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Long term study on the effects of housing C57BL/6NCrl mice in cages equipped with wireless technology generating extremely low intensity electromagnetic fields

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Title page

Long term study on the effects of housing C57BL/6NCrl mice in cages equipped with wireless technology generating extremely low intensity electromagnetic fields.

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Effects on mice of housing in DVC

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Keywords page

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Abstract

The recent development of mouse cages equipped with monitoring wireless technology raised questions on the potential effects on animals induced by electromagnetic fields (EMF) generated by electronic boards positioned underneath the cages. The aims of this study were to characterize the EMF produced by Digital Ventilated Cages (DVC) and perform a clinicopathological study on mice maintained in DVC for up to one year. EMF were measured in empty Individually Ventilated Cages (IVC) and DVC. Male (n=160) and female (n=160) C57BL/6NCrl mice were randomly housed in IVC and DVC in a single rack, 4 mice per cage. Body weight, feed and water consumption were recorded at 14-day intervals. At sacrifice (day 60, 120, 180, 365) body and testes weight was measured, and necropsy, hematology, bone marrow cytology, histology, and immunohistochemistry for cleaved-caspase 3 on testes were performed. DVC produced extremely low intensity electric fields in the range from 5 Hz to 3 GHz. No exposure-related clinical signs and mortality occurred. Occasional statistical differences in body weight, feed and water consumption, hematology, bone marrow, and histopathology were recorded, but considered without biological or clinical relevance. In conclusion, long term maintenance in DVC had no definite effects on C57BL/6NCrl mice.

Introduction

Electric fields are associated with the presence of electric charges, while magnetic fields are the result of the physical movement of an electric charge (electric current).[1] Exposure to electromagnetic fields (EMF) from human-made sources have increased steadily over the past century due to the increased use of electricity and new technologies. In the past decades, there has been much debate about the potential detrimental effects produced on humans and animals from exposure to EMF,[2] as well as more recently about their potential therapeutic use (e.g. pulsed electromagnetic fields, tumor treating fields),[3-5] indicating that EMF can produce biological effects, regardless of the adverse or favourable outcomes. The mechanisms implicated in the generation of biological effects by EMF are so far not fully understood, but involve non-thermal effects, resulting from ions fluxes (i.e. increased intracellular Ca^{2+}), oxidative stress, free radicals, and melatonin suppression.[6-8]

Several epidemiological studies in humans pointed towards an association between long term exposure to EMF of different frequency ranges [including extremely low frequency (ELF)-EMF, ranging from 0 to 300 Hz, and radiofrequency (RF) ranging from 100 kHz-300 GHz], and increased cancer risk,[9,10] but when all the studies are considered together the association results weak, leading to the conclusion by different panels of experts of an overall limited or inadequate evidence of causal relationship between exposure to EMF and incidence of some tumors (i.e. leukemia, brain, head and breast cancers).[2,11-13] Similarly, neurobehavior, neuroendocrine, and reproductive effects following exposure to EMF were inconsistently reported in epidemiological studies, in particular no effects were detected on hemopoietic and germinal cells.[2]

From the animal model point of view, studies performed on laboratory rodents on the impact of chronic exposure to EMF mostly failed to demonstrate an effect on tumor development,[14-20] with only one study reporting the induction of lymphoproliferative diseases in three successive generations of CPW mice exposed to very strong (25 mT) 60-Hz EMF for up to 408 days,[21] and one study reporting a slight increase in risk for myeloid leukemia in female B6C3F1 mice after 15.5 months of

exposure to 50 μT 50 Hz EMF.[22] Although a conclusive role of EMF on cancer was not yet demonstrated, evidence of biological effects of EMF on rodents were occasionally reported, in particular on nervous and neuroendocrine systems,[23-25] testes (including sperm and testicular weight abnormalities, and increased germ cell apoptosis),[26-29] and hematological parameters,[30-32] even though other studies failed to replicate these effects.[33,34]

Recently, Digital Ventilated Cages (DVC, Tecniplast, Buguggiate) equipped with wireless technologies that use extremely low intensity EMF were developed to enable continuous automated intra-cage monitoring and data capture of the animal activity and cage micro-environment.[35] In this context, the introduction of the DVC raises concerns about the potential effects on mice that could be induced by the EMF generated by the electronic boards positioned underneath the cages. Before introducing any change that may influence the animal welfare or the scientific outcomes of experiments it is essential to assess the impact of this new technology on the animals. The aim of the study was to characterize the EMF produced by the DVC and perform a long-term trial (up to one year) on C57BL/6NCrl mice of both sexes to evaluate the effects of the exposure to this recently commercialized intra-cage wireless data collection technology.

Materials and Methods

Animals and husbandry

The study was conducted in a distinct unit of the animal facility of the M. Negri Institute (Milan, Italy) not previously populated by animals and fumigated with 30% hydrogen peroxide before the beginning of the study. Specific Pathogen Free C57BL/6NCrl mice (Charles River, Calco, Italy), aged 3 weeks at arrival, for a total of 160 male and 160 female mice used in the first (main) experiment, and 40 female mice used in the second experiment. Upon arrival, mice were weighed and randomly divided into control and exposed groups on a single rack (DVC rack, Tecniplast, Buguggiate, Italy), four mice per cage. Control mice were housed in standard Individually Ventilated Cages (IVC) (GM500, Tecniplast, Buguggiate, Italy), while exposed mice were housed in standard

IVCs equipped with DVC boards positioned underneath the cage and fixed to the rack. The chessboard distribution of IVC and DVC on the rack is shown in Figure 1. In the main experiment, groups were formed at the beginning of the study according to the scheduled time point of sacrifice (day 60, 120, 180, 365). Each group included 20 male and 20 female control mice, and 20 male and 20 female exposed mice. Mice were allowed to acclimate for one week prior to the beginning of exposure (i.e. when DVC boards were switched on).

IVC ventilation was set up in positive pressure at 75 air changes per hour. Room environmental conditions were controlled with a temperature of 22°C + 2°C and a 55% + 10% relative humidity. The animal room had a controlled photoperiod of 12h:12h light:dark cycle. Mice were maintained on autoclaved Corn Cob bedding (Follador, Treviso, Italy), 150 g per cage. Routine cage changing occurred every 14 days. At cage change, random cage rotation was applied for both control and exposed groups. Mice were provided filtered autoclaved water and autoclaved diet (2014S Envigo/Teklad global diet rodent maintenance, 14% protein) *ad libitum*. Water bottles and diet were weighed at cage change for the assessment of water and food consumption. Clinical signs were assessed daily. Body weights were recorded at cage change and before the sacrifice. Body weight gain in each group was calculated as follows: (mean body weight per cage at day of sacrifice - mean body weight per cage at randomization)/mean body weight per cage at randomization*100.

Microbiological monitoring was performed on sampling dates (at day 60, 120, 180, 365). Fur, mouth swabs, and fecal pellets were sampled from one control and one exposed cage, and a swab was taken from the exhaust pre-filter of the Air Handling Unit (AHU). The pre-filter was changed at each time point after swabbing. All samples were submitted to Charles River Laboratories (Wilmington, USA) for infectious disease PCR testing. Intra-cage samples were analyzed according to the Mouse Surveillance PRIA panel (at day 60) or the Mouse FELASA Complete PRIA panel (at 120, 180, 365 days). Pre-filters were analyzed according to the Environmental (EAD) Mouse Surveillance Plus PRIA panel (at day 60, 120, 180, and 365).

Mice were euthanized by carbon dioxide inhalation using a gradual 20% vol/min displacement rate[36] at day 60, 120, 180, and 365 after the beginning of the experiment. At sacrifice, blood was drawn from the heart and immediately placed in tubes containing EDTA, stored at room temperature and transported to the laboratory, when they were employed to perform routine hematology, as described below. Bone marrow was collected from the femur using a 24G syringe needle and immediately smeared on glass slides and air dried. Then, mice underwent complete necropsy, as specified below.

The second experiment was carried out on 20 control and 20 exposed female mice, maintained for 60 days in the same conditions as aforedescribed, to verify the effects on neutrophils observed during the main experiment. At sacrifice, blood and bone marrow were collected and processed as described above.

Animals were maintained according to the guidelines set out in Commission Recommendation 2007/526/EC of 18 June 2007, for the accommodation and care of animals used for experimental and other scientific purposes, and were used in accordance with the Italian laws (D.L 26/2014), which enforces the Council Directive 2010/63/UE, on the approximation of laws, regulations, and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes. The study was approved by M. Negri Institute Ethical Committee. The Ethical Committee of M. Negri Institute uses the COST Action B-24 suggestions on Laboratory Animal science and welfare to evaluate any study involving laboratory animals.

Radiate Electromagnetic fields and temperature measurements

The DVC board is an electronic board with an array of 12 electrodes that are multiplexed by a central proximity sensor. The working principle is based on charging and discharging 4 times/sec each electrode to measure electrical capacitance of all the materials (e.g. plastic of the cage, bedding, animals) that are immersed into the generated low intensity EMF. The entire DVC board power

consumption is ~250 mW (powered at 5.4 VDC and absorbing 50 mA) distributed on a total surface of ~500 cm^2.

To assess the intensity of EMF produced by the DVC board, radiate EMF measurements were performed in 10 IVC and 10 DVC with the board on, positioned in the central region of the rack, as shown in Figure 1. An electric and magnetic field strength analyser (EHP-50, Narda STS, Italy) was used to measure EMF in the frequency range 0 Hz - 100 kHz, and an electromagnetic field strength meter (8053 2004/40 + ES330 Probe, Narda STS, Italy) was used to measure EMF in the frequency range 100 kHz - 3 GHz. The EMF were measured close to the floor of the cage, and results were reported as root mean square (rms) V/m (electric field strength) and μT (magnetic field flux).

To assess the local heating produced by the DVC board, temperature measurements were performed in 4 IVC and 5 DVC positioned in the central region of the rack, as shown in Online Figure S1. Data loggers for temperature (174H mini temperature and humidity data logger, Testo Inc., USA) were placed on the floor of the cages and measured the temperature every 3 minutes for 2 hours. One additional data logger was placed on the top of the rack to measure the environmental temperature. Temperatures were measured when DVC was switched off and on.

Clinical Pathology (Hematology, and Bone Marrow evaluation)

The complete cell blood count (CBC) was performed on whole blood collected in EDTA using a laser-based cell counter (Sysmex XT-2000iV) validated in mice.[37] Samples with evident clots were excluded from the haematological analysis. Differential leucocyte counts provided by the instrument were verified through microscopic evaluation on May Grünwald Giemsa stained smears, which allowed also the differentiation between mature (segmented) neutrophils and immature (band) neutrophils. During microscopic evaluation, particular attention was paid to any possible morphological abnormality of erythrocytes, leukocytes and platelets. The following parameters of the CBC were recorded: Erythrocyte number (RBC), Hemoglobin concentration (HGB), Hematocrit (HCT), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular

hemoglobin concentration (MCHC), Platelet number (PLT), Number of total leukocytes (WBC), and of the different leukocyte populations: neutrophils, lymphocytes, monocytes, eosinophils, and basophils.

Bone marrow smears were stained with May Grünwald Giemsa and microscopically analysed to perform a 500 nucleated-cell count, in order to estimate the myeloid:erythroid (M:E) ratio. Within each cell population, the number of cells belonging to the proliferative (P) pool (composed by blasts able to divide) and the maturation (M) pool was recorded. Based on these numbers, the P:M ratio and the percentage of precursors on the total number of cells belonging to each cell lineage (%PE = percentage of erythroid precursors and %PM = percentage of myeloid precursors) were calculated. The percentage of lymphocytes (%L) and plasma cells (%PL) on the total number of counted cells was also recorded. Any possible abnormal morphology of cells of both proliferative and maturation pool was also recorded.

Gross examination and histopathology

At sacrifice, mice underwent complete necropsy, and any gross change was recorded. The testes weight was measured. For histological examination, spleen, kidneys, adrenal glands, liver, small intestine (duodenum and jejunum), testes, mammary gland, brain (only at day 365), and organs with gross lesions were fixed in 10% neutral buffered formalin for at least 48 h at room temperature, routinely processed for paraffin embedding, sectioned at 4 µm thickness, stained with hematoxylin-eosin (H&E), and evaluated under a light microscope. Grading of histopathological lesions detected in the liver, spleen, and kidneys was performed according to the grading system reported in Online Table S1.

Immunohistochemistry

Immunohistochemistry was performed on a single section of both testes from all examined mice to assess germ cell apoptosis. Four µm sections underwent deparaffinization and heat induced

epitope retrieval for 40 min at 96°C (Dewax and HIER Buffer H, Thermo Scientific Lab Vision, cat. No. TA-999-DHBH). Endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2 for 10 min. Slides were rinsed, incubated with PBS containing 10% normal goat serum for 30 min at room temperature to reduce nonspecific background staining and then incubated for 1 hour at room temperature with a rabbit polyclonal anti-cleaved-caspase 3 antibody (clone Asp175, Cell signalling, cat. no. 9661). Sections were then incubated with a biotinylated secondary antibody (goat anti-rabbit, Vector Laboratories, USA, cat. No. VC-BA-1000-MM15), labelled by the avidin-biotin-peroxidase procedure (VECTASTAIN® Elite ABC-Peroxidase Kit Standard, Vector Laboratories, USA, cat. No. VC-PK-6100-KI01). The immunoreaction was visualized with 3,3'-diaminobenzidine (DAB, Peroxidase DAB Substrate Kit, Vector Laboratories, USA, cat. No. VC-SK-4100-KI01) substrate and sections were counterstained with Mayer's haematoxylin. Known positive control sections were included in each immunolabeling assay. For the evaluation of germ cell apoptosis, the percentage of the no. of cleaved-caspase 3-positive cells/number of seminiferous tubules per section of both testes was calculated.

Statistical analysis

Hematological, bone marrow, gross, histopathological and immunohistochemical evaluations were performed in a blinded fashion, i.e. without knowledge of exposed and control groups. Data were analysed using GraphPad Prism version 7.00 (GraphPad Software, La Jolla California USA, www.graphpad.com). The results of EMF measurements in IVC and DVC, and the results obtained in control and exposed groups, within each sex, regarding body weight, food and water consumption, hematological parameters, bone marrow cells counts, histopathological grade, and germ-cell apoptosis were compared to each other using Mann-Whitney U test. Prism 7.00 handles two identical values assigning them to the same rank and computing an exact P value. Fisher's exact test was used to compare the prevalence of mortality, gross and histopathological findings between control and exposed groups within each sex. P-values < 0.05 were considered statistically significant.

Results

Radiate electromagnetic fields and temperature measurements

Results of radiate EMF measurements are reported in Tables 1 and 2. Overall, the detected EMF values were extremely low in intensity in empty IVC and DVC. In DVC there was a significant increase of the electric field values as compared to adjacent IVC, at all the frequency ranges examined. No significant differences between DVC and IVC were observed in the induction of magnetic fields.

Results of temperatures measured in IVC and DVC are reported in Table 3. The temperature at the bottom of the DVC was not significantly different from that at the bottom of the IVC when the DVC was switched on (Mann-Whitney test, $p = 0.7857$).

Clinical signs, body weights, food and water consumption, mortality, and microbiological monitoring

No clinical signs were noted during the whole period of the experiment in control or exposed mice of either sex. The body weight evaluation resulted in no significant differences between control and exposed groups of female mice at all time points, and male mice at day 60, 120, and 365. At day 180, exposed male mice had a significantly reduced body weight compared to control male mice (control: 39.5±3.2 g; exposed: 34.3±3.9 g, Mann-Whitney U test $p = 0.0002$ (Figure 2). Despite the difference in the body weight, there was no significant difference in body weight gain between control and exposed male mice at day 180 (control: +136.12%; exposed +117.62%; $p = 0.0556$) (Table 4), and there was no significant difference in the body weight of male mice maintained up to 365 days when measured at day 180 (control: 37.4±3.3 g; exposed: 36.2±4.4 g, Mann-Whitney U test $p = 0.2110$). Significant differences between control and exposed animals were found for water consumption in females at day 180 ($p = 0.0256$) and day 365 ($p = 0.0055$), and in males at day 365 ($p = 0.0296$) (Figure 3) and for feed consumption in females at day 365 ($p < 0.0001$) and in males at day 180 ($p < 0.0001$) (Figure 4).

During the study, 6 mice died (Table 5). In three cases it was not possible to assess the cause of death due to advanced post-mortal changes or cannibalism. Two cases were related to husbandry issues (e.g. bottle obstruction), and one was a case of malocclusion. Regardless of the (determined/undetermined) cause of death, no association of mortality with treatment was found.

Health monitoring revealed the presence of *Staphylococcus aureus* and *Klebsiella oxytoca* from intra-cage sampling at day 60 (no more tested at the following time points) and in the pre-filters of the Air Handling Unit (*S. aureus* at day 60, 120, and 180; *K. oxytoca* at day 60, and 180) (Online Table S2).

Hematology and bone marrow evaluation

Complete hematology and bone marrow results are reported in Online Tables S3-S6. Statistical analysis revealed some significant differences between control and exposed mice, summarized in Table 6.

In a second replicate experiment carried out by exposing a new additional group of female mice for 60 days, no significant difference in the neutrophil count was detected between control and exposed animals (Figure 5).

Pathology

Gross lesions

At necropsy, gross changes were only occasionally observed at all time points (Table 7), and included: splenic melanosis, splenomegaly, unilateral hydronephrosis, uterus enlargement, preputial gland adenitis, submandibular lymph nodes and salivary glands enlargement, and a single case of a retroauricular mass. Barbering lesions in both aged male and female mice, and kyphosis in aged female mice were also seen. There was no significant effect of exposure to DVCs on the prevalence of the observed gross lesions in both sexes.

Histopathology

Prevalence of histopathological findings in examined organs is reported in Table 8.

In the liver, the most common finding was the presence of inflammatory cell infiltrates, either perivascular or randomly distributed throughout the liver parenchyma, the latter variably associated with single cell hepatocellular necrosis. The prevalence of these findings was similar in control and exposed groups at all examined time points. Although the prevalence of mice affected by perivascular inflammatory cell infiltration was similar regardless of the age and sex, the degree of severity increased with age, and at day 365 the infiltrates were so well organized to be consistent with tertiary lymphoid structures, but no exposure-related effects on the grading of these perivascular infiltrates was found (Online Table S7). Fatty change was observed only in male mice after day 120, and at day 180 there was a significant difference in its prevalence (control: 15/20; exposed: 5/20; Fisher's exact test: $p = 0.0038$) and severity (control: median grade = 1; exposed: median grade = 0; Mann-Whitney test: $p = 0.0023$). Other findings [pigment (hemosiderin) accumulation within Kupffer cells, hepatic extramedullary hematopoiesis, hepatocyte karyomegaly] were only occasionally present after day 180 of exposure, and exposure had no effect on the prevalence of these findings.

In the kidneys, tubular vacuolation was observed only in male mice after day 120, and presence of hyaline casts and hyaline glomerulopathy was found only in female mice after day 180 and 365, respectively. At day 365, there was a significant reduction of the prevalence of intratubular hyaline casts in exposed female mice as compared to control ones (control: 8/19; exposed: 2/20; Fisher's exact test: $p = 0.0310$). Interstitial inflammatory cell infiltrates, mainly composed of lymphocytes and plasma cells, were found in the cortex and around the pelvis. They increased in prevalence with age and were more frequent in male mice than in female mice, but no exposure-related effects on the grading of these infiltrates was found.

In the spleen, the prevalence of histopathological findings was similar in control and exposed groups at all examined time points (Table 7). Presence of macrophages with pigment accumulation (consistent with hemosiderin) was the most common finding, increased in severity with age and was

more abundant in female than in male mice. No exposure-related effect was observed in the grade of severity of follicular hyperplasia, pigment (hemosiderin) accumulation, and melanosis, while at day 120 splenic extramedullary hematopoiesis resulted significantly higher in control female mice than in exposed female mice despite the same median value (control: median grade = 2; exposed: median grade = 2; Mann Whitney test: $p = 0.0162$) (Online Table S7).

In the adrenals, testes, and mammary glands, the prevalence of histopathological findings was similar in control and exposed groups at all examined time points (Table 7). No relevant histopathological findings were observed in the small intestine of examined mice.

In the brain of animals at day 365, the most common finding was meningeal melanosis with similar prevalence in control and exposed groups. Only a single case of focal choroid plexus dysplasia was observed in the lateral ventricle of a control female mouse.

In a single exposed female mouse at day 365, a histiocytic sarcoma involving the spleen and renal lymph node was found, associated with hepatic extramedullary hematopoiesis and presence of atypical mononuclear cells detected by bone marrow cytology.

The examination of unscheduled grossly affected organs revealed the presence of sporadic cases of preputial gland suppurative adenitis, and occasional cases of uterine mucometra and cystic endometrial hyperplasia, depending on the age of examination. The single case of retro-auricular mass was an abscess with intralesional unidentified foreign body material. Submandibular lymph nodes enlargement was consistent with nodal reactive follicular hyperplasia, and salivary glands enlargement with the presence of perivascular/interstitial chronic inflammatory cell infiltrates.

Testes

No significant differences were found in testes relative weight between control and exposed male mice at all examined time points (Figure 6), while testes absolute weight was significantly reduced in exposed mice at day 180. Histologically, the most frequent finding at all time points was the presence of rare intratubular multinucleated giant cells (1-2 per section). Tubular atrophy and

interstitial cell hyperplasia were observed after day 180, and 365, respectively. Prevalence of testicular histopathological findings was similar between control and exposed animals (Table 7).

The evaluation of apoptosis through the immunohistochemical staining for cleaved-caspase 3 revealed only rare intratubular apoptotic germ cells in both groups, that were mainly located at the basal layers of the seminiferous epithelium. After quantification of cleaved-caspase 3 positive cells, no significant differences were found in the percentage of apoptotic cells per tubule between control and exposed male mice at all time points (day 60, 120, 180, 365) (Figure 7).

Discussion

The recent introduction of DVC cages for continuous automated intra-cage monitoring for data capture of the animal activity and cage micro-environment raised concerns about the potential effects on mice that could be induced by the EMF produced by the electronic board positioned underneath the cages. Thus, the aim of this study was to determine the intensity and frequency of EMF produced by DVC and whether the maintenance of mice in DVC over the long term would result in any adverse effect, as compared to mice housed in standard IVC.

The room of a facility is a complex environment from the point of view of the EMF, because there are several possible sources of EM radiations (e.g. power sockets, lighting system, extensions, changing station, Wi-Fi system). Before starting the *in vivo* study, preliminary investigations revealed that there was a background level of electric fields in the empty room of the facility, and that it varied depending on the position in the room (e.g. close or not to building power sockets), and whether these sources (e.g. lightening system) were switched off or on (Online Table S9). Considering the variability of the environmental electric fields we decided to house all the animals of the study in the same room, and within the same rack, to reduce the impact of external sources of EMF that could mask the potential effects induced on mice by the EMF generated by the DVC. Since the distance of detection of the DVC was restricted to 2.55 cm above/below and 1.5 cm laterally to the DVC board (according to internal testing performed by the manufacturer, personal communication), adjacent

cages could be not reached by the EMF generated by the DVC boards, and thus the checkboard arrangement of the two types of cage within the rack was chosen to minimize the effect on mice of the cage position within the rack.

Measurements of radiate EMF revealed that DVC boards produced EMF at a frequency ranging from 5 Hz to 3 GHz, thus including ELF, Intermediate frequency (IF), and RF EMF. Also inside IVC low levels of EMF were detected, and since EMF generated by the DVC were not able to reach adjacent cages, they were regarded as part of the background EMF present in the room due to external sources, as discussed above. Electric fields were significantly increased in DVC as compared to IVC at all ranges of frequency examined, while no significant differences between DVC and IVC were observed in the induction of magnetic fields. Although the electric fields were significantly increased in DVC, their intensity was extremely low in general terms and, in comparative terms, well below the ICNIRP reference levels recommended for general public exposure (that are based on established evidence regarding onset of acute effects on people (Online Table S8).[1] The evaluation of the temperature at the bottom of both types of the cages (IVC and DVC) revealed that DVC board did not generate heating, excluding a potential contributing role of heating on the induction of biological effects.

No clinical signs or increased mortality were observed in exposed mice of both sexes. Body weight of exposed male mice sacrificed at day 180 was significantly reduced compared to control male mice, and likely correlated to the reduced feed consumption observed in this group. The other statistically significant differences detected in food and water consumption were considered not biologically relevant because no differences were noted in the body weight of animals. Despite the difference in the body weight detected at day 180, no significant difference in the body weight gain between control and exposed male mice was observed in this group. When looking at the group of male mice that was maintained up to 365 days, no difference in body weight between control and exposed mice was present at days 180 and 365, indicating that this result was not reproducible and exposure time-dependent. At randomization of the 180-day male group there was no significant

difference in body weight between control and exposed mice, but the individual body weights of the exposed male mice were overall lower (except for one animal) than those of control mice, and we can speculate that over time this initially irrelevant difference became more evident. In literature, effects of EMF on body weight were contradictory. Gradual body weight loss was reported in male Balb/c mice after continuous whole-body exposure to 50 Hz ~1.4 mT ELF-EMF for 30 days,[32] and in male and female B6C3F1 mice exposed to 50 Hz 50 μT ELF-EMF for 15.5 months, with male mice starting to lose weight after 6 months and female mice after 4 months.[22] On the contrary, no significant effect on the body weight was found in male and female Swiss mice exposed for 90 days to 50 Hz 25 μT ELF-EMF,[38] in male and female B6C3F1 exposed for two years to 60 Hz 2 μT, 200 μT, and 1000 μT ELF-EMF,[16] in male Sprague-Dawley rats exposed to 50 Hz 25 mT ELF-EMF for 18 consecutive weeks.[39] in male albino rats exposed to 50 Hz 5 uT ELF-EMF for 32 weeks,[33] in female F344/N rats exposed to 60 Hz 2, 20, 200, 2000 uT ELF-EMF for 2 years,[14] in male and female F344 rats exposed to 50 Hz 0.5 and 5 mT magnetic fields for two years,[15] and in male and female F344/N rats exposed for two years to 60 Hz 2 μT, 200 μT, and 1000 μT ELF-EMF.[40]

Statistical analysis of hematology and bone marrow parameters revealed the presence of some significant differences between control and exposed mice in both sexes. However, results in both groups were always within the published reference intervals provided by the supplier of mice (https://www.criver.com/sites/default/files/resources/C57BL6MouseModelInformationSheet.pdf) or reported in literature.[41] Despite reference intervals reported in previous studies have been likely generated in different housing conditions and using different analyzers, the consistency of data recorded in this and previous studies suggests that most of the significant differences detected in this study did not have a biological relevance. This hypothesis is also supported by the fact that the magnitude of the changes recorded in exposed mice compared with control mice was lower than the intrinsic variability (imprecision) of the methods: for example the changes regarding HGB (day 60), HCT (day 120), MCV and MCHC (day 180) ranged from 1.6% to 5.3%, consistent with the inherent imprecision of most erythroid parameters.[42] Similarly, the differences regarding percentage of

lymphocytes (day 60 and 120), or erythroid precursors (day 365) in bone marrow, although of great magnitude (around 20%), are consistent with the intrinsic variability of microscopical cell counts, especially of cell populations that are poorly represented in the sample.[43]

The only hematological result considered biologically relevant was the decreased neutrophil count in exposed females after 60 and 365 days of exposure, given the magnitude of the difference recorded between control and exposed animals (-35.2% at day 60, and -53.4% at day 365 in exposed female mice) and the result largely lower in the exposed groups than the reference intervals.[41] Results of previous studies provided contrasting data regarding neutrophils: Çetin et al[31] reported a decrease of neutrophil~~s~~ counts in male Swiss mice after 90 and 120 days of exposure to pulsed 60 Hz 3μT ELF-EMF, while Hashish et al[32] reported an increase of neutrophils in male Swiss mice after 30 days of exposure to 50 Hz ~1.4 mT ELF-EMF. However, this latter result was referred to neutrophil percentages and not to neutrophil count and therefore it may be due to the corresponding decrease of lymphocytes rather than to a true increase of neutrophil counts, which should be considered unaffected by treatment.

A second experiment carried out by exposing a new additional group of female mice for 60 days, failed to confirm the previously observed difference in the neutrophil count between control and exposed female mice. The lack of reproducibility of this finding, along with the lack of bone marrow changes at day 60 and the lack of changes in neutrophils or bone marrow at day 120 and 180, suggests that the possible effect of DVC on neutrophils, if any, is transient and occasional. However, a decrease in the neutrophil count in exposed female was found again at day 365, when also the M:E ratio showed a significant decrease of great magnitude (-40.2%), suggesting that long term exposure may depress myeloid activity in the bone marrow, resulting in a decreased number of circulating neutrophils. Interestingly, no changes in the ratio between the proliferative or maturation myeloid pools were found, indicating that this possible effect, if any, may depend on a depressed myelopoietic activity rather than on a direct effect on a specific stage of myeloid progenitor cells. Finally, it is interesting to note that either at day 60 or at day 365 the reduction of neutrophil counts was detected

only in exposed female, suggesting a possible influence of gender in this effect. Despite the significant decrease of neutrophils observed at day 60 and 365, ~~was~~ considered of biological relevance because of its magnitude, there was no increase of ~~no~~ clinical signs or histopathological lesions indicative of infection, the main expected complication of neutropenia. ~~related to neutropenia (e.g. infections) were observed.~~

No exposure-related gross changes were observed during necropsy. Histopathological examination revealed the presence of several findings in sampled organs, but overall, all the lesions were considered spontaneous, either incidental or background lesions (strain, age and/or sex-related), with similar prevalence and severity observed in control and exposed groups, except for the reduction of hepatic fatty accumulation observed at day 180 in exposed mice (consistent with the decreased body weight observed in this group), and a significant decrease in the prevalence of renal intratubular hyaline casts in the exposed females at day 365. The biological meaning of this latter finding is doubtful, leading to the conclusion that it could be a fortuitous finding. Since only a single case of tumor was found in this study (histiocytic sarcoma in an exposed female at day 365), no conclusions can be drawn about the effect of DVC on cancer, and longer studies should be performed to investigate this issue.

According to previous studies, testis might represents one of the potential target organs of EMF in mice, where sperm abnormalities, increased or reduced testes weight, and/or increased germ cell apoptosis were previously observed.[26-29,44] In the current study, no significant differences were found in testes relative weight, histopathology, and germ cell apoptosis between control and exposed mice at all time points. The reduced absolute testes weight observed in exposed male mice at day 180 was correlated to the reduced body weight observed in this group, since no testicular histopathological lesions were identified. The number of intratubular apoptotic cells was overall very low (less than 1 per 100 tubules). At day 60, germ cell apoptosis was slightly increased ($p = 0.0760$ Mann-Whitney test) in exposed mice, but this trend was no more observed at later time points, indicating that even if a potential effect might exist it was early and transient.

Based on the results of this study, DVC electronic boards produced extremely low intensity electric fields at a wide range of frequency, from 5 Hz to 3 GHz. Maintenance of mice in DVC up to one-year exposure resulted in statistically significant differences in some parameters (body weight, water and feed consumption, some hematology and bone marrow parameters, and some histology findings), but most of them were considered likely fortuitous, resulting from biologic variability, or related to inbuilt variability of the assay method, as similarly concluded by other authors.[20,45] The only exception considered relevant from a biological point of view, and based on the results of previous studies, was the reduced neutrophil count at days 60 and 365 in exposed female mice. This finding was however not time-dependent, transient, not reproducible, and most importantly not associated with signs of infection and therefore likely not clinically relevant. Similarly to the reported controversial role of EMF on eliciting biological effects on humans and animals, also the results of this study were not completely unequivocal, and further studies are needed to unveil whether the exposure to the extremely low intensity EMF generated by the DVC might elicit unquestionable adverse effects on mice. In conclusion, the results of this study indicate that one-year maintenance in DVC for the purposes of intra-cage monitoring of the animal activity and cage micro-environment did not produce any definite clinicopathological effect in either sex of C57BL/6NCrl mice.

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Declaration of Conflicting Interests

Fondazione Unimi (formerly Fondazione Filarete) received a payment from Tecniplast S.p.a for the execution of necropsies, hematology, bone marrow cytology and histopathological examination. Fondazione Unimi assigned the execution of the service to the Mouse and Animal Pathology Laboratory and the Department of Veterinary Medicine. Camilla Recordati presented the preliminary results of this work at two meetings [AALAS 2015 (Phoenix, AZ, USA); FELASA 2016 (Brussels, Belgium)] and received reimbursement from Tecniplast S.p.a for travel expenses. Gianpaolo Milite worked as a scientific consultant in Laboratory Animal Sciences for Tecniplast S.p.a. Marcella De Maglie, Gerardo Marsella, Alessandro Rigamonti, Saverio Paltrinieri, Eugenio Scanziani declare that they ~~have~~ had no direct conflict of interest.

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Figure Legends

Figure 1. Chessboard distribution of standard IVC (white) and DVC (grey) on the rack. Cages included in the central area delimited by the bold black line underwent radiate electromagnetic field measurements.

Figure 2. Body weight of female (A) and male (B) mice measured at day 60, 120, 180, and 365 after the beginning of the exposure. Data are reported as individual values and median. Mann-Whitney Test was applied for statistical analysis. *** $p = 0.0002$.

Figure 3. Water consumption in female (A) and male (B) mice measured at day 60, 120, 180, and 365. Data are reported as median value and range. Mann-Whitney Test was applied for statistical analysis (n = number of cage changes). * $p < 0.05$; ** $p < 0.01$.

Figure 4. Feed consumption in female (A) and male (B) mice measured at day 60, 120, 180, and 365. Data are reported as median value and range. Mann-Whitney Test was applied for statistical analysis (n = number of cage changes). **** $p < 0.0001$.

Figure 5. Neutrophils were significantly reduced in exposed female mice at day 60 of exposure in the main experiment (**$p = 0.0072$), but in a replicate experiment no significant difference was observed ($p = 0.2594$). Data are reported as individual values and median..Mann-Whitney Test was applied for statistical analysis (n = 18-20).

Figure 6. Absolute (g) (A) and relative (% of body weight) (B) testes weight measured at day 60, 120, 180, and 365. Testes absolute weight was significantly reduced in exposed male mice at day 180 of exposure (** $p = 0.0015$). No significant differences were found in testes relative weight. Data are

reported as individual values and median. Mann-Whitney Test was applied for statistical analysis (n = 19-20).

Figure 7. Testes, immunohistochemical evaluation of germ cell apoptosis. (A) Percentage of the number of cleaved caspase-3 + cells per number of tubules per section of both testes, at day 60, 120, 180, and 365. Data are reported as individual values and median. Mann-Whitney Test was applied for statistical analysis (n = 19-20), no significant differences were found. (B) Representative image of a cleaved caspase-3 immunostained section: note the nuclear brown staining of a single apoptotic cell located in the basal layer of the seminiferous epithelium in an exposed mouse, at day 60 (immunoperoxidase staining, original objective 40X). Image of cleaved-caspase 3 immunostained mouse spleen section used as positive control is shown in Online Figure S2.

Online Figure S1. Cages identified with an asterisk and included in the central area delimited by the bold black line underwent temperature measurements.

Online Figure S2. Mouse spleen used as positive control for the cleaved-caspase 3 immunostaining: occasional apoptotic cells (with brown-stained nuclei) are visible in the germinal centre of a splenic follicle (immunoperoxidase staining, original objective 40X).

Table 1. Results of electric field measurements in empty IVC and DVC. Significant differences in the electric field values were found between IVC and DVC at all the frequency ranges examined.

Cage	**No. of examined cages**	**Electric Field r.m.s. [V/m] mean ± SD**			
		5Hz-100Hz	**12Hz-1KHz**	**1.2KHz-100KHz**	**100KHz-3GHz**
IVC	10	4.98±1.01	4.90±1.05	0.38±0.01	0.31±0.02
DVC	10	8.56±2.42	8.21±2.08	0.44±0.02	0.44±0.06
Mann-Whitney test	*p*-value	0.0004	0.0002	0.0002	0.0004

Table 2. Results of magnetic field measurements in empty IVC and DVC. No significant differences were found between IVC and DVC.

Cage	No. of examined cages	Magnetic Flux Density (B) [µT] mean ± SD			
		5Hz-100Hz	12Hz-1KHz	1.2KHz-100KHz	100KHz-3GHz
IVC	10	0.02±0.01	0.03±0.00	0.06±0.00	nd
DVC	10	0.02±0.01	0.03±0.00	0.06±0.00	nd
Mann-Whitney test	*p*-value	0.8664	0.8002	0.8935	\

nd = not detectable (overlapping with background magnetic field noise of the room)

Table 3. Results of temperature evaluation in the environment, and within IVC and DVC, measured at the bottom of the cage. Mann-Whitney Test was applied for statistical analysis. No significant differences were found between IVC and DVC.

	Temperature °C mean ± SD		
Tested Condition	**Environment**	**IVC (n = 4)**	**DVC (n = 5)**
DVC OFF	21.80 ± 0.02	21.89 ± 0.12	21.92 ± 0.11
DVC ON	21.80 ± 0.06	21.81 ± 0.16	21.87 ± 0.18

Table 4. Body weight gain in female and male mice at day 60, 120, 180, and 365. Data are expressed as % of increased body weight within each group. For statistical analysis Mann-Whitney test was applied (n = 5 cages per group).

Time point	FC	FE	*p*-value	MC	ME	*p*-value
day 60	52.81%	52.34%	>0.9999	68.13%	70.55%	0.5476
day 120	70.74%	74.31%	0.6905	110.22%	117.00%	0.4206
day 180	91.08%	112.82%	0.0317	136.12%	117.62%	0.0556
day 365	111.25%	102.90%	0.2222	154.41%	135.73%	0.5476

FC=female control; FE=female exposed; MC=male control; ME=male exposed

Table 5. Prevalence of mortality in female and male mice at day 60, 120, 180, and 365. Data are expressed as no. of dead mice/no. of total mice per group. For statistical analysis Fisher's exact test was applied (no significant differences were found) (n = 20).

Day	FC	FE	MC	ME
60	0/20	1/20	0/20	0/20
120	0/20	0/20	1/20	0/20
180	0/20	2/20	0/20	0/20
365	1/20	0/20	0/20	1/20
Total	1/80	3/80	1/80	1/80

FC=female control; FE=female exposed; MC=male control; ME=male exposed

Table 6. Summary of results of hematological and bone marrow examination at day 60, 120, 180, and 365 of exposure that resulted in a significant difference between control and exposed mice. Data are expressed as mean ± SD. Mann-Whitney Test was applied for statistical analysis.

Time point	Analysis	Parameter	FC	FE	*p*-value	MC	ME	*p*-value
Day 60	**CBC**	***n***	***19***	***18***		***18***	***18***	
		HGB (g/dL)	13.82 ± 0.34	14.05 ± 0.55	0.0171	13.86 ± 0.51	14.23 ± 0.71	0.0945
		Neutrophils x 10/µL	1.05 ± 0.39	0.68 ± 0.36	0.0072	1.02 ± 0.76	1.11 ± 1.04	0.9543
	Bone marrow	***n***	***20***	***19***		***19***	***20***	
		%L	8.54 ± 2.48	10.44 ± 2.52	0.0117	10.25 ± 3.34	8.33 ± 2.63	0.0372
Day 120	**CBC**	***n***	***17***	***20***		***17***	***19***	
		HCT (%)	54.26 ± 2.22	53.89 ± 2.05	0.4925	53.69 ± 2.15	55.53 ± 1.81	0.0112
	Bone marrow	***n***	***20***	***20***		***18***	***20***	
		%L	9.49 ± 1.61	7.78 ± 1.91	0.0075	8.07 ± 2.42	7.87 ± 2.18	0.5778
Day 180	**CBC**	***n***	***20***	***18***		***20***	***19***	
		MCV (fL)	53.04 ± 2.36	51.41 ± 2.00	0.0091	49.49 ± 4.14	51.36 ± 1.64	0.3686
		MCHC (%)	24.95 ± 0.84	25.54 ± 0.44	0.0035	26.01 ± 2.26	24.63 ± 0.47	0.0330
Day 365	**CBC**	***n***	***18***	***17***		***17***	***18***	
		Neutrophils x 10/µL	1.33 ± 0.78	0.62 ± 0.39	0.0009	1.08 ± 0.52	1.30 ± 0.78	0.4675
		Eosinophils x 10/µL	0.06 ± 0.07	0.04 ± 0.05	0.6660	0.04 ± 0.08	0.10 ± 0.09	0.0203
	Bone marrow	***n***	***19***	***19***		***20***	***19***	
		M:E	0.87 ± 0.60	0.52 ± 0.25	0.0171	1.06 ± 0.29	1.11 ± 0.39	0.7335

PE:ME	0.15 ± 0.04	0.12 ± 0.03	0.0134	0.14 ± 0.06	0.14 ± 0.03	0.9278
%PE	13.04 ± 3.32	10.53 ± 2.18	0.0081	11.99 ± 4.41	11.91 ± 2.54	0.8786

FC=female control; FE=female exposed; MC=male control; ME=male exposed

Table 7. Prevalence of gross lesions at day 60, 120, 180, and 365 days of exposure. Data are expressed as no. of affected mice/no. of total examined mice. Fisher's exact test was applied for statistical analysis.

Organ and finding	day 60				day 120				day 180				day 365			
	FC	FE	MC	ME	FC	FE	MC	ME	FC	FE	MC	ME	FC	FE	MC	ME
Spleen, apical melanosis	1/20	1/19	1/20	1/20	0/20	2/20	3/19	1/20	0/20	0/18	1/20	0/20	0/19	0/20	0/20	0/19
Splenomegaly	0/20	0/19	0/20	0/20	1/20	0/20	1/19	0/20	0/20	0/18	0/20	0/20	3/19	3/20	0/20	0/19
Kidney, hydronephrosis (unilateral)	0/20	0/19	0/20	0/20	1/20	0/20	1/19	0/20	0/20	0/18	0/20	1/20	0/19	0/20	0/20	0/19
Uterus, enlargement	6/20	8/19	na	na	9/20	4/20	na	na	3/20	2/18	na	na	3/19	3/20	na	na
Preputial gland, abscess	na	na	1/20	0/20	na	na	3/19	0/20	na	na	0/20	0/20	na	na	5/20	3/19
Retroauricular SC abscess	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	1/19	0/20	0/20	0/19
Submandibular lymph nodes, enlargement	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	1/19	0/20	1/20	0/19
Salivary glands, enlargement	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	0/19	0/20	3/20	3/19
Barbering	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	1/18	2/20	1/20	13/19	14/20	6/20	11/19
Kyphosis	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	14/19	9/20	0/20	0/19

FC=female control; FE=female exposed; MC=male control; ME=male exposed

na = not applicable

Table 8. Prevalence of histopathological findings at day 60, 120, 180, and 365 days of exposure. Data are expressed as no. of affected mice/no. of total examined mice. Fisher's exact Test was applied for statistical analysis. na = not applicable.

Organ/tissue and finding	day 60				day 120				day 180				day 365			
	FC	FE	MC	ME	FC	FE	MC	ME	FC	FE	MC	ME	FC	FE	MC	ME
Liver																
Inflammation, perivascular	10/19	8/19	11/20	13/20	14/20	14/20	12/19	12/20	15/20	17/18	14/20	14/20	17/19	18/20	19/20	16/19
Inflammation, focal	19/19	19/19	18/20	20/20	20/20	20/20	15/19	17/20	20/20	18/18	16/20	14/20	16/19	19/20	17/20	17/19
Hepatocyte, fatty change	0/19	0/19	0/20	0/20	0/20	0/20	1/19	1/20	0/20	0/18	15/20	5/20**	0/19	0/20	12/20	5/19
Pigment, kupffer cells (hemosiderin)	0/19	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	2/19	0/20	0/20	0/19
Extramedullary hematopoiesis	0/19	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	1/19	1/20	0/20	0/19
Hepatocyte, anisokaryosis/kayomegaly	0/19	0/19	1/20	0/20	0/20	0/20	0/19	3/20	0/20	0/18	0/20	3/20	0/19	0/20	4/20	5/19
Spleen																
Pigment (Hemosiderin)	15/20	10/19	5/20	7/20	20/20	19/19	16/19	18/20	20/20	18/18	20/20	20/20	19/19	20/20	20/20	19/19
Melanosis	3/20	1/19	2/20	2/20	1/20	2/19	5/19	1/20	1/20	1/18	1/20	1/20	0/19	2/20	2/20	0/19
Extramedullary hematopoiesis, increased§	3/20	3/19	4/20	2/20	4/20	1/19	1/19	1/20	2/20	1/18	0/20	0/20	5/19	1/20	0/20	2/19
Hyperplasia, follicular	1/20	2/19	1/20	1/20	5/20	2/19	11/19	8/20	1/20	1/18	3/20	2/20	6/19	8/20	0/20	2/19
Kidney																
Interstitium, infiltration, cellular	3/20	2/19	0/20	0/20	4/20	1/20	5/19	5/20	4/20	3/18	6/20	8/20	16/19	13/20	19/20	18/19
Glomerulopathy, hyaline	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	6/19	3/20	0/20	0/19
Glomerulonephritis	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	1/19	0/20	0/20	0/19
Tubule, vacuolation, cytoplasmic	0/20	0/19	0/20	0/20	0/20	0/20	15/19	15/20	0/20	0/18	16/20	14/20	0/19	0/20	18/20	15/19
Tubule, regeneration	0/20	0/19	0/20	0/20	0/20	1/20	1/19	1/20	0/20	0/18	0/20	1/20	2/19	3/20	5/20	6/19
Tubule, cast, hyaline	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	2/20	1/18	0/20	0/20	8/19	2/20	0/20	0/19

Tubule, dilation	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	0/19	0/20	1/20	1/19
Fibrosis	0/20	0/19	0/20	0/20	1/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	0/19	0/20	0/20	0/19
Mineralization	1/20	0/19	0/20	1/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	0/19	0/20	1/20	0/19
Infarct, cortical, acute	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	0/19	0/20	0/20	1/19
Pelvis, dilation	0/20	0/19	0/20	0/20	1/20	0/20	1/19	0/20	0/20	0/18	0/20	1/20	0/19	0/20	0/20	0/19
Adrenals																
Cortex, hyperplasia, subcapsular	3/18	0/17	0/20	0/16	9/15	13/18	0/16	0/16	14/17	7/12	1/18	0/17	10/15	10/16	0/14	0/9
Testes																
Intratubular multinucleated giant cells	na	na	10/20	11/20	na	na	7/19	7/20	na	na	7/20	10/20	na	na	8/20	12/19
Tubular atrophy	na	na	0/20	0/20	na	na	0/19	0/20	na	na	3/20	4/20	na	na	3/20	2/19
Interstitial cell, hyperplasia	na	na	0/20	0/20	na	na	0/19	0/20	na	na	0/20	0/20	na	na	9/20	9/19
Mammary gland																
Secretory material	0/20	0/19	na	na	13/20	11/20	na	na	7/20	6/18	na	na	7/19	10/20	na	na
Hyperplasia	0/20	0/19	na	na	0/20	0/20	na	na	2/20	1/18	na	na	6/19	3/20	na	na
Brain																
Meninges, melanosis	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne	7/19	3/20	6/20	11/19
Choroid plexus, dysplasia	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne	1/19	0/20	0/20	0/19

FC=female control; FE=female exposed; MC=male control; ME=male exposed; na = not applicable; ne = not examined

§ > grade 2

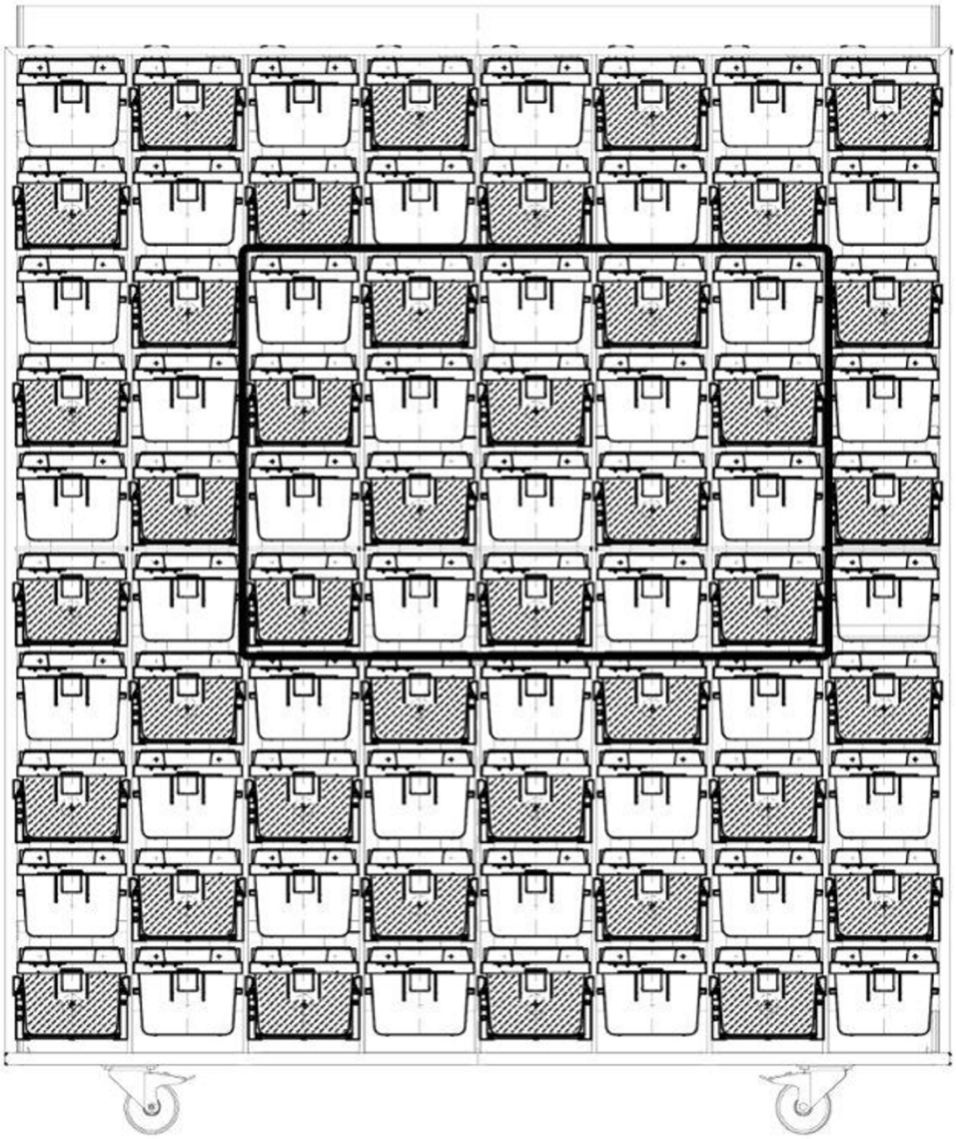

Figure 1. Chessboard distribution of standard IVC (white) and DVC (grey) on the rack. Cages included in the central area delimited by the bold black line underwent radiate electromagnetic field measurements.

88x104mm (600 x 600 DPI)

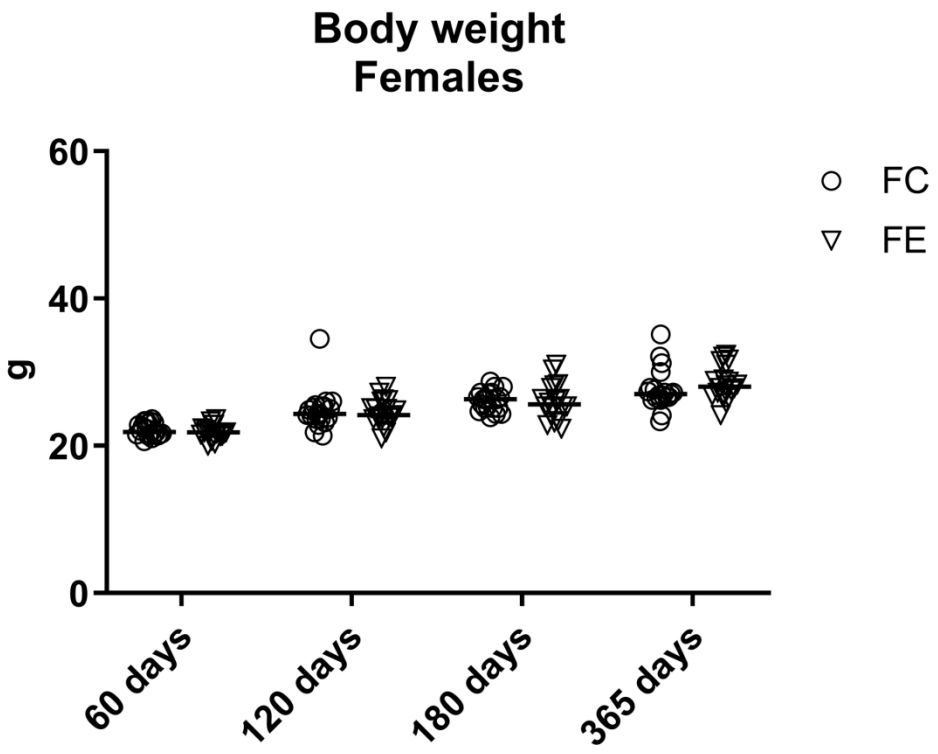

Figure 2. Body weight of female (A) and male (B) mice measured at day 60, 120, 180, and 365 after the beginning of the exposure. Data are reported as individual values and median. Mann-Whitney Test was applied for statistical analysis. *** p = 0.0002.

88x72mm (600 x 600 DPI)

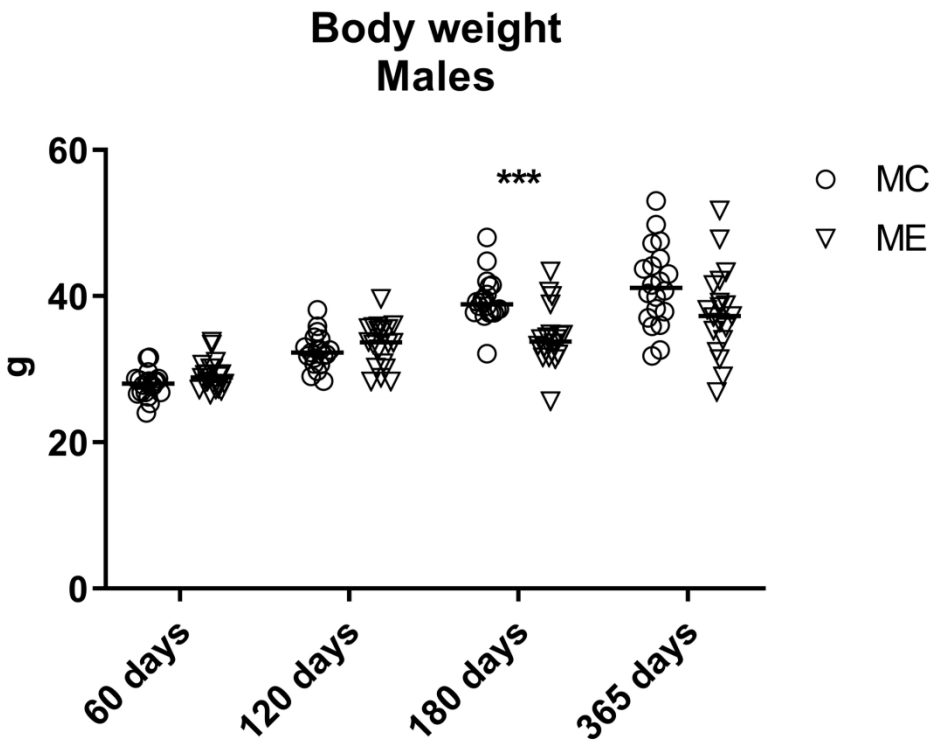

Figure 2. Body weight of female (A) and male (B) mice measured at day 60, 120, 180, and 365 after the beginning of the exposure. Data are reported as individual values and median. Mann-Whitney Test was applied for statistical analysis. *** p = 0.0002.

88x71mm (600 x 600 DPI)

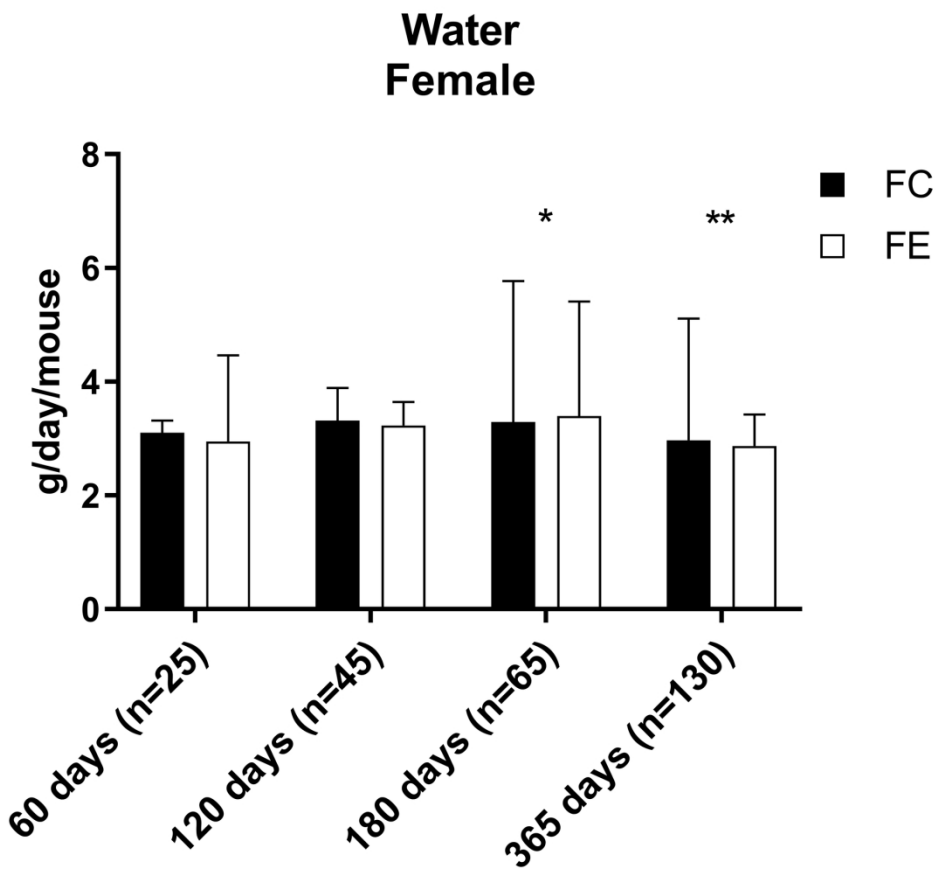

Figure 3. Water consumption in female (A) and male (B) mice measured at day 60, 120, 180, and 365. Data are reported as median value and range. Mann-Whitney Test was applied for statistical analysis (n = number of cage changes). * $p < 0.05$; ** $p < 0.01$.

88x88mm (600 x 600 DPI)

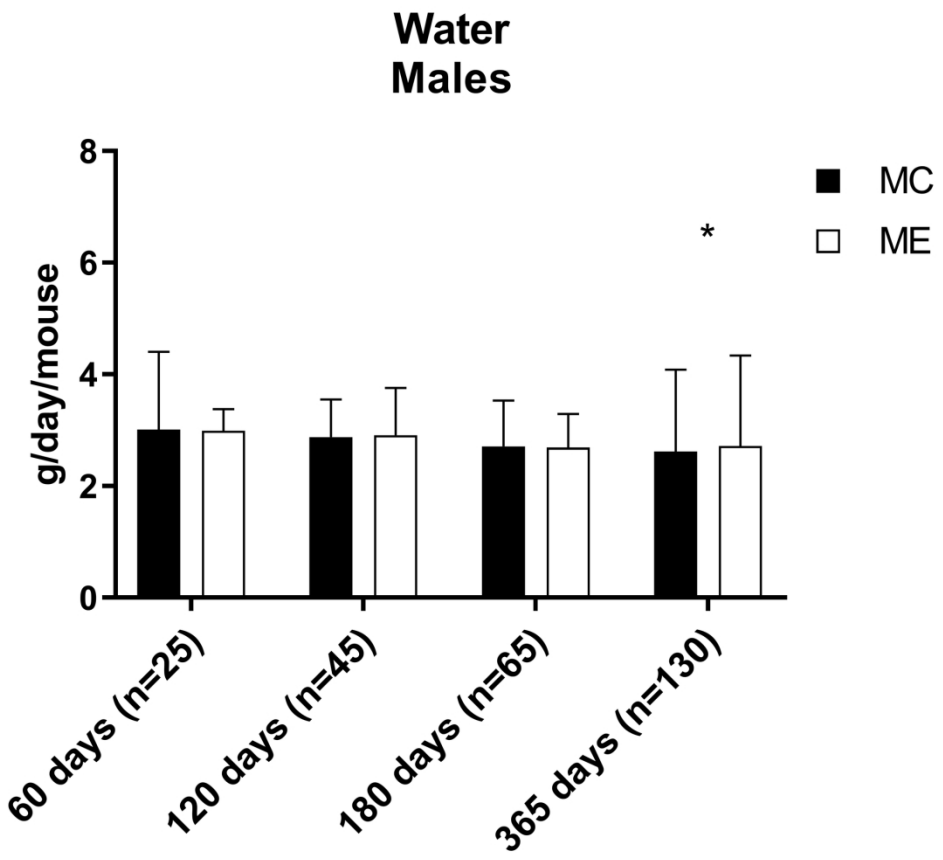

Figure 3. Water consumption in female (A) and male (B) mice measured at day 60, 120, 180, and 365. Data are reported as median value and range. Mann-Whitney Test was applied for statistical analysis (n = number of cage changes). * $p < 0.05$; ** $p < 0.01$.

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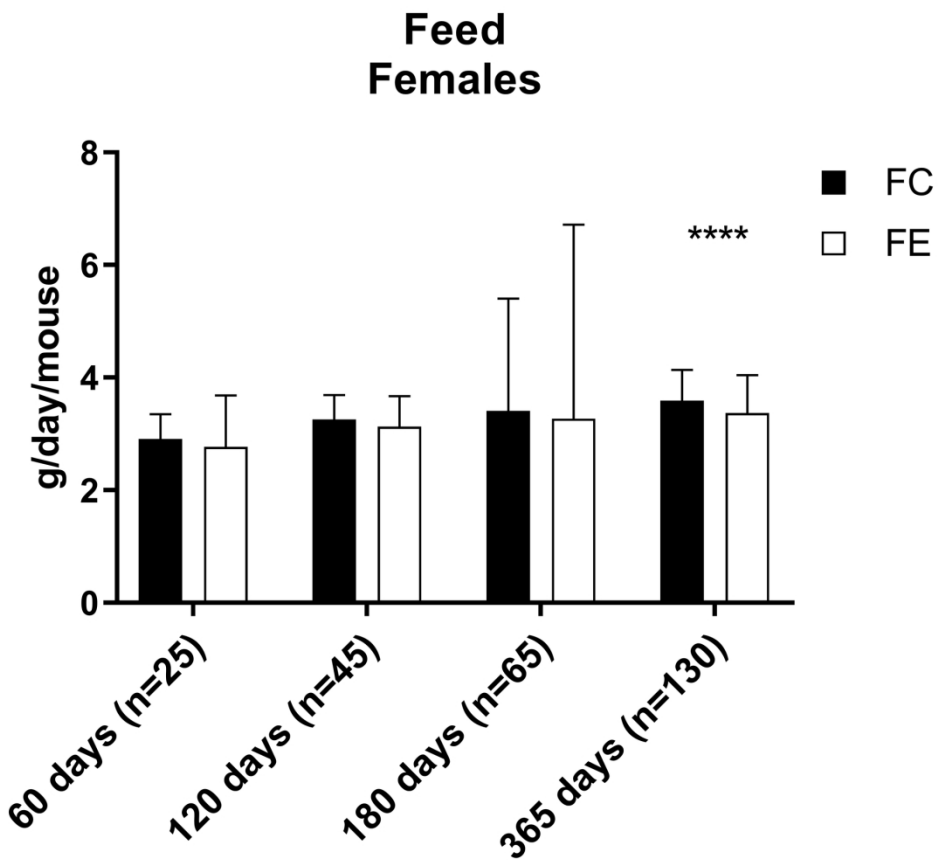


Figure 4. Feed consumption in female (A) and male (B) mice measured at day 60, 120, 180, and 365. Data are reported as median value and range. Mann-Whitney Test was applied for statistical analysis (n = number of cage changes). **** $p < 0.0001$.

88x88mm (600 x 600 DPI)

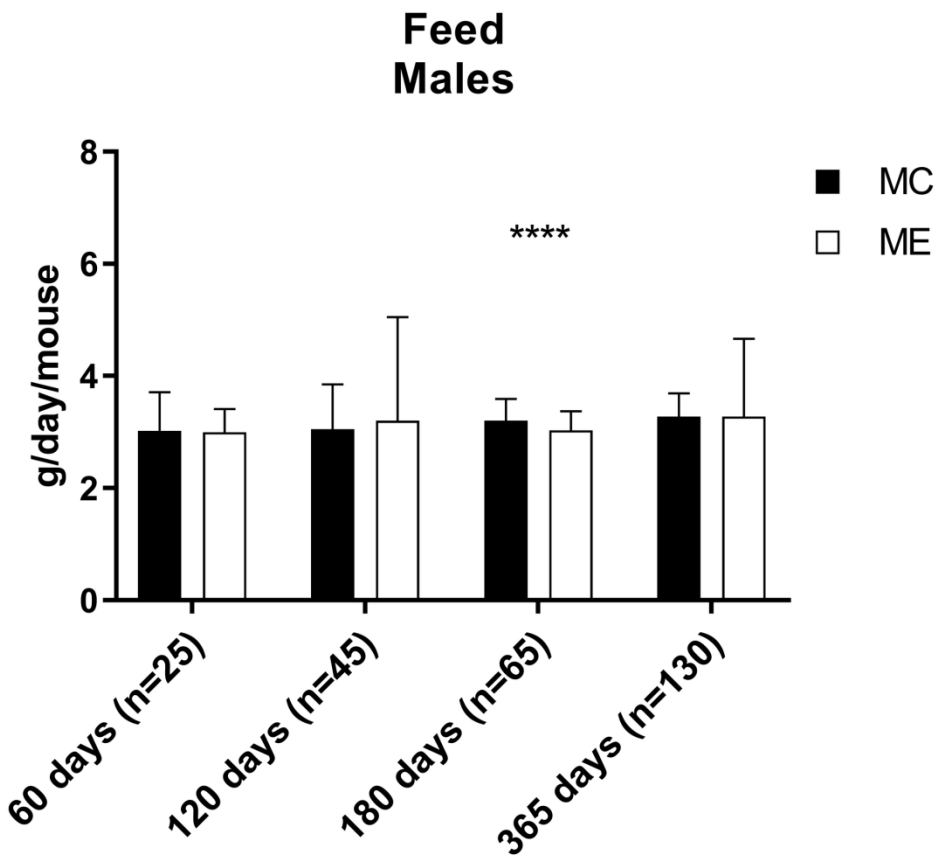


Figure 4. Feed consumption in female (A) and male (B) mice measured at day 60, 120, 180, and 365. Data are reported as median value and range. Mann-Whitney Test was applied for statistical analysis (n = number of cage changes). **** $p < 0.0001$.

88x87mm (600 x 600 DPI)

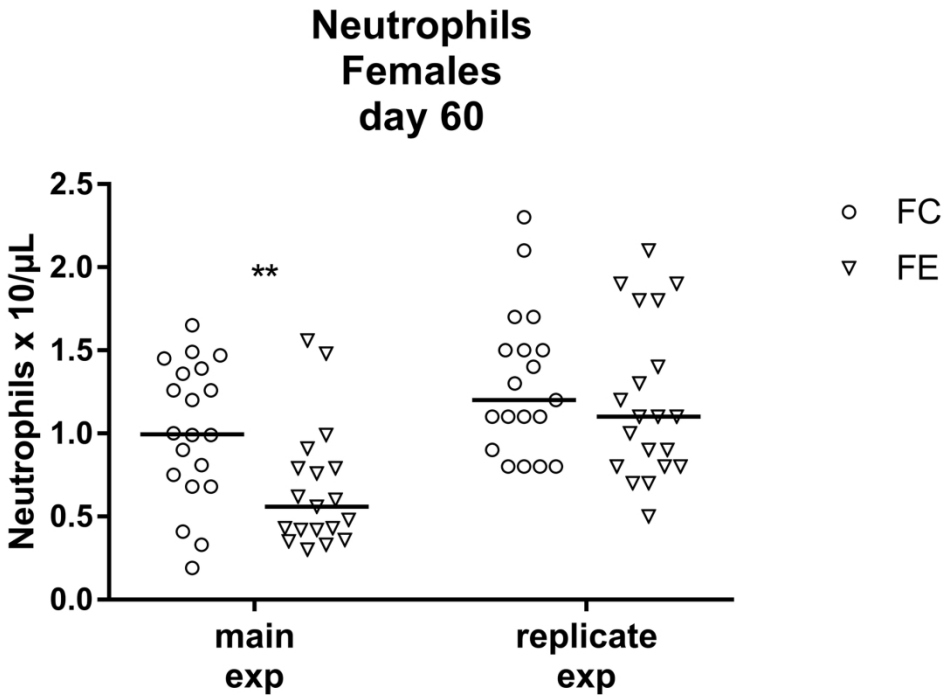


Figure 5. Neutrophils were significantly reduced in exposed female mice at day 60 of exposure in the main experiment (**p = 0.0072), but in a replicate experiment no significant difference was observed (p = 0.2594). Data are reported as individual values and median..Mann-Whitney Test was applied for statistical analysis (n = 18-20).

88x73mm (600 x 600 DPI)

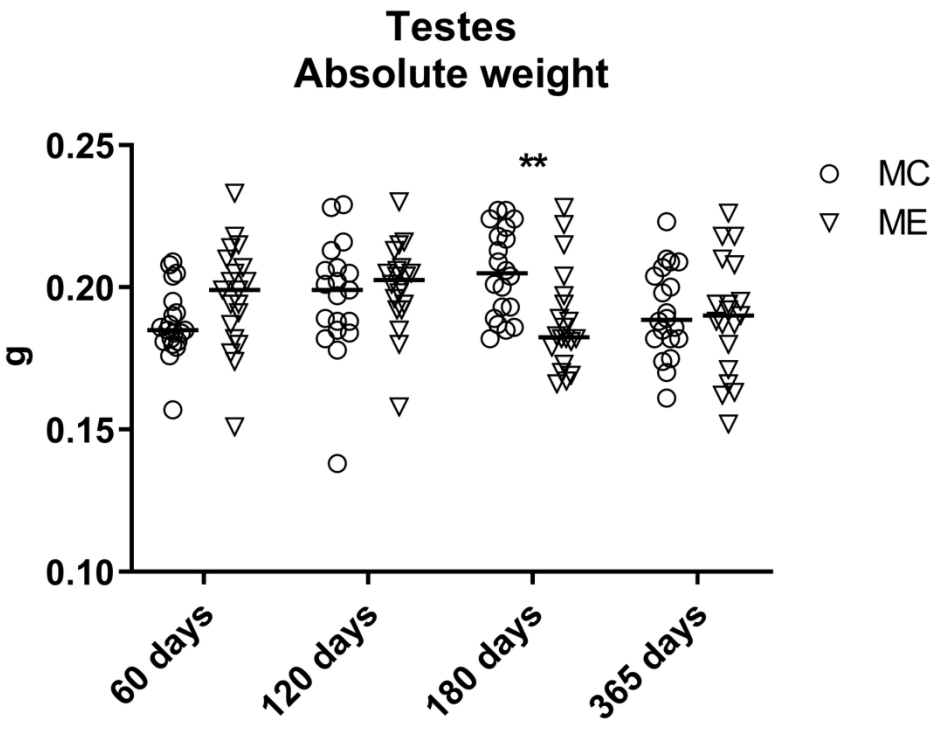


Figure 6. Absolute (g) (A) and relative (% of body weight) (B) testes weight measured at day 60, 120, 180, and 365. Testes absolute weight was significantly reduced in exposed male mice at day 180 of exposure (** p = 0.0015). No significant differences were found in testes relative weight. Data are reported as individual values and median. Mann-Whitney Test was applied for statistical analysis (n = 19-20).

88x75mm (600 x 600 DPI)

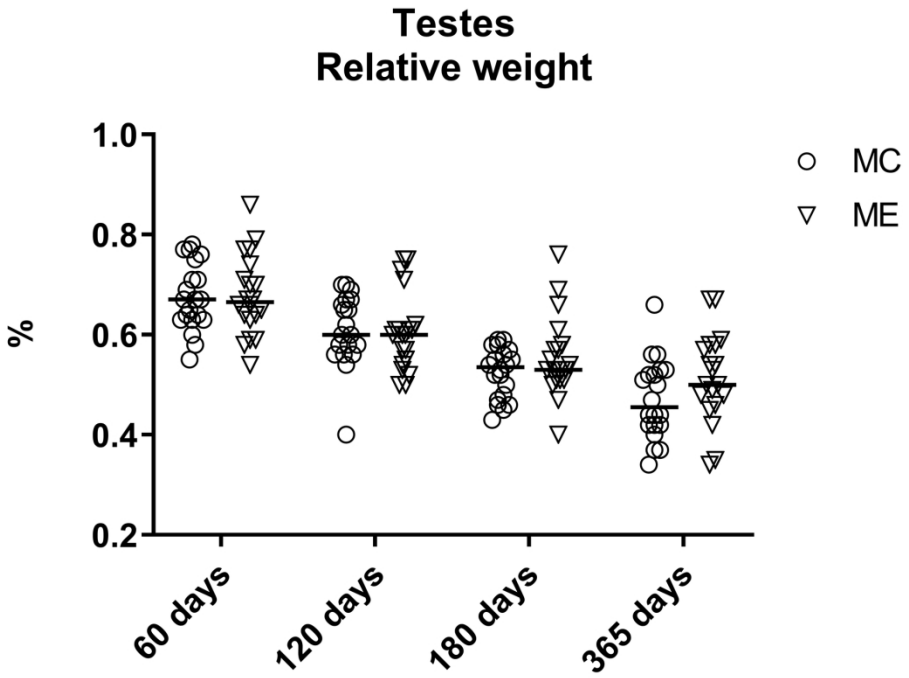


Figure 6. Absolute (g) (A) and relative (% of body weight) (B) testes weight measured at day 60, 120, 180, and 365. Testes absolute weight was significantly reduced in exposed male mice at day 180 of exposure (** p = 0.0015). No significant differences were found in testes relative weight. Data are reported as individual values and median. Mann-Whitney Test was applied for statistical analysis (n = 19-20).

88x70mm (600 x 600 DPI)

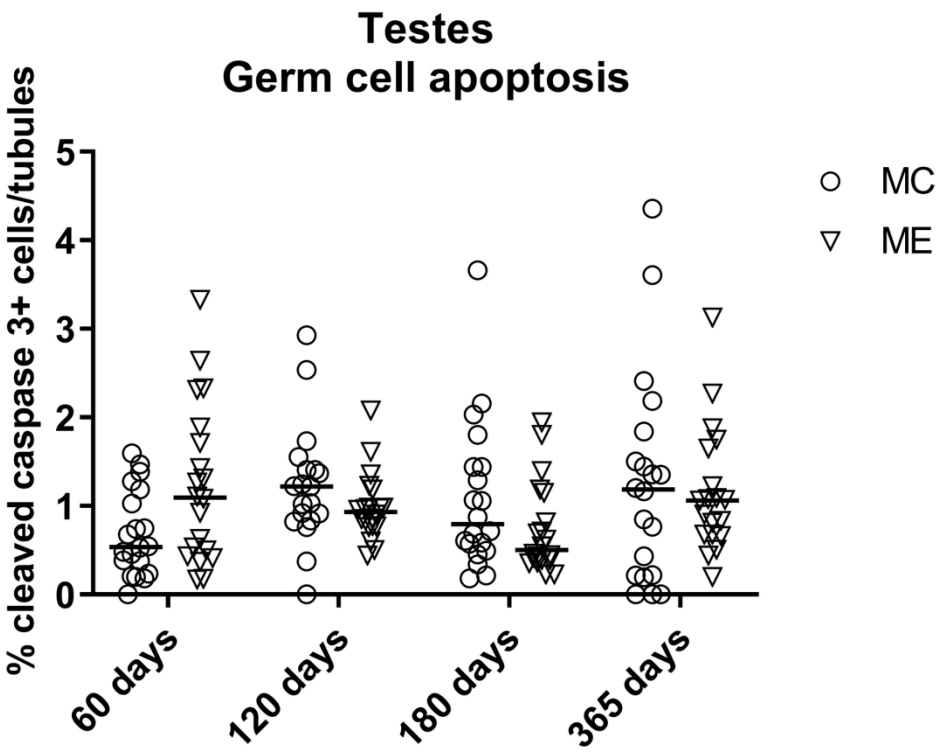
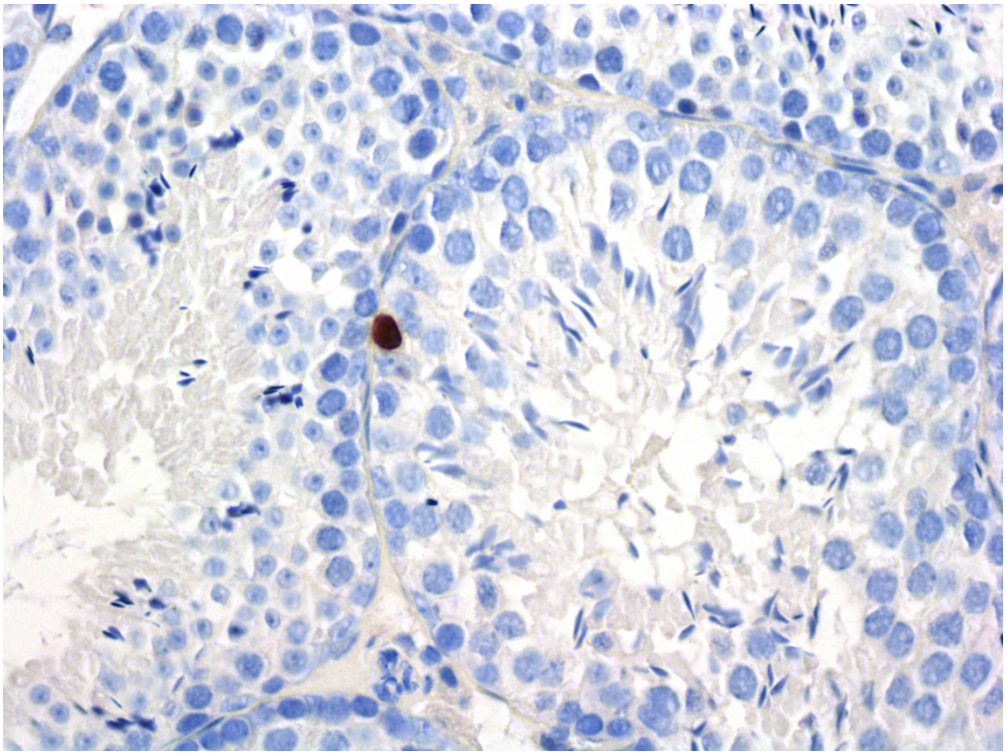


Figure 7. Testes, immunohistochemical evaluation of germ cell apoptosis. (A) Percentage of the number of cleaved caspase-3 + cells per number of tubules per section of both testes, at day 60, 120, 180, and 365. Data are reported as individual values and median. Mann-Whitney Test was applied for statistical analysis (n = 19-20), no significant differences were found. (B) Representative image of a cleaved caspase-3 immunostained section: note the nuclear brown staining of a single apoptotic cell located in the basal layer of the seminiferous epithelium in an exposed mouse, at day 60 (immunoperoxidase staining, original objective 40X). Image of cleaved-caspase 3 immunostained mouse spleen section used as positive control is shown in Online Figure S2.

88x77mm (600 x 600 DPI)



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88x66mm (600 x 600 DPI)

Online Table S1. Histopathological grading system

Organ	Finding	Grade	Description
Liver	Inflammation, perivascular (no. of aggregates)	0	no inflammatory cells aggregates
		1	aggregate of < 10 inflammatory cells
		2	aggregate of 10 - 100 inflammatory cells
		3	aggregate of > 100 inflammatory cells < field at 400x (500 µm in diameter)
		4	aggregate of inflammatory cells > field at 400x (500 µm in diameter)
		total no.	sum of no. of aggregates 1+2+3+4
		total score	no. of aggregates 1*1 + no. of aggregates 2*2+ no. of aggregates 3*3 + no. of aggregates 4*4
	Inflammation, focal (no. of aggregates)	0	no inflammatory cells aggregates
		1	aggregate of < 10 inflammatory cells
		2	aggregate of 10 - 100 inflammatory cells
		3	aggregate of > 100 inflammatory cells < field at 400x (500 µm in diameter)
		4	aggregate of inflammatory cells > field at 400x (500 µm in diameter)
		total no.	sum of no. of aggregates 1+2+3+4
		total score	no. of aggregates 1*1 + no. of aggregates 2*2+ no. of aggregates 3*3 + no. of aggregates 4*4
	Hepatocyte, fatty accumulation	0	absent
		1	rare (<5%) of hepatocytes affected
		2	some (5-25%) of hepatocytes affected
		3	numerous (26-50%) of hepatocytes affected
		4	most (> 50%) of hepatocytes affected
Spleen	Hyperplasia, follicular	0	absent
		1	rare (<5%) follicles slightly enlarged with blast cells
		2	some (5-25%) follicles moderately enlarged with blast cells
		3	numerous (25-50) follicles enlarged with blast cells

		4	most (> 50%) follicles enlarged and in contact, with blast cells
	Pigment (Hemosiderin)	0	absent
		1	few siderocytes within red pulp
		2	some siderocytes within red and white pulp
		3	moderate numbers of siderocytes within red and white pulp
		4	numerous siderocytes within red and white pulp
	Melanosis	0	absence
		1	presence
	Extramedullary hematopoiesis	0	absent
		1	<5% of the splenic area
		2	5-25% of the splenic area
		3	25-50% of the splenic area
		4	most (> 50%) of the splenic area
Kidney	Renal tubule, vacuolation, cytoplasmic	0	absent
		1	few~~x~~ (<10%) of tubules affected
		2	numerous (from 11 to 50%) of tubules affected
		3	most (>50%) of tubules affected
	Renal tubule, regeneration (basophilic tubules)	0	absent
		1	rare = 1 to 3
		2	some = 4 to 10
		3	numerous = more than 10
	Interstitium, infiltration, cellular, perivascular (cortical)	0	absent
		1	lymphocytic infiltrate < 100 cells
		2	lymphocytic infiltrate > 100 cells
		3	lymphocytic infiltrate > field at 400x (500 µm in diameter)
	Interstitium, infiltration, cellular peripelvic	0	absent
		1	lymphocytic infiltrate < 100 cells
		2	lymphocytic infiltrate > 100 cells
		3	lymphocytic infiltrate > field at 400x (500 µm in diameter)

Glomerulopathy, hyaline	0	absent
	1	few (<10%) of glomeruli affected
	2	numerous (from 11 to 50%) of glomeruli affected
	3	most (>50%) of glomeruli affected
Renal tubule, cast, hyaline	0	absent
	1	rare = 1 to 3
	2	some = 4 to 10
	3	numerous = more than 10

Online Table S2. Health monitoring results

Agent	60 days			120 days			180 days			365 days		
	Cage A[1]	Cage B[1]	Pre-filter[2]	Cage A[3]	Cage B[3]	Pre-filter[2]	Cage A[3]	Cage B[3]	Pre-filter[2]	Cage A[3]	Cage B[3]	Pre-filter[2]
LCMV PCR	-	-	-	-	-	-	-	-	-	-	-	-
MAV 1 & 2 PCR	-	-	-	-	-	-	-	-	-	-	-	-
MHV PCR	-	-	-	-	-	-	-	-	-	-	-	-
MNV PCR	-	-	-	-	-	-	-	-	-	-	-	-
Mousepox (Ectromelia) PCR	-	-	-	-	-	-	-	-	-	-	-	-
MPV PCR	-	-	-	-	-	-	-	-	-	-	-	-
MRV (EDIM) PCR	-	-	-	-	-	-	-	-	-	-	-	-
PVM PCR	np	np	-	-	-	-	-	-	-	-	-	-
REO PCR	-	-	-	-	-	-	-	-	-	-	-	-
SEND PCR	np	np	-	-	-	-	-	-	-	-	-	-
TMEV/GDVII PCR	-	-	-	-	-	-	-	-	-	-	-	-
Beta Strep Grp A PCR	np	np	np	-	-	np	-	-	np	-	-	np
Beta Strep Grp B PCR	-	-	-	-	-	-	-	-	-	-	-	-
Beta Strep Grp C PCR	-	-	-	np	np	-	np	np	-	np	np	-
Beta Strep Grp G PCR	-	-	-	np	np	-	np	np	-	np	np	-
B. bronchiseptica PCR	np	np	-	np	np	-	np	np	-	np	np	-

B. hinzii PCR	-	-	-	np	np	-	np	np	-	np	np	-
C. bovis PCR	-	-	-	np	np	-	np	np	-	np	np	-
C. kutscheri PCR	-	-	-	-	-	-	-	-	-	-	-	-
C. piliforme PCR	np	np	-	-	-	-	-	-	-	-	-	-
C. rodentium PCR	-	-	-	-	-	-	-	-	-	-	-	-
Campylobacter Genus PCR	-	-	-	np	np	-	np	np	-	np	np	-
CAR *Bacillus* PCR	np	np	-	np	np	-	np	np	-	np	np	-
Helicobacter genus	-	-	-	-	-	-	-	-	-	-	-	-
K. oxytoca PCR	+	+	+	np	np	-	np	np	+	np	np	-
K. pneumoniae PCR	-	-	-	np	np	-	np	np	-	np	np	-
M. pulmonis PCR	np	np	-	-	-	-	-	-	-	-	-	-
P. mirabilis	-	-	-	np	np	-	np	np	-	np	np	-
P. pneumotropica-Heyl PCR	-	-	-	-	-	-	-	-	-	-	-	-
P. pneumotropica-Jawetz PCR	-	-	-	-	-	-	-	-	-	-	-	-
Ps. aeruginosa PCR	np	np	-	np	np	-	np	np	-	np	np	-
S. aureus PCR	+	+	+	np	np	+	np	np	+	np	np	-
S. moniliformis PCR	-	-	-	-	-	-	-	-	-	-	-	-
S. pneumoniae PCR	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella Genus PCR	-	-	-	-	-	-	-	-	-	-	-	-
Cryptosporidium PCR	-	-	-	-	-	-	-	-	-	-	-	-

Entamoeba PCR	-	-	-	-	-	-	-	-	-	-	-	-
Mite PCR	-	-	-	-	-	-	-	-	-	-	-	-
Giardia PCR	-	-	-	-	-	-	-	-	-	-	-	-
Pinworm PCR	-	-	-	-	-	-	-	-	-	-	-	-
Spironucleus muris PCR	-	-	-	-	-	-	-	-	-	-	-	-
Pneumocystis PCR	np	np	-	np	np	-	np	np	-	np	np	-

Cage A = IVC

Cage B = DVC

[1] Mouse Surveillance PRIA Panel

[2] Environmental (EAD) Mouse Surveillance Plus PRIA Panel

[3] Mouse FELASA Complete PRIA Panel

- = negative; + = positive; np = not performed

Online Table S3. Summary of results of hematological and bone marrow examination at day 60 of exposure. Data are expressed as mean ± SD. Mann-Whitney Test was applied for statistical analysis.

CBC						
Parameter	**FC**	**FE**	***p*-value**	**MC**	**ME**	***p*-value**
n	***19***	***18***	**-**	***18***	***18***	
RBC (10^6/µL)	10.15 ± 0.32	10.29 ± 0.42	0.0996	10.49 ± 0.53	10.73 ± 0.58	0.1585
HGB (g/dL)	13.82 ± 0.34	14.05 ± 0.55	0.0171	13.86 ± 0.51	14.23 ± 0.71	0.0945
HCT (%)	54.69 ± 2.02	55.84 ± 2.05	0.0738	56.44 ± 2.25	57.39 ± 3.48	0.1887
MCV (fL)	53.91 ± 1.46	54.28 ± 1.09	0.2179	53.87 ± 1.46	53.49 ± 1.41	0.5261
MCH (pg)	13.61 ± 0.19	13.65 ± 0.20	0.4677	13.22 ± 0.25	13.26 ± 0.36	0.7328
MCHC (%)	25.28 ± 0.66	25.16 ± 0.36	0.5821	24.56 ± 0.59	24.83 ± 1.09	0.7241
PLT (10^3/µL)	1413.4 ± 207	1279.6 ± 236	0.0805	1560.6 ± 266	1523.7 ± 282	0.8820
WBC (10^3/µL)	11.27 ± 3.18	10.26 ± 2.57	0.1687	10.70 ± 4.89	10.07 ± 4.32	0.5628
Neutrophils x 10/µL	1.05 ± 0.39	0.68 ± 0.36	0.0072	1.02 ± 0.76	1.11 ± 1.04	0.9543
Lymphocytes x 10/µL	9.66 ± 2.67	9.19 ± 2.25	0.4430	8.93 ± 4.40	8.40 ± 3.35	0.5086
Monocytes x 10/µL	0.46 ± 0.25	0.36 ± 0.19	0.3017	0.47 ± 0.30	0.49 ± 0.29	0.8259
Eosinophils x 10/µL	0.10 ± 0.09	0.05 ± 0.06	0.0616	0.13 ± 0.13	0.07 ± 0.08	0.2151
Basophils x 10/µL	0.00 ± 0.00	0.00 ± 0.00	-	0.00 ± 0.00	0.00 ± 0.00	-
Bone marrow cells count						
Parameter	**FC**	**FE**	***p*-value**	**MC**	**ME**	***p*-value**
n	***20***	***19***		***19***	***20***	
M:E	1.10 ± 0.19	1.14 ± 0.28	0.8079	1.26 ± 0.27	1.22 ± 0.41	0.2734
PE:ME	0.16 ± 0.08	0.15 ± 0.06	0.8836	0.18 ± 0.07	0.17 ± 0.11	0.3093
PM:MM	0.29 ± 0.08	0.30 ± 0.10	0.8948	0.28 ± 0.13	0.26 ± 0.06	0.6912
%PE	13.54 ± 5.83	13.13 ± 4.43	0.8350	15.21 ± 5.04	14.29 ± 6.30	0.4156
%PM	22.47 ± 4.56	22.57 ± 5.70	0.8786	21.08 ± 6.94	20.39 ± 3.54	0.7284
%L	8.54 ± 2.48	10.44 ± 2.52	0.0117	10.25 ± 3.34	8.33 ± 2.63	0.0372
%PL	0.15 ± 0.18	0.11 ± 0.13	0.5817	0.10 ± 0.10	0.10 ± 0.12	0.9710

FC=female control; FE=female exposed; MC=male control; ME=male exposed

Online Table S4. Summary of results of hematological and bone marrow examination at day 120 of exposure. Data are expressed as mean ± SD. Mann-Whitney Test was applied for statistical analysis.

CBC						
Parameter	**FC**	**FE**	***p*-value**	**MC**	**ME**	***p*-value**
n	***17***	***20***		***17***	***19***	
RBC (10^6/µL)	10.46 ± 0.40	10.51 ± 0.47	0.9101	10.71 ± 0.38	10.88 ± 0.41	0.1398
HGB (g/dL)	13.64 ± 1.01	13.94 ± 0.53	0.8621	13.63 ± 0.53	13.90 ± 0.48	0.1380
HCT (%)	54.26 ± 2.22	53.89 ± 2.05	0.4925	53.69 ± 2.15	55.53 ± 1.81	0.0112
MCV (fL)	51.89 ± 1.38	51.29 ± 1.47	0.1425	50.11 ± 1.34	51.07 ± 1.79	0.1269
MCH (pg)	13.05 ± 0.97	13.28 ± 0.42	0.6534	12.71 ± 0.23	12.78 ± 0.32	0.4423
MCHC (%)	25.15 ± 1.76	25.88 ± 0.99	0.2142	25.4 ± 0.64	25.04 ± 0.85	0.1003
PLT (10^3/µL)	1295.1 ± 378.8	1322.6 ± 280.8	0.8389	1560.9 ± 426.2	1523.2 ± 339.8	0.9750
WBC (10^3/µL)	10.92 ± 2.79	9.58 ± 3.17	0.1035	10.65 ± 3.20	10.58 ± 3.23	0.9004
Neutrophils x 10/µL	1.16 ± 0.67	1.08 ± 0.39	0.9820	1.27 ± 0.68	1.41 ± 0.69	0.5359
Lymphocytes x 10/µL	9.35 ± 2.29	8.08 ± 2.83	0.0744	8.98 ± 3.15	8.67 ± 2.80	0.8266
Monocytes x 10/µL	0.30 ± 0.20	0.35 ± 0.22	0.3851	0.365 ± 0.28	0.38 ± 0.19	0.5152
Eosinophils x 10/µL	0.10 ± 0.14	0.07 ± 0.08	0.9668	0.05 ± 0.07	0.12 ± 0.12	0.0568
Basophils x 10/µL	0.00 ± 0.00	0.00 ± 0.00	-	0.00 ± 0.00	0.00 ± 0.00	-
Bone marrow cells count						
Parameter	**FC**	**FE**	***p*-value**	**MC**	**ME**	***p*-value**
n	***20***	***20***		***18***	***20***	
M:E	0.76 ± 0.36	0.85 ± 0.24	0.4986	0.96 ± 0.44	0.88 ± 0.34	0.7451
PE:ME	0.09 ± 0.04	0.10 ± 0.03	0.0849	0.13 ± 0.09	0.09 ± 0.02	0.3178
PM:MM	0.80 ± 1.18	0.53 ± 0.48	1.0000	0.87 ± 1.52	0.54 ± 0.71	0.3644
%PE	8.01 ± 3.18	9.27 ± 2.67	0.0727	11.10 ± 6.32	7.80 ± 2.04	0.2637
%PM	34.83 ± 17.01	31.60 ± 11.02	0.9893	31.50 ± 20.92	27.49 ± 18.30	0.3314
%L	9.49 ± 1.61	7.78 ± 1.91	0.0075	8.07 ± 2.42	7.87 ± 2.18	0.5778
%PL	0.09 ± 0.11	0.07 ± 0.09	0.4359	0.09 ± 0.14	0.13 ± 0.21	0.8215

FC=female control; FE=female exposed; MC=male control; ME=male exposed

Online Table S5. Summary of results of hematological and bone marrow examination at day 180 of exposure. Data are expressed as mean ± SD. Mann-Whitney Test was applied for statistical analysis.

CBC						
Parameter	**FC**	**FE**	***p*-value**	**MC**	**ME**	***p*-value**
n	***20***	***18***		***20***	***19***	
RBC (10^6/µL)	10.26 ± 0.45	10.37 ± 0.43	0.4647	10.54 ± 0.28	10.52 ± 0.32	0.8841
HGB (g/dL)	13.55 ± 0.51	13.59 ± 0.44	0.9478	13.47 ± 0.33	13.29 ± 0.45	0.1267
HCT (%)	54.33 ± 1.75	53.24 ± 1.63	0.0682	52.16 ± 4.41	54.02 ± 2.30	0.3397
MCV (fL)	53.04 ± 2.36	51.41 ± 2.00	0.0091	49.49 ± 4.14	51.36 ± 1.64	0.3686
MCH (pg)	13.21 ± 0.32	13.12 ± 0.35	0.1733	12.79 ± 0.28	12.64 ± 0.29	0.0727
MCHC (%)	24.95 ± 0.84	25.54 ± 0.44	0.0035	26.01 ± 2.26	24.63 ± 0.47	0.0330
PLT (10^3/µL)	1763.5 ± 336.9	1831.3 ± 594.1	0.5151	1959.2 ± 464.4	2251.4 ± 584.1	0.2736
WBC (10^3/µL)	9.16 ± 2.44	9.12 ± 2.31	0.9137	10.74 ± 3.09	9.77 ± 3.53	0.4484
Neutrophils x 10/µL	0.86 ± 0.37	0.93 ± 0.42	0.9137	1.15 ± 0.56	1.03 ± 0.55	0.3054
Lymphocytes x 10/µL	7.91 ± 2.16	7.80 ± 2.07	0.6804	9.22 ± 2.72	8.30 ± 3.01	0.2796
Monocytes x 10/µL	0.31 ± 0.20	0.33 ± 0.20	0.8340	0.32 ± 0.17	0.35 ± 0.26	0.9501
Eosinophils x 10/µL	0.06 ± 0.07	0.06 ± 0.08	0.8326	0.05 ± 0.07	0.08 ± 0.08	0.1257
Basophils x 10/µL	0.01 ± 0.02	0.00 ± 0.00	0.9137	0.00 ± 0.00	0.01 ± 0.03	0.4482
Bone marrow cells count						
Parameter	**FC**	**FE**	***p*-value**	**MC**	**ME**	***p*-value**
n	***20***	***18***		***20***	***20***	
M:E	0.68 ± 0.27	0.65 ± 0.22	0.6280	0.79 ± 0.22	0.82 ± 0.34	0.8046
PE:ME	0.06 ± 0.02	0.07 ± 0.02	0.6460	0.09 ± 0.03	0.07 ± 0.02	0.3483
PM:MM	0.352± 0.26	0.40 ± 0.23	0.2192	0.27 ± 0.18	0.28 ± 0.18	0.9947
%PE	5.91 ± 2.08	6.25 ± 1.77	0.4216	7.77 ± 2.88	6.89 ± 1.92	0.3547
%PM	24.02 ± 11.35	27.21 ± 10.22	0.2279	20.39 ± 8.45	20.88 ± 8.96	0.9838
%L	7.52 ± 1.22	7.70 ± 1.26	0.4737	7.41 ± 1.27	7.73 ± 1.53	0.5785
%PL	0.05 ± 0.08	0.05 ± 0.09	0.7638	0.07 ± 0.11	0.07 ± 0.10	0.9422

FC=female control; FE=female exposed; MC=male control; ME=male exposed

Online Table S6. Summary of results of hematological and bone marrow examination at day 365 of exposure. Data are expressed as mean ± SD. Mann-Whitney Test was applied for statistical analysis.

CBC						
Parameter	**FC**	**FE**	***p*-value**	**MC**	**ME**	***p*-value**
n	***18***	***17***		***17***	***18***	
RBC (10^6/µL)	9.48 ± 1.06	9.60 ± 0.50	0.5085	9.84 ± 0.48	9.78 ± 0.88	0.8642
HGB (g/dL)	12.48 ± 1.67	12.73 ± 0.55	0.4464	12.72 ± 0.43	12.61 ± 0.73	0.5725
HCT (%)	50.77 ± 5.78	51.19 ± 2.38	0.5735	51.44 ± 1.69	49.86 ± 4.93	0.7136
MCV (fL)	53.67 ± 3.69	53.40 ± 2.22	0.9156	52.36 ± 2.24	51.12 ± 4.21	0.3816
MCH (pg)	13.16 ± 0.95	13.28 ± 0.34	0.6178	12.95 ± 0.73	12.96 ± 0.95	0.6644
MCHC (%)	24.52 ± 1.16	24.88 ± 0.73	0.3212	24.74 ± 0.80	25.45 ± 2.03	0.8257
PLT (10^3/µL)	1791.3 ± 367.5	1768.2 ± 384.0	0.9806	1740.3 ± 468.4	1684.3 ± 656.1	0.4048
WBC (10^3/µL)	7.13 ± 2.59	6.22 ± 3.36	0.1345	8.46 ± 4.67	9.85 ± 3.69	0.1729
Neutrophils x 10/µL	1.33 ± 0.78	0.62 ± 0.39	0.0009	1.08 ± 0.52	1.30 ± 0.78	0.4675
Lymphocytes x 10/µL	5.55 ± 1.94	5.38 ± 3.09	0.3184	7.07 ± 4.06	8.16 ± 3.10	0.1434
Monocytes x 10/µL	0.19 ± 0.16	0.17 ± 0.10	0.9026	0.26 ± 0.28	0.29 ± 0.22	0.4376
Eosinophils x 10/µL	0.06 ± 0.07	0.04 ± 0.05	0.6660	0.04 ± 0.08	0.10 ± 0.09	0.0203
Basophils x 10/µL	0.00 ± 0.00	0.00 ± 0.00	-	0.00 ± 0.00	0.00 ± 0.00	-
Bone marrow cells count						
Parameter	**FC**	**FE**	***p*-value**	**MC**	**ME**	***p*-value**
n	***19***	***19***		***20***	***19***	
M:E	0.87 ± 0.60	0.52 ± 0.25	0.0171	1.06 ± 0.29	1.11 ± 0.39	0.7335
PE:ME	0.15 ± 0.04	0.12 ± 0.03	0.0134	0.14 ± 0.06	0.14 ± 0.03	0.9278
PM:MM	0.42 ± 0.24	0.42 ± 0.24	0.8681	0.21 ± 0.11	0.23 ± 0.11	0.5822
%PE	13.04 ± 3.32	10.53 ± 2.18	0.0081	11.99 ± 4.41	11.91 ± 2.54	0.8786
%PM	28.05 ± 10.01	27.63 ± 10.99	0.8203	17.00 ± 7.07	17.88 ± 6.44	0.5877
%L	10.84 ± 2.71	10.65 ± 2.83	0.5109	9.69 ± 1.69	9.72 ± 1.84	0.7705
%PL	0.17 ± 0.26	0.37 ± 0.49	0.2762	0.13 ± 0.24	0.02 ± 0.06	0.0744

FC=female control; FE=female exposed; MC=male control; ME=male exposed

Online Table S7. Results of grading of histopathological findings at day 60, 120, 180, and 365 days of exposure. ~~Except for liver inflammation,~~ For liver inflammation data are reported as mean total score and standard deviation (SD). For the other findings, the no. of affected mice/no. of total examined mice for each grade, the no. of total affected mice/no. of total examined mice (no. pos/total), and the median score are reported~~data are expressed as no. of affected mice/no. of total examined mice~~. Fisher's exact Test was applied to compare the prevalences (no. pos/total) (* $p < 0.05$; ** $p < 0.01$) and Mann Whitney Test was applied to compare the scores (§ $p < 0.05$; §§ $p < 0.01$)~~were applied for statistical analysis~~. ~~* $p < 0.05$; ** $p < 0.01$.~~

Organ and finding		day 60				day 120				day 180				day 365			
		FC	FE	MC	ME	FC	FE	MC	ME	FC	FE	MC	ME	FC	FE	MC	ME
Liver																	
Inflammation, perivascular	mean total score	0,95	0,68	0,95	1,85	2,20	2,50	1,05	1,45	1,45	2,06	2,30	1,55	5,94	6,63	5,60	5,11
	SD	1,08	1,09	1,05	2,32	2,61	2,37	0,91	1,64	1,15	0,94	2,20	1,43	5,18	5,33	3,27	4,40
	n	19	19	20	20	20	20	19	20	20	18	20	20	19	20	20	19
Inflammation, focal	mean total score	7,47	8,16	5,50	8,25	9,35	7,20	3,05	5,25	4,25	4,89	3,90	3,50	5,50	6,21	3,65	5,16
	SD	4,93	6,09	4,56	6,75	7,85	5,04	3,36	4,25	4,12	2,27	3,37	4,16	5,43	5,30	3,38	5,28
	n	19	19	20	20	20	20	19	20	20	18	20	20	19	20	20	19
Hepatocyte, fatty accumulation	Grade 0	19/19	19/19	20/20	20/20	20/20	20/20	18/19	19/20	20/20	18/18	5/20	15/20	19/19	20/20	8/20	15/19
	Grade 1	0/19	0/19	0/20	0/20	0/20	0/20	1/19	0/20	0/20	0/18	13/20	5/20	0/19	0/20	4/20	2/19
	Grade 2	0/19	0/19	0/20	0/20	0/20	0/20	0/19	1/20	0/20	0/18	2/20	0/20	0/19	0/20	8/20	2/19
	Grade 3	0/19	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	0/19	0/20	0/20	1/19

	Grade 4	0/19	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	0/19	0/20	0/20	0/19
	no. pos/total	0/19	0/19	0/20	0/20	0/20	0/20	1/19	1/20	0/20	0/18	15/20	5/20**	0/19	0/20	12/20	5/19
	median score	0	0	0	0	0	0	0	0	0	0	1	0§§**	0	0	1	0
Spleen																	
Hyperplasia,	Grade 0	8/20	8/19	8/20	8/20	2/20	3/19	3/19	1/20	9/20	11/18	6/20	2/20	1/19	1/20	5/20	7/19
lymphocyte	Grade 1	11/20	9/19	11/20	11/20	13/20	14/19	5/19	11/20	10/20	6/18	11/20	15/20	12/19	10/20	15/20	10/19
(white pulp)	Grade 2	1/20	2/19	1/20	1/20	5/20	2/19	11/19	8/20	1/20	1/18	3/20	2/20	6/19	8/20	0/20	2/19
	Grade 3	0/20	0/19	0/20	0/20	0/20	0/19	0/19	0/20	0/20	0/18	0/20	1/20	0/19	1/20	0/20	0/19
	Grade 4	0/20	0/19	0/20	0/20	0/20	0/19	0/19	0/20	0/20	0/18	0/20	0/20	0/19	0/20	0/20	0/19
	no. pos/total	12/20	11/19	12/20	12/20	18/20	16/19	16/19	19/20	11/20	7/18	14/20	18/20	18/19	19/20	15/20	12/19
	median score	1	1	1	1	1	1	2	1	1	0	1	1	1	1	1	1
Pigment	Grade 0	5/20	9/19	15/20	13/20	0/20	0/19	3/19	2/20	0/20	0/18	0/20	0/20	0/19	0/20	0/20	0/19
(Hemosiderin)	Grade 1	14/20	10/19	5/20	7/20	3/20	3/19	14/19	17/20	0/20	1/18	10/20	8/20	1/19	1/20	5/20	2/19
	Grade 2	1/20	0/19	0/20	0/20	13/20	13/19	2/19	1/20	4/20	3/18	10/20	7/20	3/19	2/20	13/20	11/19
	Grade 3	0/20	0/19	0/20	0/20	4/20	3/19	0/19	0/20	16/20	13/18	0/20	5/20	12/19	14/20	2/20	6/19
	Grade 4	0/20	0/19	0/20	0/20	0/20	0/19	0/19	0/20	0/20	1/18	0/20	0/20	3/19	3/20	0/20	0/19
	no. pos/total	15/20	10/19	5/20	7/20	20/20	19/19	16/19	18/20	20/20	18/18	20/20	20/20	19/19	20/20	20/20	19/19
	median score	1	1	0	0	2	2	1	1	3	3	1,5	2	3	3	2	2
Melanosis	Grade 0	17/20	18/19	18/20	18/20	19/20	17/19	14/19	19/20	19/20	17/18	19/20	19/20	19/19	18/20	18/20	19/19
	Grade 1	3/20	1/19	2/20	2/20	1/20	2/19	5/19	1/20	1/20	1/18	1/20	1/20	0/19	2/20	2/20	0/19
	no. pos/total	3/20	1/19	2/20	2/20	1/20	0/19	0/19	1/20	1/20	1/18	1/20	1/20	0/19	2/20	2/20	0/19
	median score	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Extramedullary	Grade 0	0/20	0/19	0/20	0/20	0/20	0/19	0/19	0/20	0/20	0/18	0/20	0/20	0/19	0/20	2/20	0/19

hematopoiesis	Grade 1	3/20	2/19	3/20	6/20	2/20	8/19	13/19	13/20	5/20	7/18	20/20	17/20	7/19	11/20	11/20	13/19
	Grade 2	14/20	14/19	13/20	12/20	14/20	10/19	5/19	6/20	13/20	10/18	0/20	3/20	7/19	8/20	7/20	4/19
	Grade 3	3/20	3/19	4/20	2/20	4/20	1/19	1/19	1/20	2/20	1/18	0/20	0/20	3/19	0/20	0/20	2/19
	Grade 4	0/20	0/19	0/20	0/20	0/20	0/19	0/19	0/20	0/20	0/18	0/20	0/20	2/19	1/20	0/20	0/19
	no. pos/total	20/20	19/19	20/20	20/20	20/20	19/19	19/19	20/20	20/20	18/18	20/20	20/20	19/19	20/20	18/20	19/19
	median score	2	2	2	2	2	2*	1	1	2	2	1	1	2	1	1	1
Kidney																	
Renal tubule,	Grade 0	20/20	19/19	20/20	20/20	20/20	20/20	5/19	5/20	20/20	18/18	4/20	6/20	19/19	20/20	2/20	4/19
vacuolation,	Grade 1	0/20	0/19	0/20	0/20	0/20	0/20	4/19	3/20	0/20	0/18	5/20	2/20	0/19	0/20	3/20	2/19
cytoplasmic	Grade 2	0/20	0/19	0/20	0/20	0/20	0/20	10/19	11/20	0/20	0/18	8/20	10/20	0/19	0/20	8/20	7/19
	Grade 3	0/20	0/19	0/20	0/20	0/20	0/20	1/19	1/20	0/20	0/18	3/20	2/20	0/19	0/20	7/20	6/19
	no. pos/total	0/20	0/19	0/20	0/20	0/20	0/20	15/19	15/20	0/20	0/18	16/20	14/20	0/19	0/20	18/20	15/19
	median score	0	0	0	0	0	0	2	2	0	0	2	2	0	0	2	2
Renal tubule,	Grade 0	20/20	19/19	20/20	20/20	20/20	19/20	18/19	19/20	20/20	18/18	20/20	19/20	19/19	20/20	15/20	13/19
regeneration	Grade 1	0/20	0/19	0/20	0/20	0/20	1/20	1/19	1/20	0/20	0/18	0/20	1/20	0/19	0/20	5/20	6/19
(basophilic	Grade 2	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	0/19	0/20	0/20	0/19
tubules)	Grade 3	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	0/19	0/20	0/20	0/19
	no. pos/total	0/20	0/19	0/20	0/20	0/20	1/20	1/19	1/20	0/20	0/18	0/20	1/20	0/19	0/20	5/20	6/19
	median score	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Interstitium,	Grade 0	20/20	19/19	20/20	20/20	18/20	20/20	18/19	19/20	20/20	18/18	19/20	19/20	14/19	18/20	16/20	14/19
infiltration,	Grade 1	0/20	0/19	0/20	0/20	1/20	0/20	1/19	0/20	0/20	0/18	0/20	0/20	1/19	0/20	1/20	0/19
cellular,	Grade 2	0/20	0/19	0/20	0/20	1/20	0/20	0/19	1/20	0/20	0/18	1/20	1/20	4/19	2/20	3/20	5/19
perivascular	Grade 3	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	0/19	0/20	0/20	0/19
(cortical)	no. pos/total	0/20	0/19	0/20	0/20	2/20	0/20	1/19	1/20	0/20	0/18	1/20	1/20	5/19	2/20	4/20	5/19

	median score	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Interstitium,	Grade 0	17/20	17/19	20/20	20/20	17/20	19/20	15/19	15/20	16/20	15/18	15/20	13/20	7/19	0/20	1/20	2/19
infiltration,	Grade 1	1/20	1/19	0/20	0/20	2/20	0/20	1/19	2/20	1/20	0/18	0/20	0/20	3/19	0/20	1/20	0/19
cellular peripelvic	Grade 2	2/20	1/19	0/20	0/20	1/20	1/20	3/19	3/20	3/20	3/18	2/20	5/20	7/19	8/20	9/20	11/19
	Grade 3	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	3/20	2/20	2/19	3/20	9/20	6/19
	no. pos/total	3/20	2/19	0/20	0/20	3/20	1/20	4/19	5/20	4/20	3/18	5/20	7/20	12/19	11/20	19/20	17/19
	median score	0	0	0	0	0	0	0	0	0	0	0	0	1	2	2	2
Glomerulopathy,	Grade 0	20/20	19/19	20/20	20/20	20/20	20/20	19/19	20/20	20/20	17/18	20/20	20/20	13/19	17/20	20/20	19/19
hyaline	Grade 1	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	1/18	0/20	0/20	3/19	2/20	0/20	0/19
	Grade 2	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	2/19	1/20	0/20	0/19
	Grade 3	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	1/19	0/20	0/20	0/19
	no. pos/total	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	6/19	3/20	0/20	0/19
	median score	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Renal tubule, cast,	Grade 0	20/20	19/19	20/20	20/20	20/20	20/20	19/19	20/20	18/20	17/18	20/20	20/20	12/19	18/20	20/20	19/19
hyaline	Grade 1	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	2/20	1/18	0/20	0/20	5/19	1/20	0/20	0/19
	Grade 2	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	3/19	1/20	0/20	0/19
	Grade 3	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	0/20	0/18	0/20	0/20	0/19	0/20	0/20	0/19
	no. pos/total	0/20	0/19	0/20	0/20	0/20	0/20	0/19	0/20	2/20	1/18	0/20	0/20	8/19	2/20*	0/20	0/19
	median score	0	0	0	0	0	0	0	0	0	0	0	0	0	0*§	0	0

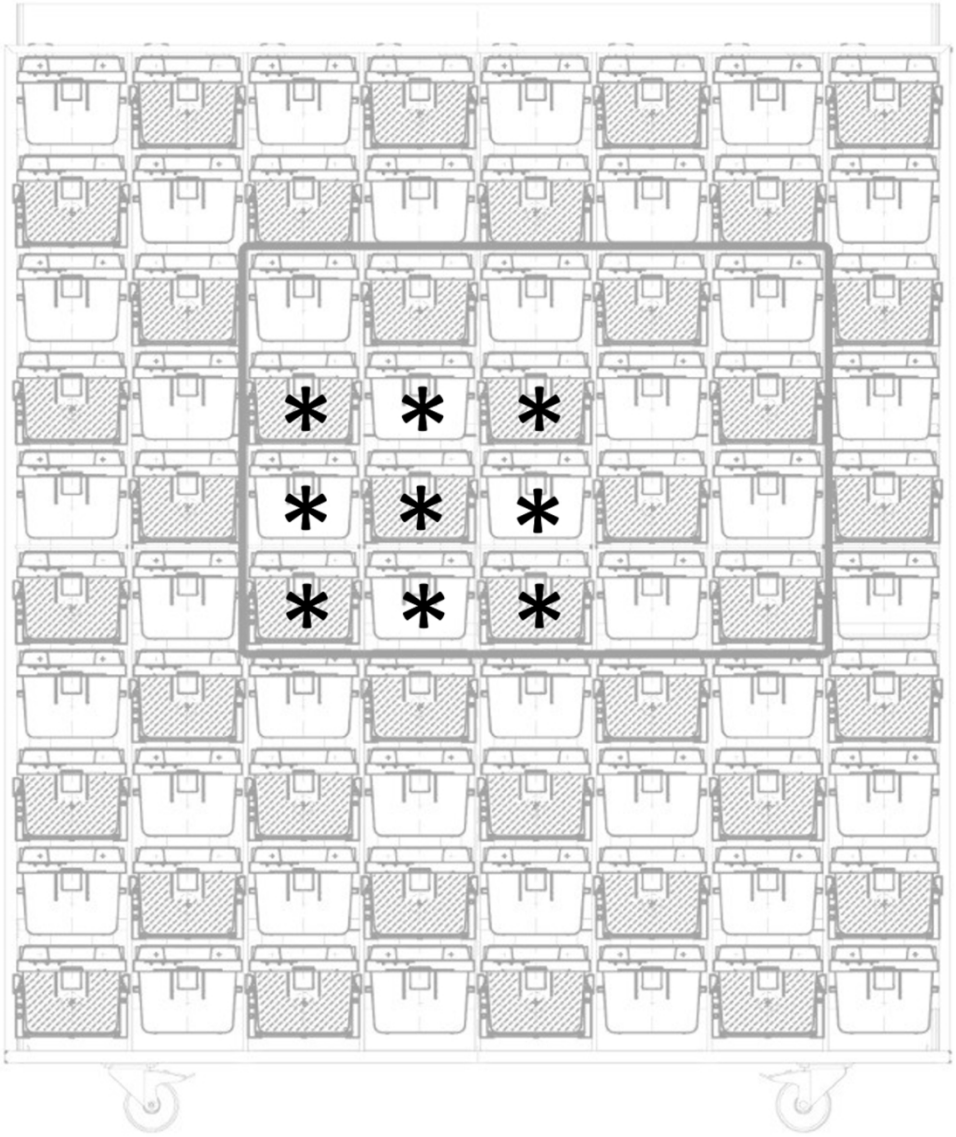
Online Table S8. ICNIRP reference levels for general public exposure to time varying electric and magnetic fields (modified from International Commission on Non-Ionizing Radiation Protection, 2010).

Field type	**Field frequency**			
	5Hz-100Hz	**12Hz-1KHz**	**1.2KHz-100KHz**	**100KHz-3GHz**
Electric Field r.m.s. [V/m]	2500-5000	250-5000	83-250	83
Magnetic Flux Density (B) [μT]	200-1600	80-400	27-66	27

Online Table S9. Impact of external sources on environmental (background) and intra-cage levels of EMF.

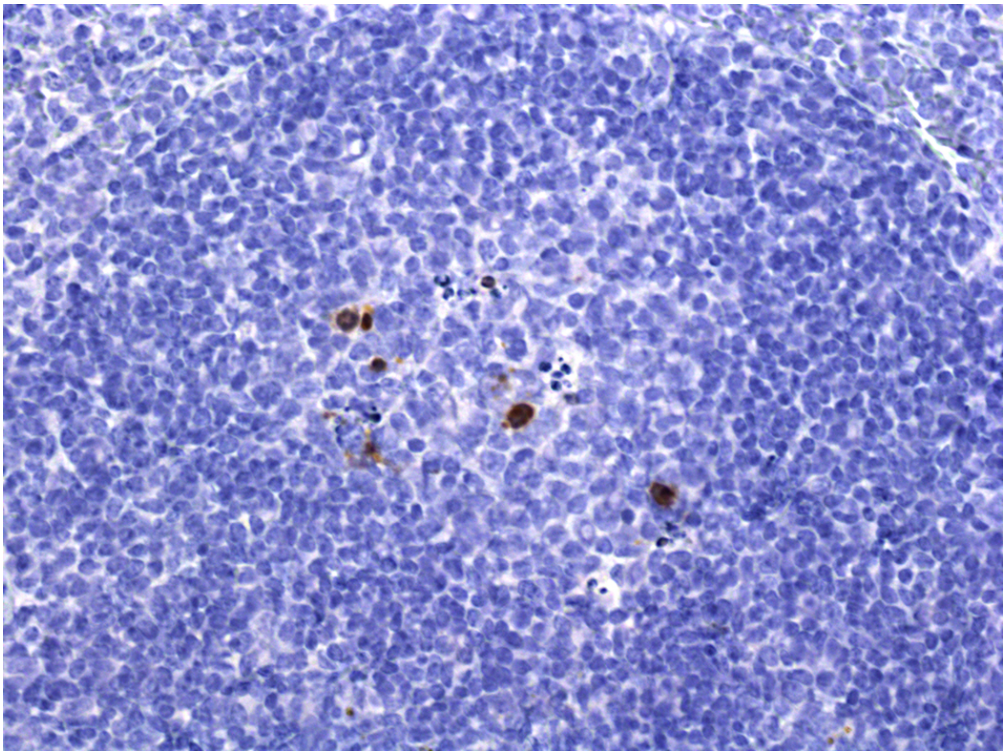
ROOM	MEASUREMENT POINT	ROOM LIGHT	DVC	Changing station*	Electric Field r.m.s. [V/m]			
					5Hz-100Hz	12Hz-1KHz	1.2KHz-100KHz	100KHz-3GHz
TEST 1 – IMPACT OF EXTERNAL SOURCES ON BACKGROUND EMF								
EMPTY	A - close to the wall (no industrial power socket)	OFF	\	\	1,81	0,35	0,38	1,02
		ON	\	\	3,90	0,52	0,37	1,36
	B - close to the wall, near industrial power socket no. 1	OFF	\	\	9,33	2,64	0,46	1,03
		ON	\	\	8,80	2,46	0,41	1,31
	C - close to the wall, near industrial power socket no. 2	OFF	\	\	32,47	7,99	0,61	1,13
		ON	\	\	40,47	7,67	0,41	1,52
TEST 2 – IMPACT OF EXTERNAL SOURCES ON INTRA-CAGE EMF								
WITH RACK	Cage A9 (with DVC)	ON	OFF	OFF	0,70	0,15	0,36	1,48
		ON	ON	OFF	1,77	0,60	0,42	1,48
		ON	ON	ON	2,75	0,71	0,43	1,54
	Cage D6 (with DVC)	ON	OFF	OFF	0,43	0,12	0,36	1,90
		ON	ON	OFF	1,71	0,62	0,51	1,53
		ON	ON	ON	2,60	0,68	0,37	1,76
	Cage H2 (with DVC)	ON	OFF	OFF	0,76	0,17	0,34	1,83
		ON	ON	OFF	3,24	0,86	0,45	1,54
		ON	ON	ON	4,00	1,02	0,46	1,78

*2 metres away from the rack



Online Figure S1. Cages identified with an asterisk and included in the central area delimited by the bold black line underwent temperature measurements.

88x104mm (600 x 600 DPI)



Online Figure S2. Mouse spleen used as positive control for the cleaved-caspase 3 immunostaining: occasional apoptotic cells (with brown-stained nuclei) are visible in the germinal centre of a splenic follicle (immunoperoxidase staining, original objective 40X).

88x66mm (300 x 300 DPI)

Table R1- Results of electric field measurements in empty IVC and DVC cages with DVC turned off and on.

Cage type	DVC status (OFF/ON)	No. of examined cages	Electric Field r.m.s. [V/m] mean ± SD			
			5Hz-100Hz	12Hz-1KHz	1.2KHz-100KHz	100KHz-3GHz
IVC	OFF	10	4.36±0.59	4.28±0.75	0.38±0.01	0.31±0.03
	ON	10	4.99±1.02	4.90±1.05	0.38±0.01	0.31±0.03
	Mann-Whitney test	*p*-value	0.1655	0.1903	0.2725	0.5919
DVC	OFF	10	6.32±0.90[§]	6.18±0.98[§]	0.38±0.00	0.32±0.11
	ON	10	8.56±2.42	8.21±2.08	0.44±0.02	0.44±0.06
	Mann-Whitney test	*p*-value	0.0185	0.0089	<0.0001	0.0048

[§]In DVC cages with DVC turned off the EF was increased as compared to EF measured within IVC in the lower frequency range (< 1KHz). DVC boards are electronic parts that can be coupled with the metallic part of the standard IVC rack and producing/distributing residual amount of EF originating from the common electrical building sources. In the range above 1KHz this residual effect is not present anymore.