Reviewer's report

Title: Anti-proliferative effect of extremely low frequency electromagnetic field on preneoplastic lesions formation in the rat liver

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Reviewer: Ronald J Midura

Reviewer's report:

The title and abstract of this manuscript accurately conveys the novel findings in this study. The overall question posed by this study is fairly well defined and considered highly relevant. For the most part, the research design, experimental approach, and methods are well described except for one significant omission. The manuscript should provide a detailed diagram of dB/dt versus the time domain for the chosen electro-magnetic field (EMF) treatment. The current description included in the manuscript does not allow a reader to compare this EMF treatment with other existing EMFs. For the most part, with the exceptions noted below, the data provided are sound and of high quality. Though the discussion and conclusions are for the most part well balanced and supported by the data, some limitations in the work are not discussed and are identified below. The manuscript adheres to relevant standards for scientific reporting and data deposition. The manuscript is well written and well organized, and the authors clearly acknowledge previously published and unpublished work in this field. Authorship roles in this study are clearly defined.

Major Compulsory Revisions:

1) Figure 1 shows that EMF treatments began a week before the first drug treatment to induce the modified resistant hepatocyte model (MRHM) of pre-neoplastic lesions. A rationale for starting EMF treatments PRIOR to induction of MRHM is not described. Furthermore, from a relevance view point, it is hard to justify a biophysical treatment prior to diagnosis of a disease state from ethical and clinical standpoints. What would have happened if EMF treatments started after DEN treatment, or after partial hepatectomy? Without an adequate rationale for this research design, the biological and clinical significance of this work is jeopardized. For example, would the EMF treatments prior and during subsequent drug treatments have had an effect on the pharmacokinetics of these drugs and thus alter their bioavailability in liver tissue? If so, then one would conclude that EMF reduced pre-neoplastic lesions in the liver because it reduced the negative influences of these drugs via altering their accessibility to the liver.

2) The immunohistochemistry data in Table 1 need to be broken down with regard to signal detection within the boundaries of the pre-neoplastic lesions versus signal detection outside lesion boundaries. Only with this kind of assessment can one determine whether the EMF effects are specific to the cells within the lesion as opposed to having generalized effects on all cells within the
3) Figure 3 TUNEL results are of good quality, but the authors need to realize that TUNEL detects free 3’-OH in genomic DNA via DNA strand breaks resulting from damaging insults. Thus, TUNEL is best interpreted as an assay of DNA damage, and can only be interpreted regarding apoptosis when additional corroboration is made. The study shows activated (cleaved) caspase 3 Western blots for NC, CT, and CTF liver samples. Close inspection of these Western blots demands that more sampling of liver tissues from additional animals needs to be obtained in order to provide a truly representative sampling. Specifically, in the CTF group two of the sample signals were noticeably lower in intensity than even the NC signals; only one CTF sample exhibited relatively high signal intensity. Does this one specimen skew the data erroneously, and if so, then the CTF would actually be shown to reduce overall activated caspase 3 levels. This would lend an interpretation that EMF treatment might have reduced overall apoptosis levels in the liver tissue. Closer inspection of the TUNEL results support this claim as the intensity of the TUNEL signal per nucleus seems greater in the CT group versus NC or CTF groups. In fact, the TUNEL signal of he CT group seems closer to the DNaseI digestion positive control for the assay. The authors are suggested to re-analyze their activated caspase 3 and TUNEL results and seek a higher level of confidence regarding the quantifiable attributes of their data. Given a lack of a statistical power analysis in the Methods section, it is unclear at present whether a sufficient sample population for each outcome was definitively established.

4) The above mentioned limitations in the TUNEL and activated caspase 3 analyses reported in this manuscript are also impacted by a re-analysis of the PCNA versus Ki-67 immunostaining results for nuclei in liver tissue sections. Ki-67 is strictly a marker of replication fork presence due to proliferation, while PCNA has been demonstrated to positively stain both proliferating nuclei and those actively undergoing DNA repair [Nucleic Acids Res 27:4476, 1999; Circulation 99:2757, 1999]. Given that serial sections were used to immunostain the liver tissue sections, this study should be able to assess how many nuclei stained positive for both Ki-67 & PCNA, as opposed to those nuclei that stained positive only for PCNA. This would provide an assessment of cell nuclei that are not actively engaged in replication, but rather DNA damage repair. This type of assessment would be able to cross referenced against the TUNEL/caspase3 data in Figure 3 in order to provide a more definitive assessment of whether EMF was altering apoptosis outcomes. For example in Table 1, if one assumes that all nuclei staining positive for Ki-67 were also staining positive for PCNA, then one could perform a simple subtraction [PCNA results – Ki-67 results = # nuclei undergoing DNA damage repair] to provide an assessment of the number of liver cells attempting to repair DNA damage. This type of calculation would yield the following data:

<table>
<thead>
<tr>
<th>Group</th>
<th>PCNA – Ki67 # DNA damage repair cells # cyclin D1 positive</th>
</tr>
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<tbody>
<tr>
<td>NC</td>
<td>22 - 7 cells/mm² = 15 cells/mm² 49 cells/mm²</td>
</tr>
<tr>
<td>CT</td>
<td>492 - 192 cells/mm² = 300 cells/mm² 265 cells/mm²</td>
</tr>
</tbody>
</table>
CTF 59 - 25 cells/mm² = 34 cells/mm² 45 cells/mm²

There is a remarkable similarity in the proportions of cells exhibiting DNA damage repair and cyclin D1 detection. I do not believe this similarity is a coincidence, and this possibility needs to be more rigorously investigated. If this holds true, then EMF altered the amounts of DNA damage to liver cells in the MRHM model.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** Yes, and I have assessed the statistics in my report.

**Declaration of competing interests:**

Do you have any other financial competing interests?

Yes, I have received honorarium from Orthofix, Inc. for speaking at a basic science summit on bone growth stimulators sponsored by this company. One of Orthofix's product lines is pulsed electromagnetic field stimulators.