Ethics on Animal Experiments of the University of Novi Sad.

EMF Exposure system

The EMF exposure system consisted of a single solenoid type coil (Electronic Equipment Factory "Novkabel", Novi Sad, Serbia) equipped with a cooling system and energized from 50 Hz, 220 V and 10 A via an autotransformer, which provided 100 V output. Cages with animals were placed on both sides of the coil, perpendicular to the coil axis, at 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 EMF produced by the coil was in the horizontal direction with respect to the geomagnetic field. The EMF was non-homogeneous and of decaying intensity along the animal cages. In this experiment, two fields of different intensity were used: 1) an intensity of 500 μT on the side of the cage near the coil and 50 μT on the opposite side; and 2) an intensity of 1000 μT and on the side of the cage near the coil and 100 μT on the opposite side. The value of the electric field at any other point in the room was less than 10 V/m. 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.

Experimental procedures

Animals with similar initial body weights were divided into three groups. Group 1 (9 rats) was subjected to 4 h daily exposure (from 10.00 to 14.00 h) to the weaker EMF (50 Hz, 500-50 μT). Group 2 (9 rats) was subjected to 4 h daily (from 10.00 to 14.00 h) exposure to the stronger EMF (50 Hz, 1000-100 μT). Group 3 (10 rats) was untreated and defined as the control group.

Light microscopy

Animals were sacrificed in the morning by decapitation. Pancreases of all 28 animals were taken, fixed in Bouin's solution, embedded in paraffin, and sectioned into 5 μm thick serial slices. For histopathological and morphometrical examinations, pancreatic sections were performed by Hematoxillin-Eosin (HE) and Malory-Azan staining.

Immunohistochemical preparation

Localization of individual endocrine cells of Langerhans islets (alpha, beta, D and PP) was performed by testing for expression of insulin, glucagon, somatostatine and pancreatic polipeptide positive cells using the DAKO LSAB^{+}/HRP kit, following the manufacturers instructions. Briefly, paraffin-embedded pancreatic tissue specimens were consecutively sectioned into 4 μm thick slices. After deparaffinization in xyline and rehydration through decreasing concentrations of ethanol, slides were immersed in citrate buffer (pH 6.0). After antigen retrieval using a microwave oven at 98 °C for 20 min, slides where incubated in 3% hydrogen peroxide for 10 min at room temperature to block endogenous peroxidase activity. Then the sections were incubated with primary rabbit antibodies: to insulin (1:100; DakoCytomation), to glucagon (1:75; DakoCytomation), somatostatine (1:200; DakoCytomation) and to pancreatic polipeptide (1:600; DakoCytomation) at 4 °C over night in a humid atmosphere. Negative control staining slides were incubated in the absence of primary antibody. Slides were washed and chromogen was developed for 5 min using liquid 3,3'-diaminobenzidine; the slides was then counter-stained with Mayer's hematoxylin, dehydrated, and mounted with Canada balsam for examination.

All pancreatic sections were analyzed on a Leica DMRB Photo Microscope and photographs were taken using a JVC TK 1280E Video Camera (Leica) and the QWin program (Leica).

Stereological analysis

Hematoxiline-eosin, Mallory Azan stained pancreas sections and sections stained according to the immunohistochemistry protocol were used for quantitative estimation of Langer-

hans islets and endocrine cells. Analysis of pancreatic sections was performed using the "point counting technique", with Weibel's multi-purpose test grid (M 42) (Weibel et al. 1966). The total volume density of Langerhans islets and the number and volume density of alpha, beta, D and PP cells were determined. In addition, the diameter and number of Langerhans islets per section was estimated. Conventional morphometry was used to calculate islet diameter. Using a graticule from a calibrated linear scale, major axes (a), minor axes (b), and axes of islets at right angles to (a) were measured and the mean islet diameter was calculated. The number of islets per unit area (NA) was determined by the formula NA = N/AT, where N is the number of sectioned profiles of islets and AT is the area of the section. In addition, the volume density of blood capillaries and connective tissue in and around Langerhans islets were determined.

Statistical analysis

All data were presented as the mean ± standard error (SE). Statistical comparisons for all examined stereological parameters between the three groups were made and compared with one-way analysis of variance (ANOVA) and Tukey's t-test. Differences in values were considered significant if $p < 0.05$.

RESULTS

Animals exposed to ELF EMFs of either low or high intensity displayed a reduced number of Langerhans islets, as can be seen in sections of pancreas stained with hematoxyline and eosin (Fig. 1). This result was confirmed by estimating the number of islets per section of pancreas (Fig. 2). The observed decrease in the number of islets per section was particularly pronounced in animals exposed to the stronger ELF EMF field (Figs 1, 2). In contrast, exposure to ELF EMFs had no significant influence on the total volume density of islets. In exposed animals, the volume density of islets did not change significantly compared to controls (Fig. 3), but ELF EMF exposure induced significantly increase in the volume density of connective tissue and increase, but not significantly blood capillaries volume density in Langerhans islets, upon treatment with ELF EMFs of higher intensity (Fig. 3).

Size distribution analysis indicates that in control animals the diameters of Langerhans islets were between 30 and 350 μm, with the greatest number of islets having a diameter between 40 and 80 μm (Fig. 4). Exposure to ELF EMFs shifted the distribution of islet size toward populations of larger islets (Fig. 4, Table 1). The mean diameter of islets was significantly higher in both ELF EMF exposure groups ($p < 0.01$, respectively) compared to the control. In fact, in exposed animals, islets with diameters greater than 380 μm were observed, which were not present in the control group (Fig. 4).

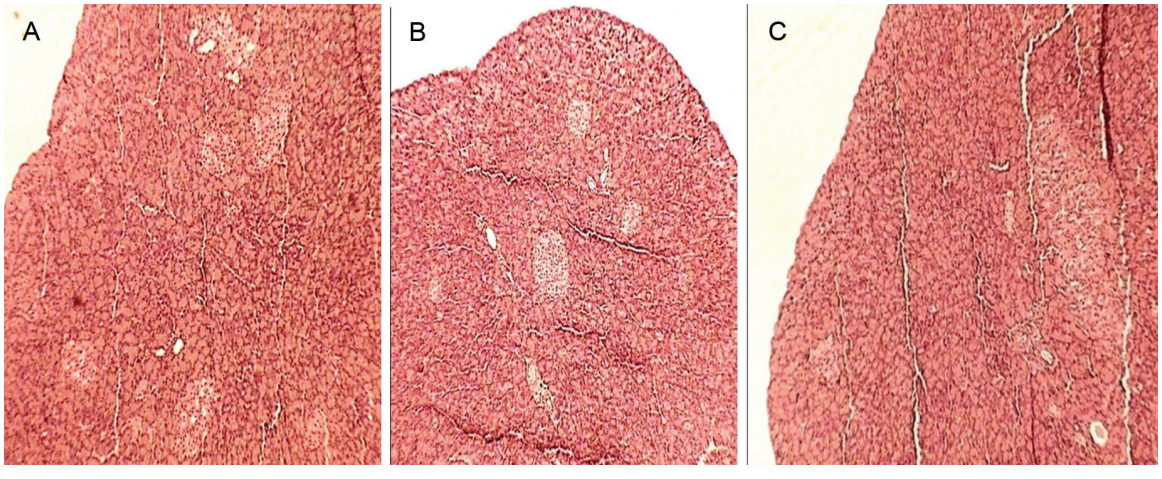

Fig. 1. Rat pancreas. Langerhans islet. **A**, control animals; **B**, group 1 (lower intensity of ELF EMFs); **C**, group 2 (higher intensity of ELF EMF). HE, x100.

Immunohistochemical study for alpha, beta, D and PP cells of Langerhans islets

Alpha cells. In control animals (in the majority of islets), alpha-cells were located at the periphery, however, in some islets they were found to be located in the centre. ELF EMF exposure induced strong immunostaining intensity to glucagon (Fig. 5). ELF EMFs of lower intensity increased the percentage and volume density of these cells, while ELF EMFs of higher intensity decreased both the number and volume density of these cells ($p < 0.01$, respectively) (Fig. 9, Table 2) vs. controls. Both large and small islets displayed a typical distribution of glucagon positive cells on the periphery of the islets (Fig. 5A-F).

Beta cells. Immunohistochemical identification of insulin-positive beta cells. In all animals (control as well as animals exposed to ELF EMFs of both intensities) the smallest islets showed much stronger immunoreactivity to insulin vs. large

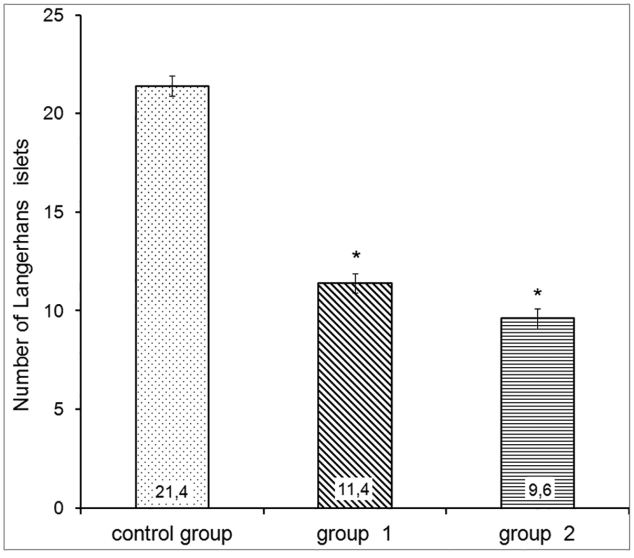

Fig. 2. Number of Langerhans islets per section (mean ± SE) of pancreas in the control group, group 1 (lower intensity ELF EMF), and group 2 (higher intensity of ELF EMF). *statistical significant at $p \leq 0.05$ compared to the control.

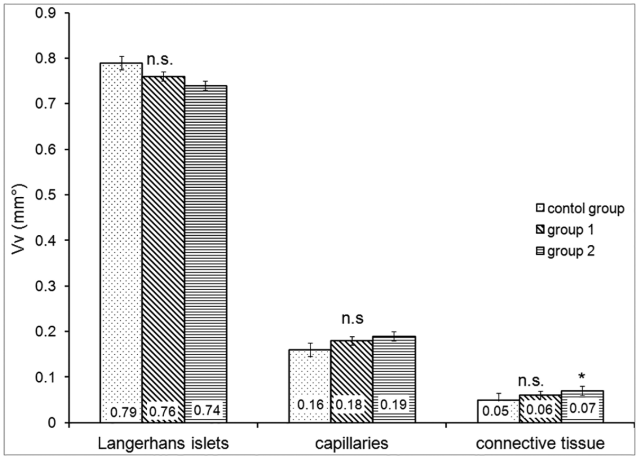

Fig. 3. Volume density (Vv) (mean ± SE) of: **A**, Langerhans islets, **B**, capillaries of Langerhans islets and **C**, connective tissue of Langerhans islets of the control group, group 1 (lower intensity of ELF EMFs) and group 2 (higher intensity of ELF EMFs). *statistical significant at $p \leq 0.05$ compared to the control, n.s. statistical non- significant compared to the control.

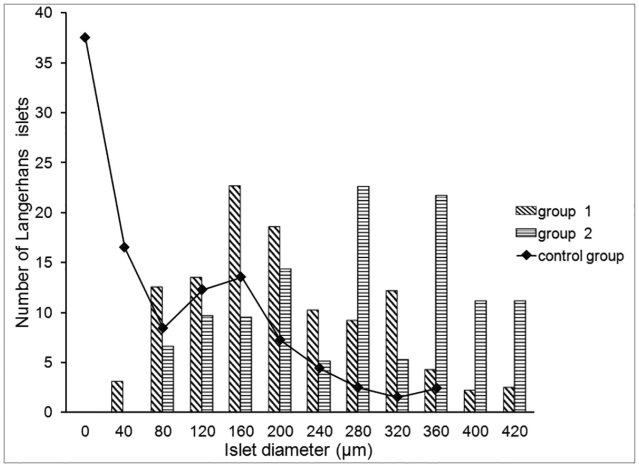

Fig. 4. Langerhans islets. Size distribution of islets diameters: control group, group 1 (lower intensity of ELF EMFs); group 2 (higher intensity of ELF EMFs).

Table 1. Diameter of Langerhans islets of control group, group 1 (lower intensity of ELF EMF) and group 2 (higher intensity of ELF EMF).

	Diameter of islets (µm) (mean ± SE)
control group	136 ± 1.21
group 1	166 ± 9.7**
gorup 2	248 ± 35.02**

** statistically significant at $p \leq 0.01$compared to the control.
n.s. non-statistically significant compared ro the control.

Table 2. Volume density of endocrine cells (alpha, beta, D, PP) of Langerhans islets of control group, group 1 (lower intensity of ELF EMF) and group 2 (higher intensity of ELF EMF).

	Volume densityof cells of Langerhans islets (mean ± SE) (mm^0)		
	control group	group 1	gorup 2
alpha cells	0.33 ± 0.042	0.36 ± 0.065**	0.35 ± 0.073**
beta cells	0.48 ± 0.00034	0.48 ± 0.0063*	0.48 ± 0.0058*
D cells	0.07 ± 0.011	0.05 ± 0.0072*	0.04 ± 0.014*
PP cells	0.13 ± 0.011	0.11 ± 0.0064*	0.13 ± 0.014*

**$p \leq 0.01$, *n.s.

islets (Fig. 6A-F). The percentage of beta cells in the total number of Langerhans islets endocrine cells increased in both ELF EMFs exposure groups (Fig. 9), but no changes in the volume density of these cell was observed vs. controls (Table 2).

D cells. Delta-cells were located at the periphery of Langerhans islets. Immunoreactivity to somatostatin (SST) was slightly increased in islets of animals exposed to ELF EMFs of lower intensity, while in animals from group 2, a

pronounced decrease in immunoreactivity to SST was observed compared to controls (Fig. 7). The percent of delta cells among the total number of Langerhan's islets was decreased. In addition, the volume density was slightly decreased in both ELF EMF treated groups vs. controls (Fig. 9, Table 2).

PP cells. PP cells were found to be arranged singly or in clusters at the periphery of the Langerhans islets. ELF EMF exposure induced decreased immunostaining intensity for pancreatic polypeptide (Fig. 8), and reduced the percent and volume density of PP cells in both treated groups vs. control (Fig. 9, Table 2).

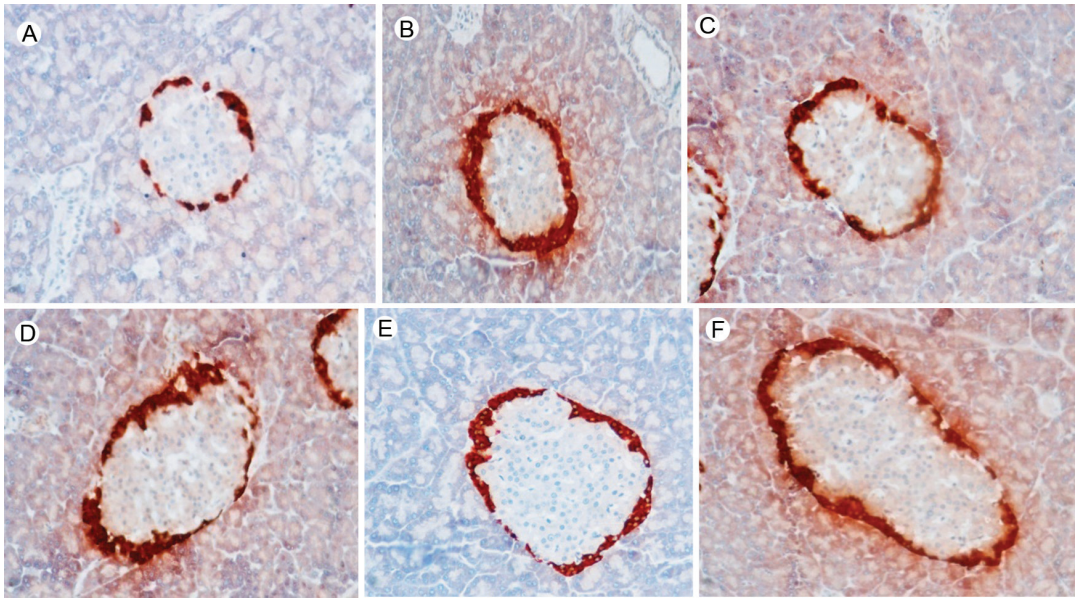

Fig. 5. Rat pancreas. Langerhans islet. Glukagon-positive cells (alpha cells) on the periphery of small islets (**A-C**) and large inselts (**D-F**). Control group (**A**, **D**); group 1 (lower intensity of ELF EMF) (**B**, **E**); group 2 (higher intensity of ELF EMF) (**C**, **F**). x400.

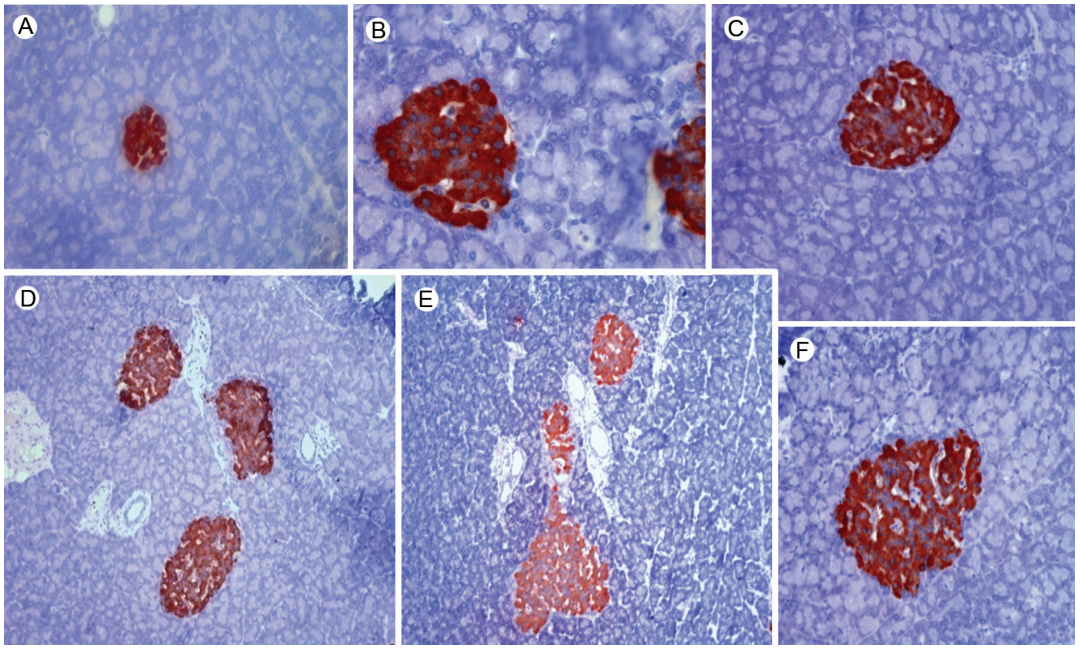

Fig. 6. Rat pancreas. Langerhans islet. Insulin-positive cells (beta cells) among small (**A-C**) and large (**D-F**) islets. **A**, **D**, control group; **B**, **E**, group 1 (lower intensity of ELF EMF); **C**, **F**, group 2 (higher intensity of ELF EMF); **A**, **C**, x200; **B**, **F**, x400; **D**, **E**, x100.

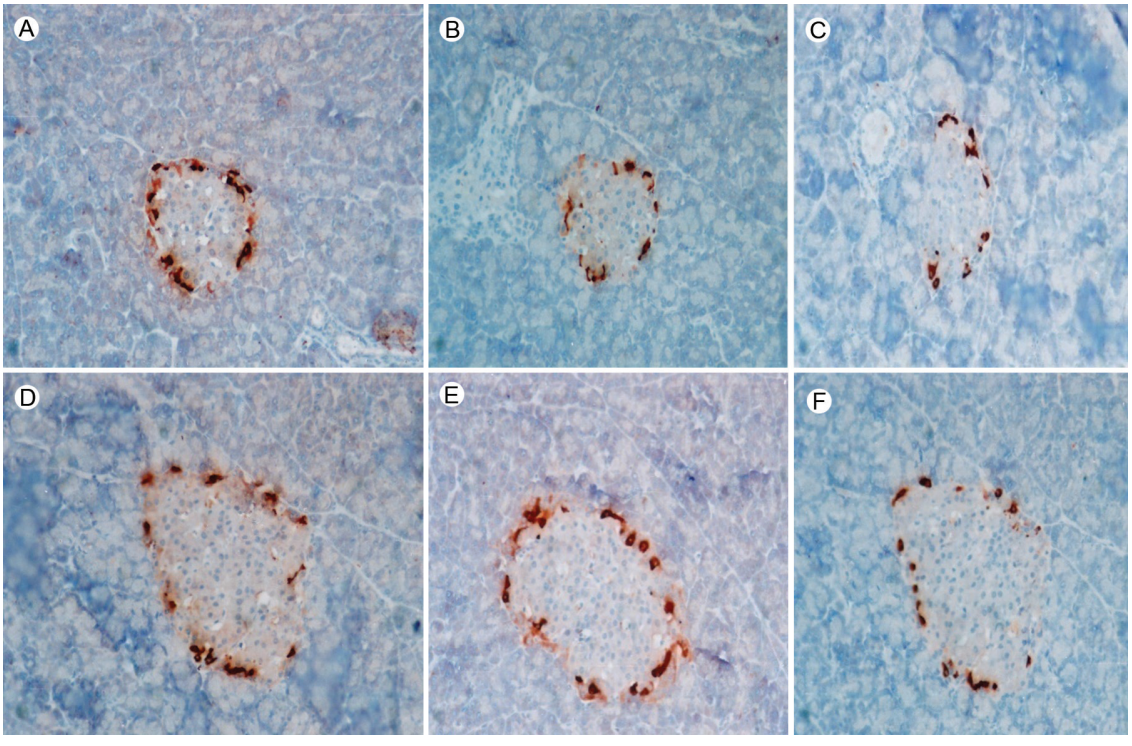

Fig. 7. Rat pancreas. Langerhans islet. Somatostatin-positive cells (D cells) of small (**A-C**) and of large (**D-F**) islets. **A**, **D**, control group; **B**, **E**, group 1 (lower intensity of ELF EMF); **C**, **F**, group 2 (higher intensity of ELF EMF). x250.

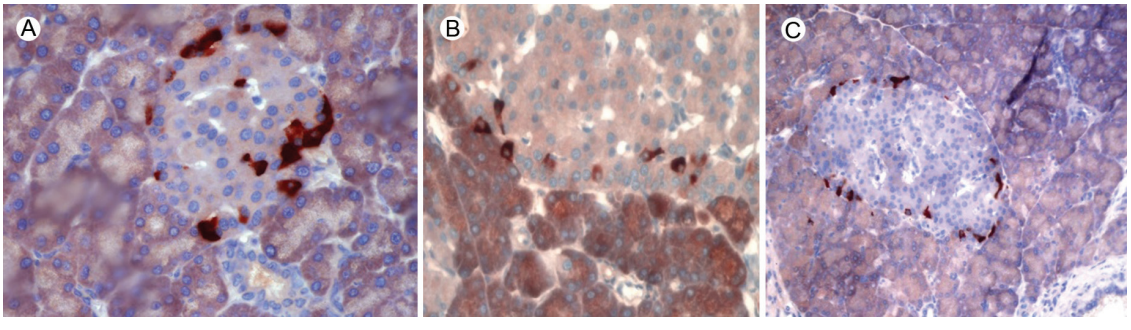

Fig. 8. Rat pancreas. Langerhans islet. Pancreatic polipeptide-positive cells (D cells). **A**, control group; **B**, group 1 (lower intensity of ELF EMF); **C**, group 2 (higher intensity of ELF EMF). **A**, x1000; **B**, x400; **C**, x200.

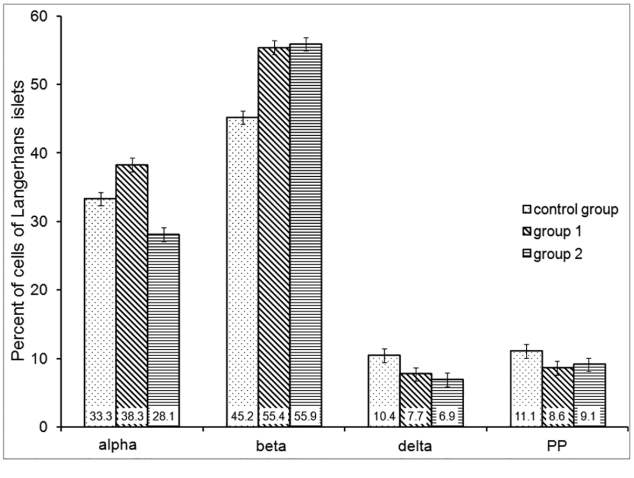

Fig. 9. Percentage of alpha, beta, delta and PP cells with respect to the total number of Langerhans islet endocrine cells: control group, group 1 (lower intensity of ELF EMF), group 2 (higher intensity of ELF EMF).

DISCUSSION

Our results suggest that long term exposure to ELF-EMFs alters the size distribution of Langerhans islets, as well as the number, volume and immunoreactivity of endocrine cells in the rat pancreas. Both intensities of applied ELF-EMFs induce a dramatic increase in islet diameter in a dose-dependent manner. We founded that small islets constitute the majority of the number of islets, while EMF treatment induced the appearance of larger islets: up to 380 μm, which were not present in controls. This result is particularly striking, given that the bulk of endocrine functions in the pancreas are carried out by large islets, whereas the so-called extra-islets (very small islets) are likely to be their precursors (Kaihow et al. 1986). According to Gholampour et al. (2011), two mechanisms may be responsible for this enlargement of the islets of Langerhans. First, the accumulation of a greater quantity of insulin in beta cells, and second, an increase in beta cell population. It seems that the interplay between glucose and insulin may be important for the control of islet cell proliferation in vivo (Koiter et al. 1995). With regard to the cellular structure of the islets, we found that the smaller the islet, the smaller the percentage of islets containing alpha cells: with the vast majority of cells in the smallest islets (often single cells) being beta cells (Kaihoh et al. 1986). Pancreatic islets, in general, grow proportionally to the number of cells in the islet; *i.e.*, each islet may grow in response to external demand, independently of the others (Jo et al. 2007). Jo et al. also reported that in cases where islets do grow, the ratio of the total surface area to the total volume of all islets in a pancreas decreases; indicating that the relative population of beta cells tends to increase.

The actual growth of islets is governed by a dynamic process consisting of gain due to replication and loss due to apoptosis (Finegood et al. 1995; Bonner-Weir 2001; Teta et al. 2005). Similar growth of islet size, as seen in the present study, was observed in the pancreas of dexamethasone-treated rats (Rafacho et al. 2007; Rafacho et al. 2008), during normal postnatal growth (Bonnevie-Nielsen et al. 1983; Dor et al. 2004), pregnancy (Parsons et al. 1995), or obesity (Bock et al. 2003), when preexisting islets proliferate more to meet demands for additional B-cell mass; however no additional neogenesis occurs. Thus, as the demand for growth increases (e.g., due to pregnancy or obesity) islets continue to grow (Hellman et al. 1961; Parsons et al. 1995) and the relative population of beta cells becomes dominant. This can cause (among other problems), obese-hyperglycemic syndrome where most islets indeed become abnormally large (He et al. 2010), similar to our findings in ELF EMFs treated animals.

It is well known that pancreatic islets are one of the most vascularized organs of the body. While islets occupy only a small volume of the pancreas, they receive a disproportionally greater fraction of pancreatic blood (Jansson and Hellerström 1983; Lifson et al. 1985). This dense vasculature is therefore likely to play a role in normal physiology as well as diseases of the pancreatic islets (Olsson and Carlsson 2006; Zanone et al. 2008). Islet endothelial cells are involved not only in the delivery of oxygen and nutrients to endocrine cells, but produce a number of vasoactive, angiogenic substances and growth factors. In addition, these cells have a physical and functional relationship with beta cells. They induce insulin gene expression during islet development, affect adult beta cell function, and promote beta cell proliferation (Alismail and Jin 2014). In our experiments, treatment with both intensities of ELF EMF induces increasing volume density of blood capillaries. This is in agreement with previous findings concerning the angiogenic potential of ELF EMFs (reviewed by McKay et al. 2007). Tepper et al. (2004) have shown that magnetic fields of 15 Hz increased angiogenesis in vitro and in vivo through release of a known mitogenic growth factor: fibroblast growth factor. Also, pulsed electromagnetic fields were shown to induce vascular endothelial cell growth and angiogenesis via influencing vascular endothelial growth factor (VEGF)-related pathways (Okano et al. 2006; Delle Monache et al. 2008). However, Chunhua et al. (2013) have shown that the increased blood capillary volume density which we observed in the present study, may be a consequence of pancreatic islet vasculature adaptation to insulin resistance through dilation and not angiogenesis. In our experiments, the volume density of connective tissue in islets was also significantly increased in both ELF EMFs treatment groups. These findings are in agreement with reports showing that a frequency of 25 Hz with peak intensity of 2 mT can significantly enhance collagen synthesis in rat skin when applied for 2.5 h/day for a duration of 8 days (Ahmadian et al. 2006).

In our long term experiments, ELF EMFs induced a biphasic response in alpha cells. ELF EMFs of lower intensity increase the number and volume density of these cells, while ELF EMFs of higher intensity decrease their number and volume density. Thus, in our experiments, a negative correlation was found between the number and volume of beta and alpha cells, in agreement with the fact that insulin is an important suppressor of glucagon secretion (Ungera and Orci 2010). Similarly, Gortzinska and Wegrzynowicz (1991) have shown that electromagnetic fields (1 mT and 10 mT, 1 h/day for up to 10 days) cause increased glucagon levels in rats. It is also known that glucose is important for control of alpha cell secretion (Wang et al. 2012), although catecholamine may be as important as glucose in the control of glucagon secretion. Glucagon secretion is stimulated by epinephrine and norepinephrine secretion (Weir et al. 1974). Taking into account the fact that ELF EMF stimulation alters catecholamine metabolism (Yim and Jeong 2006), some authors have postulated that long-term exposure to ELF EMFs prevents an increase in glucagon secretion due to low glucose level via decreasing catecholamine levels (Laitl-Kobierska et al. 2002; Gholampur et al. 2011).

The endocrine pancreas is a plastic organ, especially considering the ability of beta cells to change mass in response to insulin demands. The population of these cells is dynamic and undergoes compensatory changes to maintain euglycemia (Bonner-Weir 2000, 2001). It is well known that in pancreatic islets in rodents, the core is occupied only by beta cells, whereas others non-beta cells including alpha, D, and PP-cells, are present in the mantle (Brissova et al. 2005). It has also been reported that there is a population of extra-islet beta cells scattered over the exocrine tissue, representing 15% of all beta cells (Bouwens and Rooman 2005).

In both of our experimental groups, ELF EMF treatment caused an increase in the percentage of beta cells, while their volume density remained unchanged compared to controls. Results of similar experiments by Gholampur et al. (2011), where rats were exposed to 50 Hz ELF EMF, 1 mT for 24 h daily over 135 days, showed that in exposed animals insulin levels were increased ($p < 0.01$), which was associated with increased pancreatic islet size and decreased glucose levels ($p < 0.01$). Similarly, exposure of cultured beta cells to ELF EMFs (60 Hz, 5 mT) for 5 days in the absence of glucose induced increased cell numbers, while treatment for 5 days with 100 ml/dl glucose increased insulin secretion to the culture medium (Sakurai et al. 2008). Moreover, long term exposure of rats to ELF EMFs with therapeutic parameters (110 Hz and induction of 1.8-3.8 mT or a sinusoidal magnetic field at a frequency of 40 Hz and induction of 1.3-2.7 mT), produced changes in the ultrastructure of B cells, leading to increased synthesis and secretion of insulin and secondary hypoglycemia in the initial phase of exposure (Laitl-Kobierska et al. 2002).

In fact, ELF EMF exposure increases glucose uptake, leading Sieron et al. (2007) to hypothesize that ELF EMFs may influence glucose uptake not only via increased pancreatic secretion of insulin, but also by increasing the affinity of insulin receptors and/or increasing signal transduction in target cells and insulin transporter function.

The ELF EMFs (of both intensities) applied in our experiments significantly decreased the number, volume density and immunoreactivity of D cells in pancreatic islets. These cells produce somatostatin and have a tonic inhibitory influence on insulin and glucagon secretion. SST receptors have been identified on alpha and beta cells (Hauge-Evans et al. 2011). Decreasing of D cell activity, which is associated with the decreased SST levels observed in our experiments, can further influence alpha and beta cells such that changes in their activity may be the result of the direct and indirect influence of ELF EMF exposure.

Similar to D cells, PP cells responded to ELF EMF exposure with a decrease in cell number, volume and immunoreactivity. PP cells are pancreatic polypeptide producing cells. Pancreatic polypeptide inhibits somatostatin secretion (Kim et al. 2014), but long term exposure to ELF EMFs decrease the activity of both cells type, probably changing their relationship.

In conclusion, the results of the present study demonstrate that long-term exposure to ELF EMFs of low and high intensity leads to an increase in the mean diameter and causes changes in diameter size distribution in Langerhans islets, in dose dependent manner. Moreover, ELF EMF exposure directly increases the volume density of connective tissue and blood capillaries in islets, and likely directly and indirectly effects islet beta and non-beta-cells. According our results, alpha cells have the greatest sensitivity to changes in ELF EMF intensity, while all other non-beta cells, as well as the beta cells, do not alter their response profile depending on the intensity of the ELF EMFs. However, further investigation is required to elucidate intra-islet interactions between different endocrine islet cells types during ELF EMF exposure.

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