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Progression of arteriovenous bypass restenosis in mice exposed to a 50 Hz magnetic field

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Abstract The controversy over whether magnetic fields (MF) produced by electrical wiring and appliances contribute to diseases such as cancer has been debated in the literature for more than 2 decades. These extremely low frequency fields at 50 or 60 Hz are omnipresent in the industrialized world and have been linked to various forms of cancer by epidemiological studies. Little has been published investigating any possible role of MF and cardiovascular disease, and this is the first study looking specifically at the effect of exposure to high-intensity MF on the development and progression of restenosis. A mouse arteriovenous bypass model was used, and mice were exposed to MF for periods of 1, 2, or 3 weeks. Neointima formation, infiltration of mononuclear cells, and heat shock protein 60 expression were all studied at the conclusion of the exposure regimen. Animals exposed to the MF for 1 week showed significantly smaller neointima formation compared with control mice exposed to a null field, although this difference was not observed in mice exposed for 2 or 3 weeks. No difference was found between mice exposed to MF and controls in any of the other parameters investigated.

INTRODUCTION

The magnetic fields (MFs) emanating from sources of electrical power (50–60 Hz) have been implicated as playing a role in a variety of disorders, including cardiovascular disease (Savitz et al 1999), Alzheimer disease (Sobel et al 1996), and most commonly reported, cancer (the landmark publication linking exposure to MF and cancer was from Wertheimer and Leeper 1979). A number of epidemiological studies have reported higher rates of cancer in people exposed to high-intensity (measured, calculated, or implied) MFs (Feychting and Ahlbom 1993; Olsen et al 1993). The literature is complex, however, due to the lack of consistency in study design, methodology, and the results reported (see Ahlbom et al 2001 for a review). In an effort to resolve this problem, carefully controlled experiments have been performed both in vivo and in vitro. Here, the literature is also discordant, with the majority of studies finding no influence from exposure to the MFs, whereas others report significant effects on various parameters (reviewed in Loscher and Liburdy 1998). It can be assumed therefore, that any effects from MFs are weak, although they may be still present.

We have published recently an in vitro study looking for any effect of MFs on the expression of the sensitive indicator of cellular stress heat shock protein 60 (Hsp60), in human umbilical vein endothelial cells (HUVECs; Henderson et al 2003). Hsp60 was also interesting because of its role as an autoantigen in the development of atherosclerosis (Xu et al 1992; Wick and Xu 1999). After exposure of HUVECs to a 50 Hz, high-intensity field (700 μT) under tightly controlled conditions, Hsp60 expression was investigated, on both the messenger ribonucleic acid and protein levels, and found to be unchanged. In addition, when cells were subject to a mild heat stress before or after exposure to the 700 μT MF, no additional effect was observed.

Because restenosis and atherosclerosis are complicated multifactorial diseases, the use of an animal model enables a much wider range of parameters to be investigated that cannot be reproduced by cell culture experiments.

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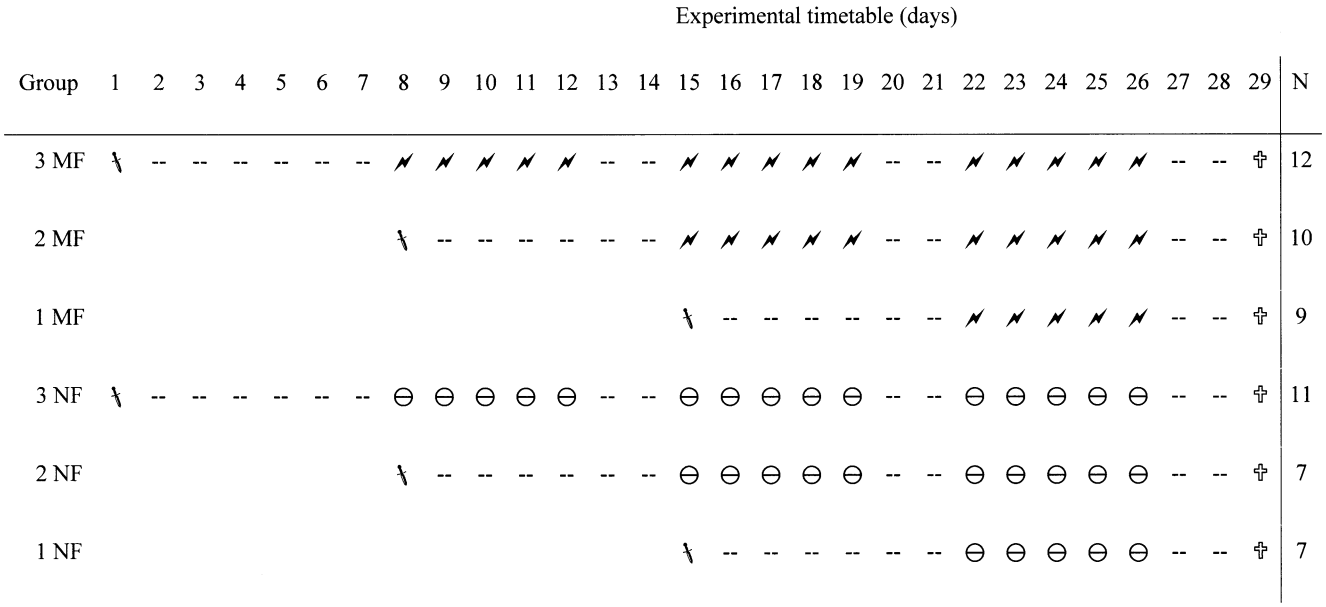


Fig 1. Schedule of the magnetic field (MF) exposure experiments. Mice were allowed to recover (––) for 6 days after an arteriovenous bypass graft operation (↯) before being exposed for 2 hours per day, for 5 days to a 50 Hz, 700 μT MF (↯, MF), or sham exposed to a 0 μT magnetic field (⊖, null field [NF]) for periods of 1 (1MF or 1NF), 2 (2MF or 2NF), or 3 (3MF or 3NF) weeks, respectively. The mice were not treated for 2 days (––) before being sacrificed or undergoing repeated exposure to NF or MF. At the conclusion of the exposure regimen, the mice were sacrificed under anesthesia by heart puncture (✝), before the transplanted vein and a collateral autologous control artery and vein were removed. The numbers of mice able to be analyzed in each group are indicated (N).

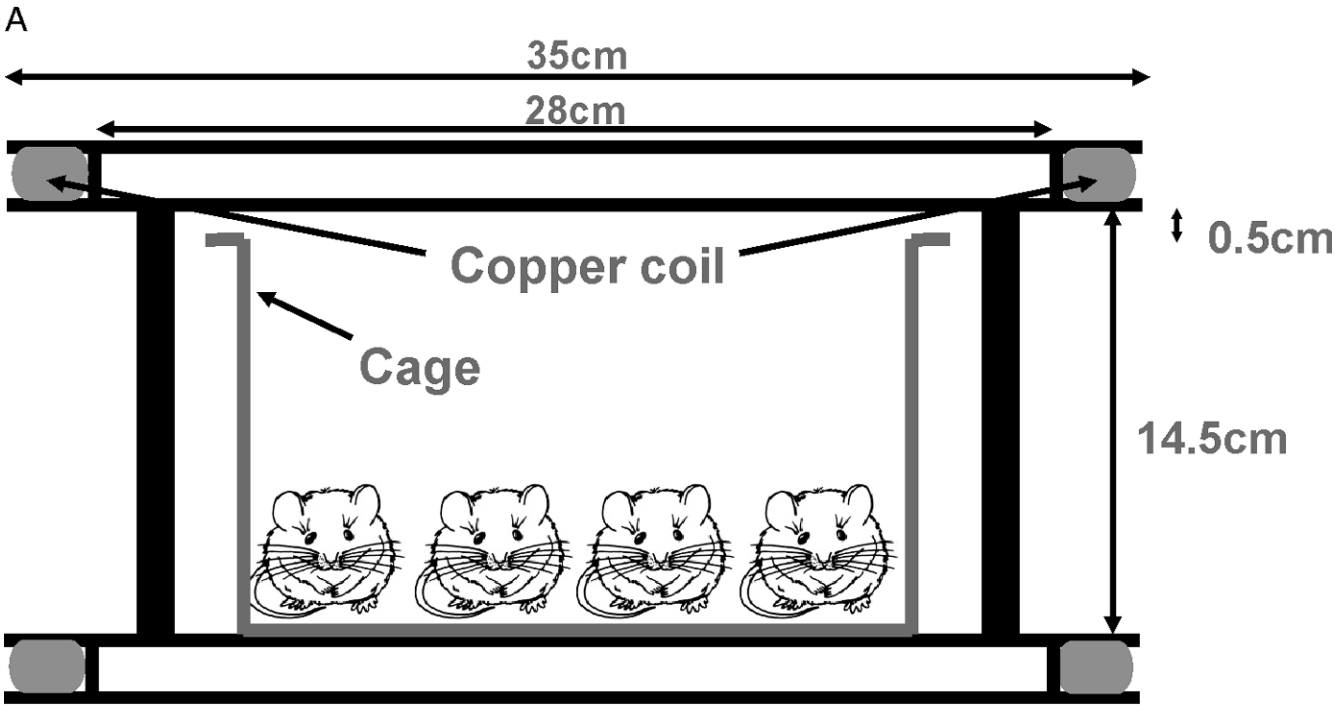
A model of venous bypass arteriosclerosis, mimicking many of the properties of the human disease, was established recently by our group to allow detailed investigations of the mechanisms involved in restenosis (Zou et al 1998). In a surgical procedure, autologous or syngenic veins (the jugular vein or vena cava) are grafted into the common carotid artery and so are exposed to arterial blood pressure. Under these conditions, the transplanted veins show greatly accelerated arteriosclerosis development compared with nonoperated contralateral carotid artery or jugular vein. Thickening of the intima is observed as early as 1 week after the bypass, whereas the intimal thickness increases an average of 10-fold after just 4 weeks and 15-fold after 8 weeks (Zou et al 1998).

Because of the unphysiological nature of cell culture, we were also interested in performing controlled MF exposure experiments on a mouse model of restenosis in vivo. In this study, carotid bypass operations were performed on groups of mice using the vena cava from donor mice, before exposure to a 50 Hz, 700 μT MF for 2 hours per day, 5 days per week for 1, 2, or 3 weeks as used previously for in vitro investigations (Henderson et al 2003). This intensity of MFs is higher than the time-averaged residential exposures (usually ≤1 μT) but is within the range of occupational exposures, eg, for electrical, railway, and arc-welder workers. A number of parameters were studied at the conclusion of the exposure regimen on histological preparations, including the thickness of the intima and media and luminal diameter, infiltration by T cells and macrophages, and the levels of Hsp60 present in the cells of the wall of the bypass graft.

MATERIALS AND METHODS

Arteriovenous bypass operation

Bypass operations were performed on 74 three-month-old female BALB/c mice as previously described, using the vena cava from syngenic age-matched female donor mice (Zou et al 1998). The experimental protocol was approved by the Federal Committee on Animal Ethics of the Austrian Federal Ministry of Education, Science and Culture (BMBWK). Briefly, the right carotid artery was dissected and nylon cuffs placed at each end. The vena cava vein taken from the syngenic donor animal was ligated to the cuffs, thereby anatomizing the artery. Mice surviving the surgical procedure (73 of 74) were allowed to recover post operatively for 6 days. The mice were assigned randomly into exposed (700 μT MF) and control ''sham'' exposed (0 μT, null field [NF]) groups, each consisting of 35 subjects. Mice were then exposed to a 700 μT (50 Hz) MF for 2 hours per day, 5 days per week, for 1, 2, or 3 weeks (Fig 1), whereas control animals were exposed in an identical sham exposure system (where the MF is effectively cancelled out by antiparallel wiring of the coils) to a 0 μT MF (NF) at the same time (Fig 2). The intensity of the MFs produced by the coils was controlled using a gaussmeter (C.A 40, Chauvin Arnoux, Paris, France). At



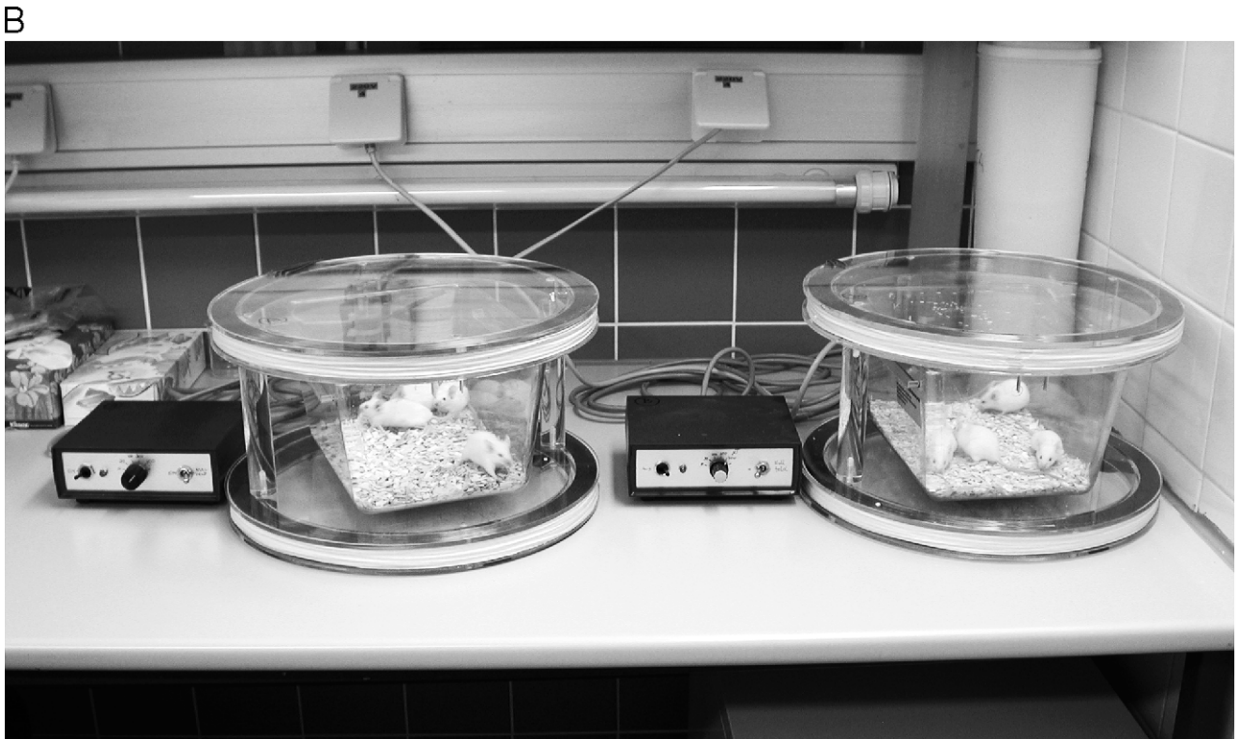


Fig 2. Magnetic-field (MF) exposure. (A) Dimensions of the Helmholtz coils for the generation of 700 μT MFs, or null fields (NF) used during the experiment. (B) The 2 identical Helmholtz coils used to generate the MFs during the experiments. Copper wire was wound around the coils in an antiparallel fashion, allowing the generation of a NF (sham exposure, 0 μT, right coil) when current was applied, because of the opposing coils effectively canceling each other out. Generation of a MF (700 μT) was achieved by switching the direction of the current in the top coil, so that the direction of flow in both coils was the same. The plastic cages (4 mice per cage) were completely covered by the coils, with 1 cm between the top of the cage and the coil to allow adequate air exchange.

Table 1 Results of histological and immunohistochemical analysis of transplanted veins following 1-, 2-, or 3-wk exposure to a 700 μT or sham (0 μT) magnetic field

				Immunohistochemical analyses				
				HSP60		Mononuclear cell infiltration		
Exposure time	Field strength (μT)	*n*	Restenosis	EC	Vessel wall	Total	T cells	Macrophages
1 week	0	4	+–+++	++	++	++–+++	+–+++	+++
	700	5	+–+++	++	++	+–+++	+–+++	+–++++
2 weeks	0	3	++–++++	++	++	++–+++	++–+++	+–++++
	700	5	++–++++	++	++	++–++++	++–+++	++–++++
3 weeks	0	4	++–++++	0–++	+	++–++++	++–++++	++–+++
	700	4	+++–++++	0–++	0–+	+++–++++	++–++++	++–+++

Two slides from each of 3 to 5 animals (N) were scored for restenosis, heat shock protein 60 (Hsp60) expression by endothelial cells (EC) and the rest of the vessel wall (intima and media), and infiltration of mononuclear cells in the intima and media (total). The identity of the infiltrating cells was established by staining with T cell- and macrophage-specific markers. Parameters were scored semiquantitatively as being nonexistent (0), low (+), medium (++), high (+++) and very high (++++), respectively. Complete restenosis, designated by (++++), was found in several animals after just 2 wk treatment, and in the majority of the vein grafts after 3 wk. Levels of Hsp60 were consistently high over the first 2 wk of exposure, before dropping in the last week, as complete restenosis occurred. The numbers of mononuclear cells increased, with T cells being the main participant. As the initial inflammatory response began to subside after 2 wk, the number of macrophages present in the intima was found to be reduced. No difference in any of the parameters was found among mice exposed to the 700 μT magnetic field, and those sham exposed to a 0 μT field.

the conclusion of the exposure period to the MF, the mice were placed under barbiturate anesthesia (Vetanarcol®, 40–50 mg/kg body weight intraperitoneal) before being sacrificed by blood withdrawal through heart puncture. The transplanted vena cava (in the position of the arteria carotis), the autologous arteria carotis communis, and vena cava caudalis (both nonoperated controls for histochemistry) were harvested using a stereomicroscope (Olympus SZH10, Olympus Optical Co. Ltd, Tokyo, Japan) and processed for routine histology analyses. The blood vessels were embedded in autologous liver to facilitate positioning for later preparation of cryosections before being stored in liquid nitrogen.

Histological and immunohistological analysis

The arterial and venous tissues were prepared for histological analysis by taking serial transversal (4 μm) and frozen sections as described previously for histological and immunohistological analysis, respectively (Kleindienst et al 1993). Tissue slices were fixed using phosphate-buffered formaldehyde (4%, pH 7.2) and stained with hematoxylin-eosin (HE) for conventional histological analysis and measurement of vessel wall thickness. Lumen, intima, and media areas were measured from HE-stained tissue sections. Images were captured using a transmission scanning microscope (Zeiss LSM 510, Zeiss, Jena, Germany). Images were first scanned and then overlaid by different linings to trace the lumen, the internal elastic lamina, and external elastic lamina. The intima was defined as the region between the lumen and the internal elastic lamina. The media was defined as the region between the internal and external elastic laminas. The (neo)intimal area was determined by subtracting the area of the lumen from the area enclosed by the internal elastic lamina. The medial area was determined by subtracting the area enclosed by the internal elastic lamina from the area of the external elastic lamina. Four to 5 cross sections were reviewed from each graft, nontransplanted artery and vein. In the statistical analyses, the individual values for the area from each animal at each time point (1, 2, and 3 weeks of MF treatment) were used.

Alternatively, sections were stored at −80°C until stained for immunohistochemistry, as described previously (Zou et al 1998). Sections were labeled with rabbit anti-Hsp60 antibody (Stressgen, Vancouver, Canada), biotin-labeled hamster anti-mouse CD3 antibody (clone 145-2C11, Pharmingen, San Jose, CA, USA), or a CD68 antibody (TIB-128 hybridoma cell line). Antibody binding was detected using AP-conjugated goat anti-rabbit (Dako, Glostrup, Denmark), AP-conjugated streptavidin (Dako), or AP-goat anti-rat Ig, (Chemicon, Temecula, CA, USA) respectively. Infiltration of mononuclear cells into the intima and evaluation of Hsp60 levels were determined by a single operator, using a transmission laser scanning microscope (Zeiss LSM 510, Zeiss).

Statistical analysis

Statistical analysis was performed using the statistical software SPSS (version 10) for Macintosh. Study groups were compared for statistical significant differences using the 2-tailed Mann-Whitney *U*-test. P values <0.05 were considered to indicate statistical significance.

RESULTS

A total of 70 mice were taken for the exposure phase of the experiment, of which 35 were taken for MF (700 μT)

and 35 for sham (NF, 0 μT) exposure, respectively. After postoperative recovery (6 days), the mice were exposed for 1 week (20; 10 at 700 μT, 10 sham), 2 weeks (20; 10 at 700 μT, 10 sham), or 3 weeks (30 mice; 15 at 700 μT, 15 sham), respectively. Microscopically visible signs of fibrosis or thrombosis (blocking the flow of blood) in the transplanted vein led to the exclusion of the animal from further investigation. The numbers remaining in each group (with the number discounted in brackets) were: 1 week: 7 sham and 9 MF exposed (4); 2 weeks: 7 sham and 10 MF exposed (3); 3 weeks: 11 sham and 12 MF exposed (7).

Neointima size and restenosis

The luminal area of the transplanted veins was found to be reduced at each of the time points investigated, from 1 to 3 weeks of exposure to the 700 μT MF or sham (0 μT) field. This was mainly as a result of hyperplasia of the intima of the transplanted vessels, associated predominantly with infiltration of $CD3^+$ T cells but also by $CD68^+$ macrophages (Table 1). This indicates the beginning of an inflammatory response typical of restenosis and atherosclerosis, which is further driven by the release of proinflammatory cytokines from infiltrating mononuclear cells.

The level of restenosis displayed by the transplanted vena cava increased with the time elapsed from the surgical procedure, from low to medium after 1 week of exposure, to high to very high after 3 weeks exposure. This is consistent with the time scale of restenosis in this model as reported previously (Zou et al 1998). However, no significant difference was seen between animals exposed to the high-intensity MF (700 μT) and those sham exposed (to 0 μT). In addition, no hyperplasia was detected in the intima or media of the autologous, nonoperated aorta carotis or vena cava, whether from 700 μT-MF–exposed or sham-exposed animals (data not shown).

Intimal and medial areas were measured and compared between the groups exposed to 700 μT (MF) and those sham exposed to 0 μT (NF) (3–5 mice per time group, Table 1). Mice exposed to the 700 μT MF for 1 week displayed a significantly smaller intimal area compared with those exposed to the sham field (0 μT) (Mann-Whitney *U*-test, $P = 0.003$, Fig 3B). No difference was seen in the medial ($P = 0.274$) or lumen ($P = 0.237$) sizes of the same animals. However, in each of the remaining 2 time periods investigated, there were no significant differences in the intimal or medial area (2 weeks exposure: intima $P = 0.515$, media $P = 0.360$; 3 weeks exposure: intima $P = 0.536$, media $P = 0.336$).

T cell and macrophage infiltration

The infiltration of mononuclear cells was evaluated from tissue sections stained with T cell (anti-CD3)- and macrophage (anti-CD68)-specific markers. In mice exposed to the MF for just 1 week, infiltration was apparent from both T cells (low to medium level) and macrophages (low to high, Fig 4; Table 1). Infiltration by macrophages was generally reduced in the mice exposed to the 700 μT MF, although this effect was not significant. After exposure for 2 or 3 weeks, infiltration was scored as medium to very high for both T cells and macrophages (Fig 4; Table 1). Interestingly, after 3 weeks exposure, the number of macrophages was reduced compared with shorter exposures of 1 or 2 weeks, whereas the T cell infiltration increased with time to very high levels after 3 weeks exposure.

Hsp60 expression

Hsp60 was found to be moderately to highly expressed in tissue sections from transplanted veins harvested 1 or 2 weeks of MF exposure (Table 1). However, after 3 weeks, expression levels were reduced drastically. There appears to be a negative correlation between the extent of restenosis displayed and the expression level of Hsp60, where much lower levels of Hsp60 are found where restenosis is highly advanced. No disparities in the levels of Hsp60 were seen between transplanted veins from mice exposed to 700 μT or 0 μT. On the molecular level, Western blotting did not reveal a significant difference in Hsp60 expression levels between the different exposure groups (data not shown).

DISCUSSION

This is the first investigation to specifically focus on the influence of exposure to 50 Hz MFs (''electrostress'') and the development and progression of bypass restenosis. We have exposed a mouse model of arteriovenous bypass restenosis to 700 μT MFs at 50 Hz and evaluated the progression of bypass restenosis, including neointima size, infiltration of mononuclear cells, and levels of Hsp60 in the developing lesions. Although a field intensity of 700 μT is high by residential standards (where 'spikes' in MF may exceed 100 μT close to some household appliances), it is entirely appropriate in an workplace setting. Mice were exposed for periods of up to 3 weeks, starting 6 days after a section of vena cava vein was grafted into the common carotid artery. After 1 week of exposure to the MF, a highly significant decrease in the intimal thickness compared with sham-exposed (0 μT) mice was found. At all other time points, there was no evidence of increased or decreased intimal or medial thickness of the

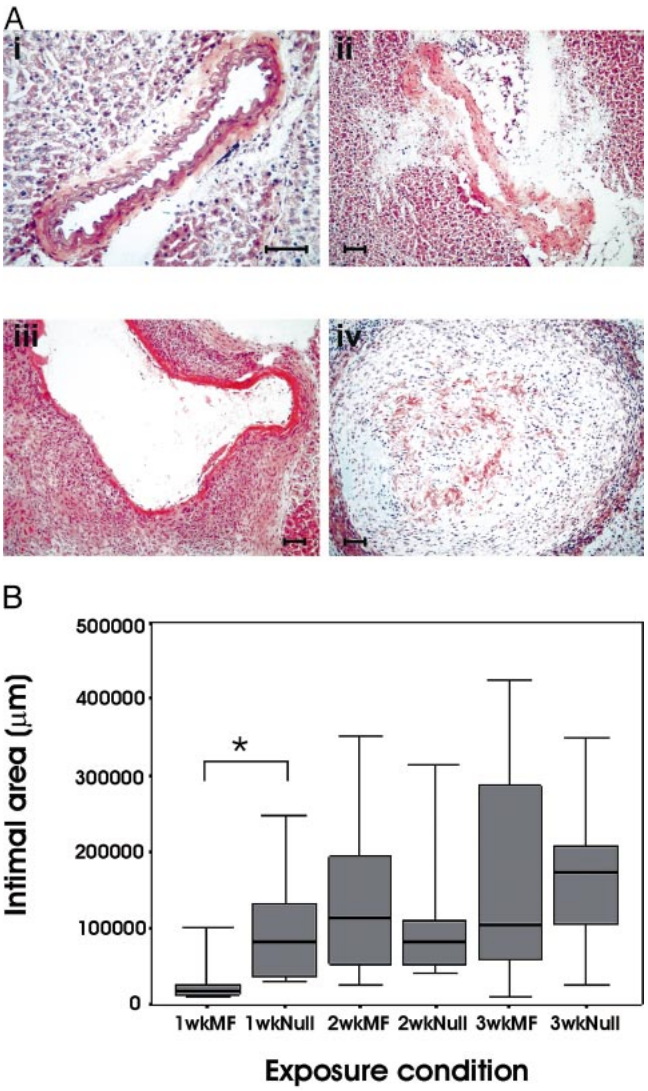


Fig 3. Intimal media thickness of transplanted bypass veins in mice exposed to 700 μT magnetic fields (MF) or to 0 μT null fields (NF). (A) Histological analysis of hematoxylin-eosin–stained tissue sections. Sections i and ii show nontransplanted carotid artery and vena cava vein (embedded in autologous liver), respectively, after 1 week of treatment, displaying no alteration in intima or media thickness. In contrast, transplanted veins showed a thickening of the intima and media after 1 week of exposure to either MF or sham fields (iii). Complete restenosis was seen as early as 2 weeks after the exposure regimen was started (iv). The scale bar represents 50 μm. (B) A box and whiskers plot of the intimal area (median and interquartile range are represented by the box, whereas the whiskers show the range of the values), measured from tissue sections of transplanted veins. The groups represented are 1 week MF exposure (1 week MF), 1 week NF exposure (1 week Null), 2 weeks MF exposure (2 weeks MF), 2 weeks NF exposure (2 weeks Null), 3 weeks MF exposure (3 weeks MF), and 3 weeks NF exposure (3 weeks Null). After 1 week of exposure to the MF, the intimal area was significantly reduced (indicated by the *) compared with sham-exposed control animals (Mann-Whitney *U*-test, $P = 0.003$). However, after 2 or 3 weeks exposure significant differences were no longer present.

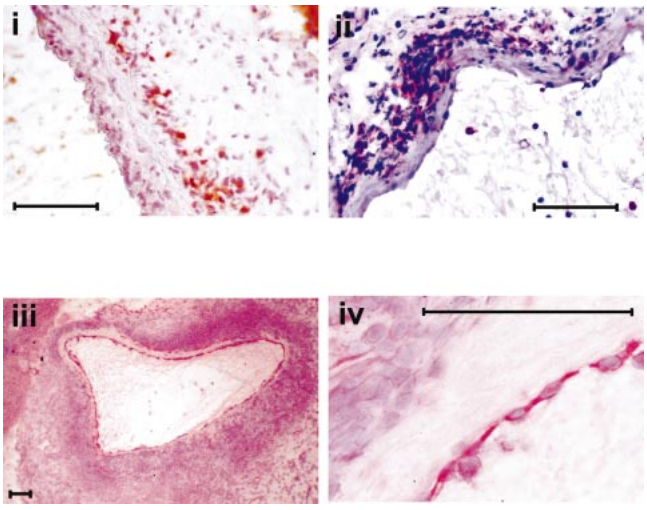


Fig 4. Immunohistochemical staining of tissue sections from vein grafts. (i) $CD3^+$ T cells infiltrated quickly into the intima and were still present after 3 weeks of treatment with either a 700 μT or 0 μT magnetic field. A section is shown from a mouse sham exposed for 3 weeks, with $CD68^+$ cells observed using horseradish peroxidase (HRP) (brown). (ii) $CD68^+$ macrophages were found in high numbers in the intima of bypass vessels after only 1 week of exposure (tissue section from a 1 week exposure to 700 μT, developed with alkaline phosphatase [AP] and counterstained with hematoxylin). Levels were similarly high after 2 weeks, before diminishing during the third week. (iii) and (iv) Hsp60 was stained in transplanted veins by an AP reaction after incubation of the sections with anti-human Hsp60 antibodies. After just 1 week of exposure, Hsp60 is clearly present throughout the vessel (iii), and above all in endothelial cells (iv). The scale bar represents 50 μm.

transplanted vein. The observation that the intimal thickness of those exposed for 2 or 3 weeks showed no effect may indicate that exposure to the MF was able to delay attachment and subsequent infiltration by mononuclear cells by as yet unknown mechanisms. If this was indeed the case, this effect was not persistent, because by the second week of exposure no difference in intimal thickness was detected, implying that the observed reduction in neointima size is of limited clinical relevance. Although the reduction in neointima formation was highly significant ($P = 0.003$), the high variability seen in the intimal area and the loss of this effect after a further 1 or 2 weeks of exposure dictates that this result should be interpreted with caution. Infiltration of mononuclear cells into the intima or media of the transplanted vein was observed in all cases after just 1 week of exposure to the MF or sham field. No significant difference could be discerned between animals exposed to the 700 μT field or the sham field (0 μT) at this point, nor later at 2 or 3 weeks of exposure. Levels of the 2 main infiltrating cell types, $CD3^+$ T cells and $CD68^+$ macrophages, were evaluated separately at each of the time points. The frequency of infiltrating T cells increased with time after the bypass procedure, with no differences seen between exposed or sham-exposed mice. The occurrence of macrophages in

the intima and media increased over the first 2 weeks of exposure, before decreasing again in the third week. Again, no variation was seen in macrophage numbers between animals exposed to 700 μT or 0 μT.

The expression of Hsp60 in endothelial cells and in the surrounding vessel was also determined on tissue sections and by Western blotting using protein extracted from the entire graft. Levels of Hsp60 were found to be high after 1 and 2 weeks in both exposure groups in endothelial cells but also in the intima and media, whereas they were sharply decreased in mice exposed for 3 weeks. No difference in Hsp60 expression levels was found between MF- and sham-exposed mice.

A relationship between atherosclerosis or bypass restenosis and exposure to MFs from domestic electrical supplies has not yet been investigated explicitly in a published epidemiological study. The extent of investigations has been limited to the endpoints of cardiovascular disease, commonly myocardial infarction, chronic coronary heart disease, and stroke. Although some early studies found a correlation between MF exposure and the risk of acute myocardial infarction (Asanova and Rakov 1972), recent studies have failed to reproduce these results (Savitz et al 1999; Sahl et al 2002).

There are several facts that may limit the complete extrapolation of this study to the human pathophysiological situation. Although mice, along with rabbits, are the best animal models available for restenosis and atherosclerosis, development and progression of the disease are not exactly as seen in humans (Rekhter 2002). For example, many mouse strains are quite resistant to the formation of atherosclerotic lesions unless fed a high cholesterol diet or are, in addition, immunized with microbial Hsp60/65 (George et al 1999). In addition, the mechanism of restenosis seen in this bypass model, although very similar in many ways to that found in humans, is not identical (Zou et al 1998). The highly accelerated intimal hyperplasia and restenosis found in these animals is many times faster than seen in humans, where only around 20% of bypasses fail after 1 year (Dalman 1994).

Because of the global presence of MFs emanating from all electrical supplies and appliances, any detrimental influence on cellular processes would have serious and wide-reaching consequences. A recent study by our group found no effect on the expression of Hsp60 in HUVECs after exposure to 700 μT, 50 Hz MFs (Henderson et al 2003). The results from the current study confirm and extend these findings by using a complex in vivo system allowing investigation of the main components involved in the very early stages of restenosis including monocyte infiltration, Hsp60 levels, and the absolute size of neointima formation. Although mice exposed to the MF for only 1 week showed a significantly reduced intimal thickness, longer exposure times failed to maintain this result. Further, the numbers of infiltrating mononuclear cells or the level of Hsp60 in endothelial cells or the entire vessel were not affected. This is evidence against any effect on the formation or progression of bypass restenosis from exposure to MFs resulting from domestic power supplies.

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