PULSE

Effects of Pulsed Electromagnetic Fields (PEMF) on Mouse Bone Marrow-Derived Macrophages

Study with the Frank Reidy Research Center for Bioelectrics on the Pulse XL Pro's effect on the body's curative mechanisms

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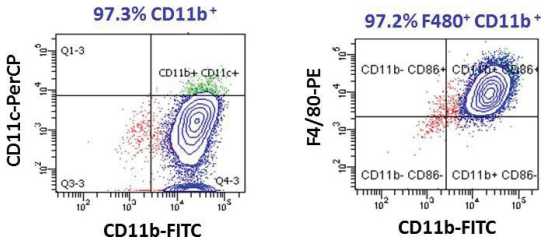
We have analyzed several macrophage responses to determine effects of pulsed electromagnetic fields (PEMFs) on macrophage function using mouse bone marrow-derived macrophages with typical macrophage phenotypic characteristics as positive for CD11b and F4/80 and negative for CD11c. Macrophages are cells of the innate or inborn immune system that are one of the first responders to internal dangers represented as infections from bacteria or virus, damaged cells and tissues, cellular debris or dead cells due to injury or aging. These analyses include determining effects on cell survival, plasma membrane permeability, calcium influx, cytokine production, and binding and endocytosis of particulate cargo. Results indicate that for PEMFs with 50% or 100% Field Strength output and 5 Pulses per Second for up to 30 minutes of exposure, there are no effects on survival, Magnetic plasma membrane permeability, calcium influx, calcium mobilization or reactive oxygen species (ROS) generation. However, there are significant enhancements of binding and endocytosis of particulate cargo by mouse macrophages, increases in anti-inflammatory cytokines and decreases in inflammatory cytokines. Together these results indicate that there are no deleterious effects of PEMFs on macrophage viability and no effects of plasma membrane permeability. Consistent with the absence of ROS production, PEMFs enhance macrophage anti-inflammatory functions and facilitate the binding and endocytosis of particulate dextran cargo. Effects on binding and phagocytosis are long-lasting, suggesting durable changes like those seen for PEMF enhancement of rat muscle mitochondria oxygenation. (See Part 2 study Correlation to *Enhanced Energy, Endurance and Performance)*

- PEMFs activate macrophages by increasing binding and endocytosis of particulate cargo up to 56.2%.
- The PEMF-induced macrophage activation is long-lasting, observable 8 and 24 h after PEMF treatment, indicating it is a relatively "durable" effect that is not readily reversible.
- PEMF-treated macrophages significantly down regulate inflammatory cytokines including TNF- associated with systemic inflammation which was decreased 300-fold.
- PEMFs also increased at least one anti-inflammatory cytokine protein, IL-6 (which likely functions as a myokine that is increased during muscle contraction and exercise).
- PEMFs are not cytotoxic and have no effects on cell viability, calcium influx, calcium mobilization, plasma membrane permeability or ROS generation.
- PEMF effects on macrophages can enhance the body's curative mechanisms to possibly clear infections and tissue damage, dissipate inflammation, and promote healing.

Studies of PEMF treatment of macrophages provide analyses of intact primary cells derived from mouse bone marrow stem cells. Primary cells are isolated from the bone marrow of mouse femurs and incubated for 7 days in the presence of M-CSF (macrophage colony stimulating factor). This promotes development of macrophages from hematopoietic stem cells. These stem cells can give rise to all blood cell types, but treatment with M-CSF produce bone marrow derived macrophages with typical macrophage phenotypes. This is demonstrated in Figure 1 showing common markers for macrophages as CD11b+, F4/80+ and CD11c-. This macrophage phenotypes provided the basis for the following studies to determine effects of PEMFs on primary mouse macrophages.

Figure 1- *Differentiated bone marrow-derived macrophage phenotype – Stem cells were isolated from mouse bone femurs, differentiated for 7 days by incubation with M-CSF. On day 7 after isolation, macrophages were characterized with CD11c labeled with PerCP (peridinin chlorophyll protein complex), CD11b labeled with FITC (fluorescein isothiocyanate) and F4/80 labeled with PE (Phycoerythrin).*
Labeled macrophages were then analyzed by flow cytometry.



In the panel on the left of Figure 1, 97.3% of the cell population appeared in the lower right quadrant as positive for CD11b and negative or low for CD11c. In the panel on the right of Figure 1, 97.2% of the cell population appeared in the upper right quadrant as positive for F4/80 and positive for CD11b. The differentiated bone marrow-derived macrophages were consistently positive for CD11b and F4/80 and negative for CD11c.

This is the typical phenotype for bone marrow-derived macrophages [Zhang et al., 2008].

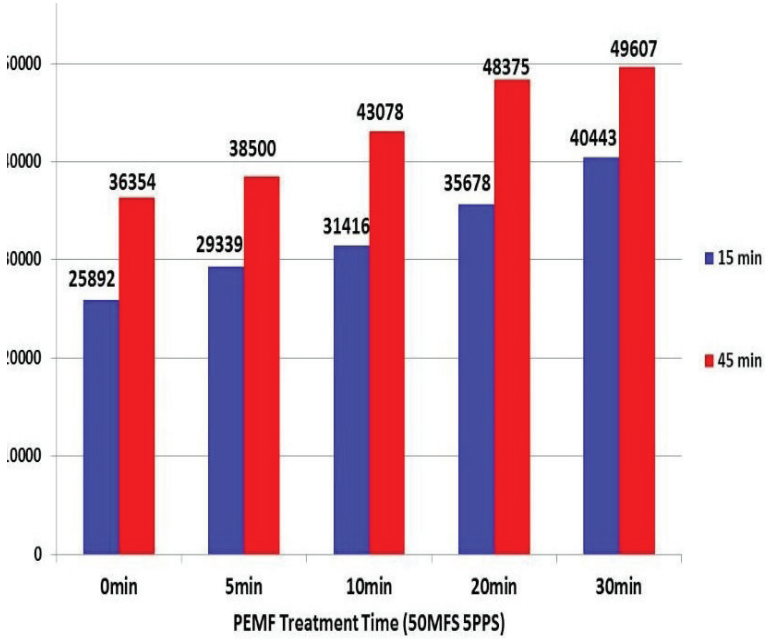


Figure 2A: *PEMFs induce macrophage binding and endocytosis of fluorescent dextran cargo in a PEMF-time dependent manner. Mouse bone marrow-derived macrophages were differentiated in vitro for 7 days in the presence of M-CSF. These differentiated macrophages were then exposed to PEMFs at 50% power (50MFS) at 5 pulses per second (5PPS) for 0, 5, 10 20, and 30 minutes, as indicated on the X-axis. Immediately after treatment macrophages were "fed" cargo of small dextran particles (3 kDa) labeled with pacific blue and fluorescence was detected by flow cytometry. This stimulates macrophages to bind the cargo and increase endocytosis of the labeled dextran, which is shown by increased fluorescence of the dextran-pacific blue on the Y-axis. Macrophages were incubated with labeled dextran for 15 minutes (blue bars) or 45 minutes (red bars) before analysis of binding and endocytotic activity (Y-axis). Values on the y-axis are arbitrary units of mean fluorescence intensity (MFI).*

When mouse macrophages were exposed to PEMFs for 0 to 30 minutes as indicated on the x-axis, there was a time-dependent increase in binding and endocytosis of the fluorescent dextran cargo as indicated on the Y-axis. After a 30-minute PEMF treatment, there was a 56.2% increase in binding and endocytosis in the first 15 minutes (see Figure 3 and associated Table below) and a 36.6% increase in binding and endocytosis over the 45 minute incubation period.

Figure 2B linearizes data in Figure 2A so that effects of PEMF can be seen in a linear manner. As seen in Figure 2A and as expected, macrophages take up more particles in 45 minutes than they do in 15 minutes.

However, the time-dependent effect of the PEMFs are essentially the same. It can also be seen that there is a direct linear relationship between increases in PEMF treatment time and increases in binding and endocytosis of the cargo. Furthermore, this can be seen after 15 and 45 minutes of incubation with label dextran cargo.

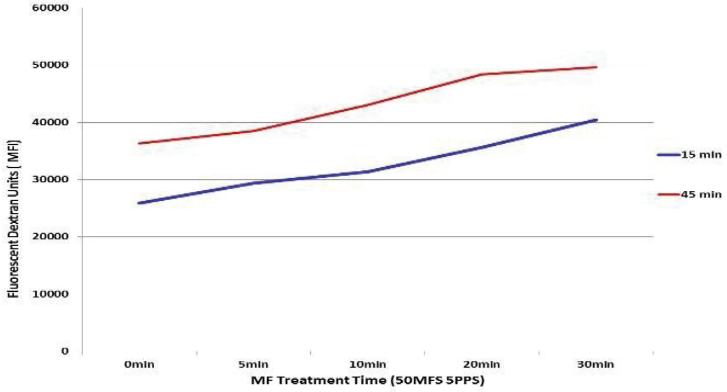


Figure 2B: *PEMFs induce mouse macrophage binding and endocytosis in a PEMF- dependent manner after 15 or 45 minutes of incubation with dextran – These data are taken from each PEMF treatment time on Figure 2A. Macrophages were treated with PEMFs for times indicated on the X-axis. Then labeled dextran was added and changes in binding and endocytosis of fluorescent dextran cargo were observed 15 minutes (blue line) and 45 minutes after later (red line).*

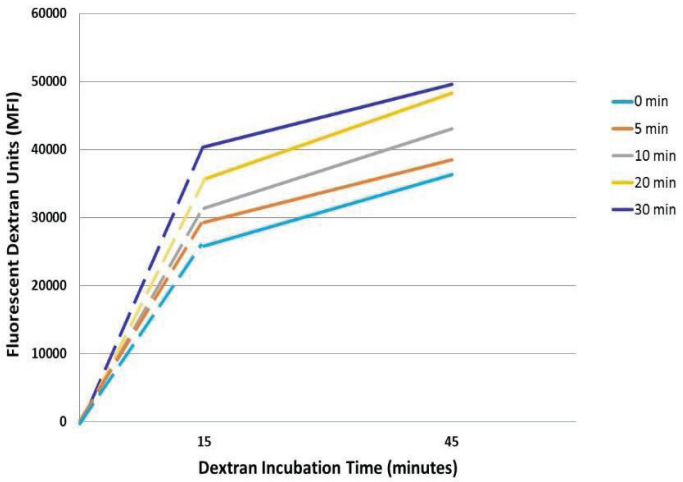


Figure 3: *Effect of endocytosis rate after PEMF treatment - Macrophages were exposed to PEMF for various times (colored lines) and then incubated with labeled dextran for 15 or 45 minutes before fluorescence was determined by flow cytometry. Solid lines were drawn between 15- and 45-minute time points, indicating fluorescence rates between those times. Dotted lines were drawn between 0 and 15 minute incubations with dextran to estimate initial rates of binding and endocytosis between 0 and 15 minutes.*

Figure 3 shows that after each PEMF treatment time, binding and endocytosis increased at near the same rates for each PEMF treatment time between 15 and 45 minutes of endocytosis; that is the slopes of the lines between 15 and 30 minutes are similar. This suggests that a primary effect of PEMFs occur in times before 15 minutes of incubation with labeled-dextran. Althoughendocytosis is rapid, these rates include a significant effect of binding the cargo before endocytosis. By assuming initial binding and endocytosis rates between 0 and 15 minutes of incubation with labeled-dextran, it is clear that there is an incremental increase in endocytosis rates as the PEMF treatment time increases. The table shows those estimated initial rates for each PEMF treatment time as fluorescent units /minutes during the first 15 minutes of incubation with labeled-dextran. After a 30-minute treatment with PEMF, mouse macrophage binding and endocytosis rates increased by about 56% [2696/min at 30 treatment divided by 1726/min at 0 treatment].

PEMF Treatment Time	Initial Slope
0 min	1726 Units /min
5 min	1956 Units /min
10 min	2094 Units /min
20 min	2379 Units /min
30 min	2696 Units /min

Given that PEMFs enhanced binding and endocytosis rates immediately after treatment, it was of interest to determine how long macrophages could express these endocytotic advantages. The next series of experiments begins to answer this question.

In the experimental design in Figure 4, macrophages were treated with PEMFs for 5, 10, 20 and 30 minutes. Then for each of the incubation times, binding and endocytosis was determined immediately that is 0 h or 5-10 minutes after these treatment (blue bars), 8 hours after treatment (red bars) and 24 hours after treatment (green bars). Figure 4B shows the same data as linear rates after PEMF treatment. Immediately after treatment with PEMFs, binding and endocytosis increases in a PEMF-dependent treatment time (this data is repeated from Figure 2A). After 30 minutes of PEMF treatment, binding and endocytosis was 36% greater than in untreated macrophages after 45 minutes of binding and endocytosis [49607/45 min after 30 mins PEMF treatment divided by 36354/45 min after 0 PEMF treatment]. As seen in Figures 4A and 4B, macrophage activity falls with time in culture, yet PEMF effects are still evident 8 h after treatment, especially for treatment times 10 - 30 minutes and evident 24 hours after treatment, especially for treatment times 20 and 30 minutes. Even 8 and 24 hours after PEMF treatment, binding / endocytosis is 18% greater than in untreated, control macrophages. This indicates that changes induced by PEMFs are long-lasting effects, suggesting that the effects are not readily reversible.

Figure 4A: *Effects of PEMFs on activation of mouse macrophage binding / endocytosis are still evident 8 to-24 hours after treatment – Mouse macrophages were exposed to PEMFs for various times (X-axis). After PEMF exposure, macrophages were incubated in a cell incubator for 0, 8 or 24 hours before treatment with labeled dextran. Macrophages were then incubated with labeled dextran for 45 minutes and binding and endocytosis (Y-axis) were determined as mean fluorescent intensity (MFI) by flow cytometry. The numbers above each bar indicate the MFI units at each PEMF treatment time of the 0, 8 and 24 h incubation*

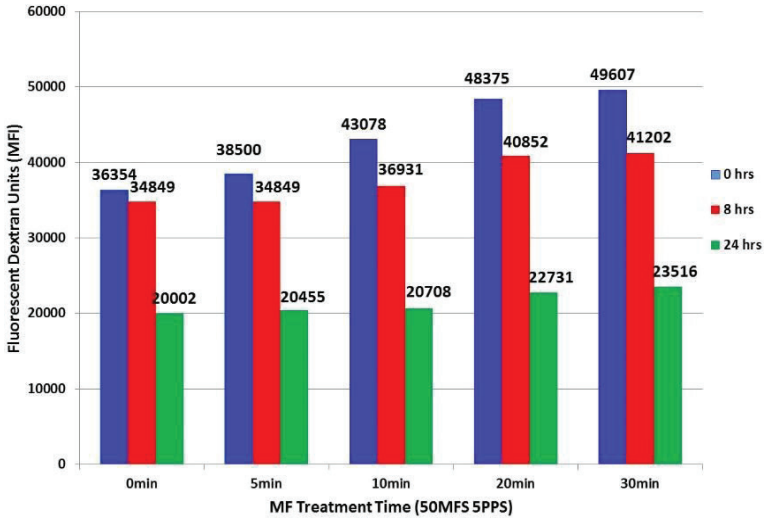
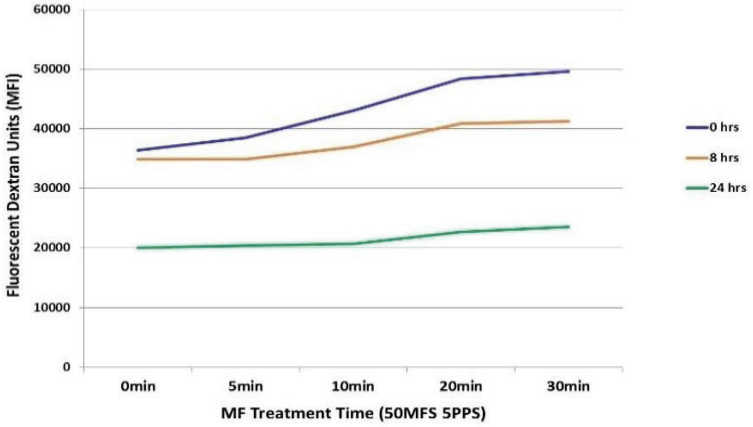


Figure 4B- *Mouse macrophage binding/endocytosis activity after PEMF treatment. The data in Figure 4A is expressed in a linear fashion for each of the three incubation times after PEMF treatment.*

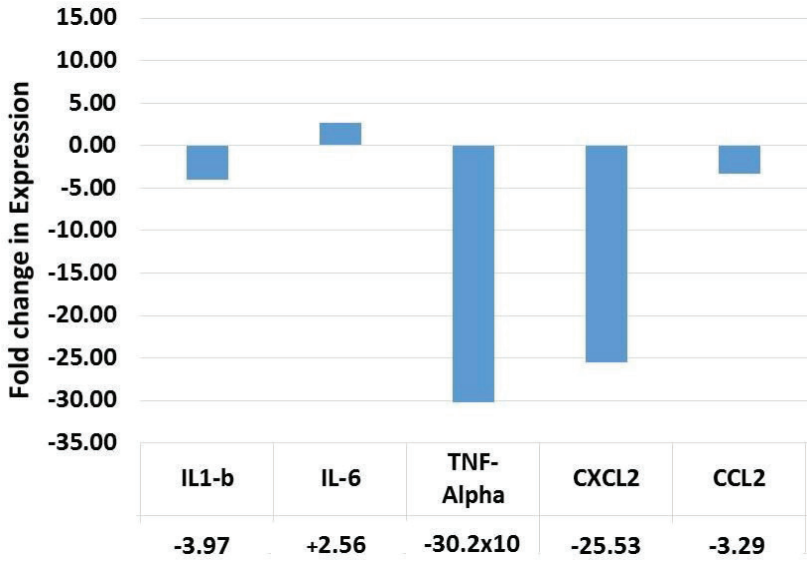


These results may underestimate the tenure of PEMF effects. As shown for macrophages that were analyzed 24 hours after treatment, binding / endocytosis is much lower. The glucose and other nutrients in the media are likely depleted after 8 and even more after 24 h. Activated macrophages depend on glycolysis instead of oxidative phosphorylation for their ATP supply, so they depend on glucose. Also after activation, macrophages not only use ATP for their endocytosis activity, but they also secrete ATP as an autocrine factor to facilitate endocytosis and maybe binding cargo.

Given these observations, it was of interest to determine what gene expression changes might take place in response to PEMF treatment of macrophages. We used RTqPCR (reverse transcription quantitative polymerase chain reaction) to determine changes in cytokine gene expression in response to PEMF at a setting to 100 MFS and 5 pulses per second for 30 minutes. One hour after PEMF treatment, total RNA was isolated and prepared for RT-qPCR of IL-1, IL-6, TNF-, CXCL2, and CCL2 as shown in Figure 5. RT-qPCR is used when the starting material is RNA. In this method, RNA is first transcribed into complementary DNA (cDNA) by reverse transcriptase from total RNA or messenger RNA (mRNA) isolated from treated and untreated macrophages. The cDNA is then used as the template for the qPCR reaction. Expression of these genes were of interest given their roles in inflammatory / anti-inflammatory reactions and mechanisms. Of these 5 cytokines, 4 are inflammatory and one, IL-6 can be participate in either inflammatory or anti-inflammatory mechanisms. The most striking changes were observed as decreases in expression of tumor necrosis factor alpha (TNF-) and CXCL2, both well-characterized inflammatory cytokines. TNF- was decreased several hundred-fold and CXCL2 was decreased more than 20-fold in these experiments. TNF- is a well-known inflammatory cytokine. It is involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction, which is a well-characterized systemic reaction to local

or systemic disruption of normal homeostasis, such as those caused by tissue injury, trauma, infections or immune disorders [Gordon and Koy, 1985]. CXCL2 is also an inflammatory cytokine. It is a macrophage inflammatory chemokine secreted to recruit neutrophils and other inflammatory cells to inflammatory sites. This essentially enhances the inflammation process. There was also a 4-fold decrease in CCL2, which is another chemokine protein that recruits several immune cells to site of inflammation produced during infections or tissue injury, again reinforcing inflammation. There was nearly a 5-fold decrease in IL-1, another key mediator of the inflammatory response. This cytokine is activated by the inflammasome, which is a molecular complex that is formed in response to bacteria, virus and other pathogens. Decreases in all of the inflammatory cytokines significantly reduces the potential for inflammation, fever, swelling, and pain of inflammation.

Figure 5 *- PEMFs effects on mouse macrophage cytokine expression determined by RTqPCR – Mouse macrophages were treated with PEMFs at 100 MFS and 5 pulses per second for 30 minutes. One hour after PEMF treatment, RNA was isolated, reverse transcribed (RT) and analyzed by real-time PCR. Gene expression was analyzed for IL-1, IL-6, TNF, CXCL2 and CCL2 using specific primers for these mouse genes. The values for each cytokine as determined by the ΔΔCT method. The 2-ΔΔ CT method is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments [Livak and Schmittgen, 2001]*



In contrast to the inflammatory cytokines analyzed, IL-6 was upregulated about 3-fold. IL-6 is a cytokine that can act as both a pro-inflammatory factor or as an anti-inflammatory myokine. Certainly, given the decreases in these other inflammatory cytokines, IL-6 is highly likely to act as an anti-inflammatory cytokine. The role of IL-6 as an anti-inflammatory myokine is mediated through its inhibitory effects on TNF-. A myokine is a cytokine that is produced in muscle cells in response to muscle contraction [Febbraio and Pedersen, 2005]. Not surprisingly, IL-6 is elevated during exercise. During exercise it is believed to mimic a hormone that mobilizes extracellular substrates and/or enhances substrate delivery [Petersen and Pedersen, 2005].

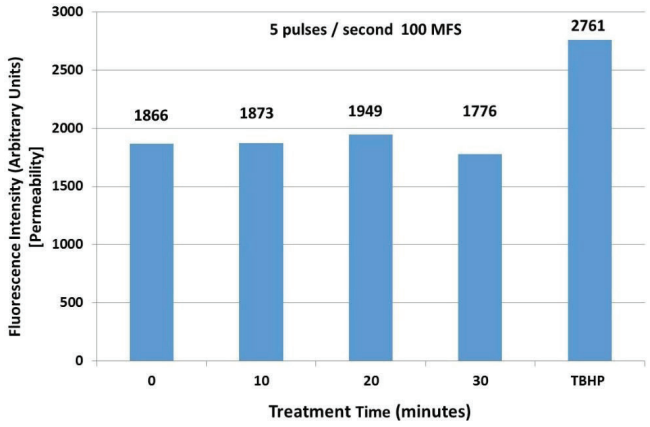
In all, this cytokine pattern is especially anti-inflammatory. It is interesting that there are several amplification cytokines that are downregulated by PEMFs. CXCL2 and CCL2 are inflammatory chemokines, which are downregulated by PEMFs that recruit more inflammatory cells. Thus, PEMFs downregulate expression of these inflammatory amplifiers. In addition, the increased expression of IL-6 functions to inhibit TNF-.

To determine if PEMFs affected calcium mobilization by permeabilizing the plasma membrane or modulating calcium channels, we use Fluo-3 loaded macrophages and flow cytometry. Figure 6 shows that when mouse macrophages were loaded with Fluo-3, an indicator for changes in intracellular calcium concentrations, and treated with PEMFs, there were no changes in fluorescence of this calcium indicator. Treatments with setting of 100% MFS and 5 pulses per second for 10, 20 or 30 minutes, had no effect or changes in intracellular calcium concentrations. Thus, when cells were analyzed 10-15 minutes after treatments of macrophages for as long as 30 minutes with the highest PEMF setting, there were no apparent changes in intracellular calcium levels. This indicates that PEMFs did not mobilize calcium by causing a

release of intracellular calcium from the endoplasmic reticulum or mitochondria. Since the extracellular media contains 1.6 mM calcium, there were also no changes in permeability of the plasma membrane to allow calcium to enter the cell by an influx across the plasma membrane. In contrast, when cells were treated with TBHP (tert-Butyl hydroperoxide), an agent that induces oxidative stress, as a positive control, there were increases in intracellular calcium.

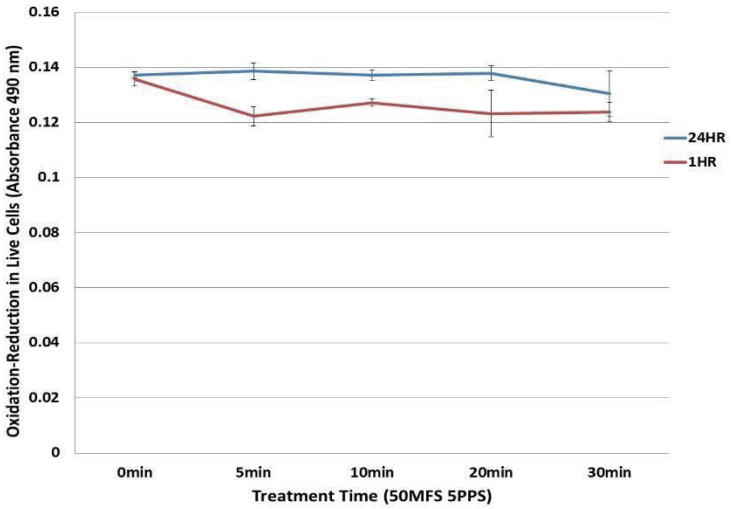
These results are in contrast to some previous reports that PEMFs increase calcium mobilization and/or activate calcium pumping mechanisms. In studies with mouse macrophages using Pulse PEMF's XLPro pulse device, there are no indications that PEMFs affect calcium mobilization, increase intracellular calcium concentrations or permeabilize the plasma membrane.

Figure 6: *PEMFs do not increase mobilization of calcium or permeabilize the plasma membrane of mouse macrophages- Mouse macrophages were loaded with Fluo-3, an indicator for calcium. The cells were then treated with PEMFs at 5 pulses per second at a magnetic field strength of 100 (100 MFS) for 0, 10 20 and 30 min. As a positive control, cell were also treated for 60 min with TBHP (tert-butyl hydroperoxide), direct-acting oxidative stress-inducing agent. The experiment represents a typical result.*



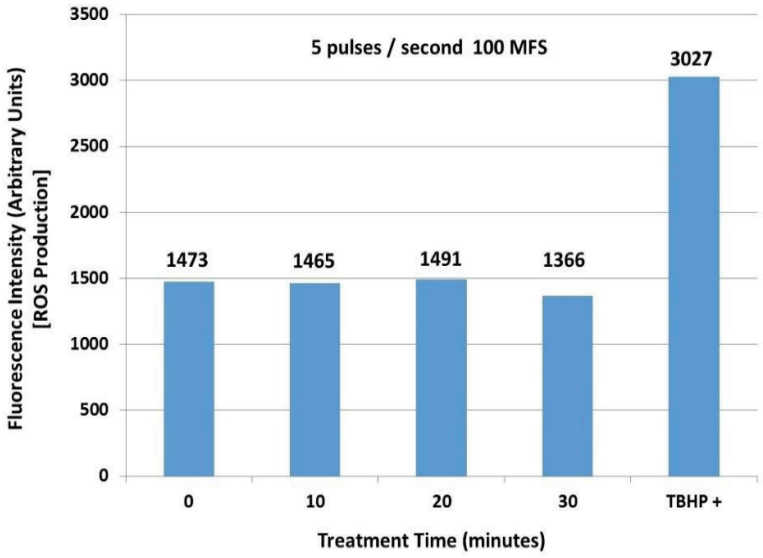
The MTS assay (Figure 7) is a colorimetric method for sensitive quantification of viable cells in proliferation and cytotoxicity assays. The method is based on the reduction of MTS tetrazolium compound by viable cells to generate a colored formazan product that is soluble in cell culture media. The oxidation – reduction (redox) activity is directly proportional to cell number and is generally used 24 h after cells have been treated with an agent to determine effects on viability. Thus, the redox activity after 24 h (**blue line**) indicates that there was no effect on cell viability after treatment for as long as 30 minutes with 50% MFS and 5 pulses per second. When cells were assayed 1 hour after PEMF treatment, there was also no differences between treated and untreated cells (0 min). This indicates that even after immediate treatment, there was no effect of PEMFs on cellular redox activity. In other studies not shown, there were no effects on cell viability with treatments with 100 MFS at 5 pulses per second for as long as 1 h.

Figure 7: *PEMFs have no effects on cell viability or on oxidation reduction (redox) reactions. Mouse macrophages were treated with PEMFs with a setting of 50 MFS at 5 pulses per second for 0, 5, 10, 20 and 30 mins. Cells were then analyzed after 1 h and 24 h in an MTS assay that measures of oxidation - reduction (redox) activity, which is generally used as an assay for quantification of viable cells. The results represent the mean and standard deviation of 3 independent assays.*



It was of interest to determine if PEMFs would increase reactive oxygen species (ROS) in mouse macrophages. In order to determine ROS levels in mouse macrophages after PEMF treatment we used CellROX green fluorescent agent. CellROX is a permeable dye that readily enters cells and is weakly fluorescent while in a reduced state. Upon oxidation by ROS and subsequent binding to DNA, the dye exhibits stable bright green fluorescence, with absorption / emission maxima of ~ 485/520 nm. Figure 8 shows that when mouse macrophages were untreated (0 min) or treated with PEMFs at a setting of 100 MFS at 5 pulses per second for 0 to 30 min, there were no changes in CellROX green fluorescence, indicating that PEMFs did not induce increases ROS under these intense conditions. Thus, PEMF treatment does not induce generation of ROS. This is an understandable result, because PEMFs downregulate expression of inflammatory cytokines (TNF, CXCL2, CCL2 and IL-1) and in fact increases expression of a potential anti-inflammatory cytokine, IL-6. Thus, PEMFs do not induce oxidative stress in mouse macrophages.

Figure 8: *PEMFs do not induce oxidative stress in mouse macrophages - Mouse macrophages were loaded with CellROX® Green Reagent, a fluorescent indicator for measuring oxidative stress in living intact cells. The cells were then treated with PEMFs at 5 pulses per second at a magnetic field strength of 100 (100 MFS) for 0, 10, 20, and 30 min. TBHP (tert-Butyl hydroperoxide), a direct-acting oxidative stress-inducing agent, was used as a positive control. he experiment represents a typical result.*



Conclusions and Summary: These studies demonstrate that there are no deleterious effects of PEMFs on cell viability, calcium influx calcium mobilization, plasma membrane permeability or ROS generation. In contrast, PEMFs activate macrophages by increasing binding and endocytosis of fluorescently labeled particulate dextran cargo. Given that increases in fluorescence is rapid, it is likely that PEMFs have effects on the plasma membrane that increase binding as well as endocytosis of dextran cargo. Importantly, PEMF-treated macrophages significantly downregulate inflammatory cytokines including TNF-, CXCL2, CCL2 and IL-1.

PEMFs also increase IL-6, which likely functions as a myokine that is increased as an anti-inflammatory protein during muscle contraction and exercise. The enhanced effects of PEMFs on macrophage binding and endocytosis of dextran cargo is long lasting, still present 8 and 24 h after treatment. Importantly, based on the PEMF-induced macrophage cytokine profile, PEMFs can reduce effects of inflammation such as swelling, irritation and pain. We can conclude from these studies that PEMF effects on macrophages can enhance the body's own mechanisms to clear infections, dissipate inflammation, and promote healing.

References:

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