RESEARCH ARTICLE

REVISED Effects of non-contact electric fields on kidney and liver histology in tumour-induced rats [version 2; peer review: 1 approved, 1 not approved]

Previously titled: Kidney and liver histology in tumour-induced rats exposed to non-contact electric fields

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Abstract

Background: A novel modality of cancer treatment based on exposure to non-contact electric fields has been developed to reduce the cancer incidence. However, the safety of this electric field exposure on vital organs has not been fully investigated. Therefore, the purpose of this study was to observe the safety of the electric field exposure on kidney and liver structures.

Methods: Female Sprague-Dawley rats were divided into one control group and three treatment groups. Animals were treated with 7,12-dimethylbenz[a]anthracene for mammary tumour induction and exposed to non-contact electric fields individually for 10 hours a day for three weeks. Fresh samples of kidney and liver were collected for observation of structural damage in both organs. Both organs were prepared for histopathological cross-sectioning using the paraffin method and Hematoxylin & Eosin staining followed by histological scoring using the post-examination masking method.

Results: Damages found in the kidney were as follows: thickening of Bowman capsule, karyolysis, karyorrexhis, pyknosis, cloudy swelling, epithelial sloughing, inflammation, haemorrhage, and congestion. The thickening of Bowman's capsule was significant, but other damages to the rest of kidney tissue were not significant. In addition, the number of inflammations and haemorrhages in the kidney structure of the placebo group was the lowest and significantly different compared to the other three groups. All damages to the

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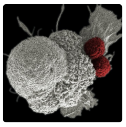
kidneys were also found in the liver, but each showed a different degree of damage. The damages to the liver caused by exposure to the electric fields were not significant.
Conclusions: Together with the effects of DMBA administration, exposure to non-contact electric fields can cause thickening of Bowman's capsule in the glomerulus of the kidney, however, in other kidney tissues and in the liver, exposure to this electric field was safe. It can even decrease the number of inflammations and haemorrhages in the organs.

Keywords
damages, histology, kidney, liver, non-contact electric field, ECCT



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REVISED **Amendments from Version 1**

The title was updated. We have created a new figure, namely the ECCT device in Figure 1, per reviewer's request. We have also corrected all figures per reviewer's request. We've also performed a new statistical analysis and presented the graphs per reviewer's request. There was a difference in the results of scoring of damage to the kidney glomeruli, where the damage in the NIT group was not significantly different from the NINT group.

Any further responses from the reviewers can be found at the end of the article

Introduction

The knowledge that electric fields can induce biological effects came to light in the 19th century. Many studies have been conducted which provide evidence that exposure to electric fields can produce alterations in living things.[1] Several studies have examined the effects produced by electric fields on cell function.[2] Kirson *et al.*[3] reported that electric field intensity inside the cell is less than 10 V/cm, but inside the cell membrane, it can reach 10^5 V/cm. At the organ level, the kidney and liver have dielectric properties that exhibit a time-temperature dependence.[4–6] Therefore, they possess both electrical conductivity and permittivity.[5,6]

Porter *et al.*[7] explained that the knowledge of dielectric properties of biological tissues is invaluable and useful in several medical device applications, including cancer detection and treatment. For example, the cell proliferations of breast cancer, oral cancer, cervix cancer, osteosarcoma, and lung carcinoma, as well as intradermal melanoma and intracranial glioma, were successfully inhibited under exposure to intermediate frequency (100, 150, and 200 kHz) and low intensity (200 V/m) alternating electric fields with the duration of exposure to the electric fields for 24 to 72 hours for cell studies, and 10-12 hours per day for 14-21 days and 24 hours for 6 days.[3,8–12] Intermediate frequency electric fields are used to treat cancer because they specifically target cancer cells and do not affect normal cells due to their higher membrane potential than that of cancer cells.[13,14] In our preliminary study using 9 mice, the intermediate frequency (100 kHz) electric fields of Electro-Capacitive Cancer Therapy (ECCT) gave good results, wherein the tumour size was reduced by more than 67%, but showed no histological alterations in mammary and skin tissues.[8] We used a 100 kHz electric fields because this frequency gave the best results in our *in vitro* studies, where 28-39% of breast cancer cells died.[8] Furthermore, we developed non-contact electric fields to avoid dermatitis due to direct contact between the electrodes and the skin, as reported by Kirson *et al.*[3] This novel modality has the potential to reduce the global cancer burden; 2.1 million people around the world were diagnosed with breast cancer in 2018, which is 11.6% of the total cancer incidence.[15]

Although non-contact electric fields-based therapy has the potential to treat cancer, the safety of such therapy in healthy tissues has to be investigated. This is because injury may occur after exposure to electric fields to organs such as the kidney and liver which have dielectric properties of the kidney and liver, which may interact with electric waves. Therefore, it is important to investigate abnormalities in the kidney and liver under exposure to electric fields during cancer treatment. The aim of this work was to investigate the safety of non-contact electric fields with a strength of 100 kHz-18 Vpp in the kidney and liver of animal tumour model, with a focus on possible histological alterations in the organs. We hypothesised that exposure to non-contact electric fields would not significantly affect the structure of the kidney and liver. According to our knowledge, this is the first study investigating the abnormalities in the kidney and liver under exposure to 100 kHz intermediate frequency and low-intensity (50-60 V/m) non-contact electric fields.

Methods

Experimental design

The experimental design and procedures, experimental animals, animal care and monitoring, housing and husbandry, sample size, inclusion and exclusion criteria, randomisation and blinding in this study were the same as our previously reported study.[9] For this study, 40 5-week-old healthy female Sprague Dawley (SD) rats (*Rattus norvegicus,* Berkenhout 1769) weighing 50−80 g were used. This rat strain is one of the animals used as animal tumour models to study human breast cancer, since it has 98% genetic homology with humans.[16] These rats were provided by the Integrated Research and Testing Laboratory (LPPT) of Universitas Gadjah Mada (UGM), and have never been used for other studies. Rats that were sick or showing symptoms of disorder were excluded from the study. The rats were placed in polypropylene cages for one week of acclimatisation. The polypropylene cage used was communal cage with a size of 50 × 40 cm^2 and the base was covered with rice hull bedding. We prepared eight communal cages with each cage consisted of 5 animals. The lighting conditions in the animal's room during the day came from lamp light, while at night it was total darkness (12L:12D photoperiod). We maintained room temperature to avoid dehydration during exposure to the electric field at 23–26°C with an average relative humidity of 81.09%.

We divided the animals into one control group (non-induction and non-therapy or NINT) and three treatment groups, namely placebo (non-induction and therapy or NIT), DMBA-induced mammary tumours without therapy (induction and

non-therapy or INT), and DMBA-induced mammary tumours with therapy (induction and therapy or IT) group. Using Federer's formula, the sample size in each group was calculated, in which 6 biological replicates were used for each group[11] and they were randomly selected to be assigned to the control and treatment groups.[9]

We administered a single dose of 7,12-dimethylbenz[a]anthracene (DMBA), 20 mg/kg body weight, to induce mammary tumours in rats in the INT and IT groups. The administration of DMBA was conducted twice a week for five weeks. This carcinogenic agent has been widely used in many mammary tumour studies using SD rats.[17,18] Furthermore, the rats in the NIT and IT groups were treated with exposure to intermediate frequency (100 kHz) and low intensity (50-60 V/m) electric fields for 10 hours daily for 21 days in modified individual cages.[9] Alternating electric fields were generated between pairs of capacitive electrodes embedded in individual cages that have been modified into ECCT devices. ECCT is called non-contact because the electrodes do not stick directly to the animal's skin. A multidirectional field was generated between pairs of capacitive electrodes and alternated every 0.5 ms (Figure 1). All individual cages were placed on the same table at the same height. The experiment was carried out in a special room which only contained experimental animal cages.[9]

The mammary tumour was palpated every two days with a digital caliper and its size (cm^2) was tabulated. Nodule size was not measured in volume due to tool limitation. All tumour measurements were performed by the same investigator (NF). The therapy was terminated once the mammary tumours enlarged to 2.25 cm^2 in size or therapy was completed on day 21. All rats were returned to their communal cages every day after the therapy was completed. Individual cages were cleaned daily by removing rat droppings and changing feed and water.[9] Rat fur was given picric acid as an individual marker to avoid potential confounders, while rat cages were labeled with a paint marker as a group marker. Each work in this study, such as DMBA administration, euthanised rat dissection, kidney and liver sample fixation, and data analysis, was carried out by a different investigator. One investigator (FA) controlled and monitored all works in this study.

Necropsy and organ harvesting

After completion of the treatment, all animals were euthanised under anaesthesia using an overdose of ketamine (150 mg/kg of body weight) via intramuscular injection. The animals were dissected ventrally side up on a dissection box by the same surgeon (AGF).[9] Two kidneys and two livers from different rats were randomly collected from each group. A total of 16 organs were used for histological examination. The number of samples used for histopathological examination was quite representative.

Renal histopathological analysis

Samples of the left kidney were taken from all groups by means of necropsy, washed with physiological saline (0.9% NaCl) and then fixed with 10% neutral buffered formalin (NBF). These organs were prepared for histopathological cross-sections using the paraffin method and hematoxylin and eosin (H&E) staining with a slightly modified protocol adapted from Bancroft and Cook.[19] A piece of organ that has been fixed was then dehydrated using graded ethanol 70%, 80%, 90%, and 100% for 2-3 repetitions, then followed by a 4 hours clearing process with xylol at room temperature.

Figure 1. ECCT device for animal study. The size of the cage is 23 cm × 18 cm × 19 cm. The electrodes are attached to the acrylic wall of the cage with opposite polarity facing each other to produce multiple field directions.

Furthermore, the organ was infiltrated by placing it in liquid paraffin at 60°C for 50 minutes with 3 repetitions. The next step was embedding, namely inserting the organ into a paraffin mold containing liquid paraffin, then cooling it to room temperature. Then the paraffin block containing the organ was cut 4-5 μm thick, and then the organ slices were placed on a glass slide and deparaffinized by dipping them in xylol for 3×5 minutes followed by dehydration using graded alcohol 96%, 90%, 80%, 70%, 50%, and distilled water for 1 minute each. The slides were then dipped in a hematoxylin dye solution for 2-5 minutes and dehydrated with 50% and 70% alcohol and subsequently dipped in eosin dye solution for 5-10 minutes, and dehydrated with 70%, 80%, 90%, and 96% graded alcohol. The last step was clearing in xylol for 15 minutes, and finally covered the slide with a cover glass.

Histopathological scoring of the kidneys was performed using the post-examination masking method combined with the ordinal scoring method.[20] The scoring referred to the endothelial-glomerular-tubular-interstitial (EGTI) system[21] which was adjusted to the needs of the study by replacing endothelial parameters with the number of congestion (Table 1). The scoring was performed on the renal cortex and medulla at 100 visual fields per group with 40× objective lens magnification. Microphotographs were taken using a Leica DM750 photomicrographic microscope. Kidney sample fixation and histopathological analysis were performed by the same researcher (NF).

Liver histopathological analysis

The liver was washed in physiological saline (0.9% NaCl) and immersed in a fixative solution (10% NBF). The histological preparations of the liver were carried out using the paraffin method, stained with haematoxylin and eosin following Bancroft and Cook[19] in the same steps as kidney preparations. Histopathological scoring was performed using the ordinal post-examination masking method. Scoring was carried out at 100 visual fields per group using a 40× objective lens magnification. Three parameters of damage, namely cellular damage, haemorrhage, and congestion were determined for the histopathological scoring system[22–24] (Table 2). Liver sample fixation and histopathological analysis were performed by the same researcher (SEDN).

Data analysis

All measured data were analysed using the appropriate methods and without any exclusion. Data were analysed qualitatively and quantitatively. Qualitative data analysis was carried out descriptively. For quantitative data analysis, the normality test was carried out first using the Shapiro-Wilk test (α=0.05). The scoring results were then analysed

Table 1. Histopathological scoring system for the kidney.

Tissue type	Injury	Score
Glomerular	No damage	0
	Thickening of Bowman capsule	1
	Retraction of glomerular tuft	2
	Glomerular fibrosis	3
Tubular	No damage	0
	Reversible damage	1
	Reversible damage with necrosis in tissue less than 25%	2
	Reversible damage with necrosis in tissue between 25% and 50%	3
	Reversible damage with necrosis in tissue more than 50%	4
Interstitial	No damage	0
	Inflammation or haemorrhage exists	1
	Inflammation or haemorrhage exists with necrosis in tissue less than 25%	2
	Inflammation or haemorrhage exists with necrosis in tissue between 25% and 60%	3
	Inflammation or haemorrhage exists with necrosis in tissue more than 60%	4
Congestion	No congestion	0
	Congestion in tissue less than 25%	1
	Congestion in tissue between 25% and 50%	2
	Congestion in tissue between 51% and 75%	3
	Congestion in tissue between 76% and 100%	4

Table 2. Histopathological scoring system for the liver.

Tissue type	Injury	Score
Cellular damage	No damage	0
	Reversible damage with necrosis in tissue less than 15%	1
	Reversible damage with necrosis in tissue between 15% and 40%	2
	Reversible damage with necrosis in tissues between 41% and 70%	3
	Reversible damage with necrosis in tissue between 71% and 100%	4
Haemorrhagic	No damage	0
	<15%	1
	15–40%	2
	41–70%	3
	71–100%	4
Congestion	No congestion	0
	Congestion in tissue less than 15%	1
	Congestion in tissue between 15% and 40%	2
	Congestion in tissue between 41% and 70%	3
	Congestion in tissue between 71% and 100%	4

statistically to determine significant differences among groups ($p<0.05$) using the Kruskal-Wallis test followed by the Mann-Whitney test ($\alpha=0.05$), since the data were not normally distributed. All data were statistically analysed using SPSS program version 16 (RRID:SCR_002865) by the same researcher (NF).

Results

The result of this study is a comparison of the histological characteristics of the kidney and liver under exposure to non-contact electric fields, which will be coherently described in the sections below.

Histopathology of kidney

The effects of non-contact electric fields exposure on renal histopathology and kidney damages scoring results are illustrated in Figure 2 and Figure 3, respectively. The main damage found in the kidney glomerulus was thickening of Bowman's capsule, which was significant in all induction groups ($p<0.05$, 1.16 ± 0.74 for INT, and 1.24 ± 0.59 for IT groups) compared to control (NINT) group (0.88 ± 0.56). In the kidney tubules, more damages were found, including karyolysis, karyorrexhis, pyknosis, cloudy swelling, and epithelial sloughing. However, the scores of these injuries were not significantly different among groups ($p>0.05$). In the renal interstitial tissues, inflammation and haemorrhage were identified and the score for both damage in the placebo (NIT) group was the lowest (1.0 ± 0.55) and significantly different from the other three groups ($p<0.05$, 1.19 ± 0.51 for NINT, 1.35 ± 0.63 for INT, and 1.31 ± 0.63 for IT groups). Congestion was found as a common injury in all parts of the kidney structure, and the number of congestions in the kidney structure in the placebo (NIT) group was also the lowest among the treatment groups, but not significantly different from the other three groups ($p>0.05$).

Histopathology of liver

The histopathological structure of the liver in the four groups had the same pattern of damage but with different levels of damage as shown in Figure 4 and Figure 5. All groups had the same type of damage, namely cellular damage (pyknosis, karyolysis, karyorrhexis), haemorrhage and congestion, and reversible damage (cellular swelling and fatty change). No significant cellular damage was found in the liver after exposure to non-contact intermediate frequency electric fields ($p>0.05$). In contrast, the scores of cellular injury and hemorrhage were highest after DMBA administration in INT group (1.96 ± 0.51 and 0.88 ± 0.46, respectively) and significantly different from the control (NINT) group ($p<0.05$, 1.75 ± 0.43 and 0.63 ± 0.48, respectively). The significant difference in hemorrhage scores between the IT group ($p<0.05$, 0.87 ± 0.56) and the control (NINT) group (0.63 ± 0.48), due to DMBA administration. Exposure to intermediate frequency electric field in the IT group slightly decreased hemorrhage, cellular injury and congestion in the liver (0.87 ± 0.56, 1.82 ± 0.48, 0.37 ± 0.56, respectively) after DMBA administration compared to the INT group (0.88 ± 0.46, 1.96 ± 0.51, 0.52 ± 0.66, respectively). The scores of congestion also were not significantly different among groups ($p>0.05$). The histology of the liver tissue in all groups did not show any fibrosis, so it can be said that the congestion that occurred was not at a chronic

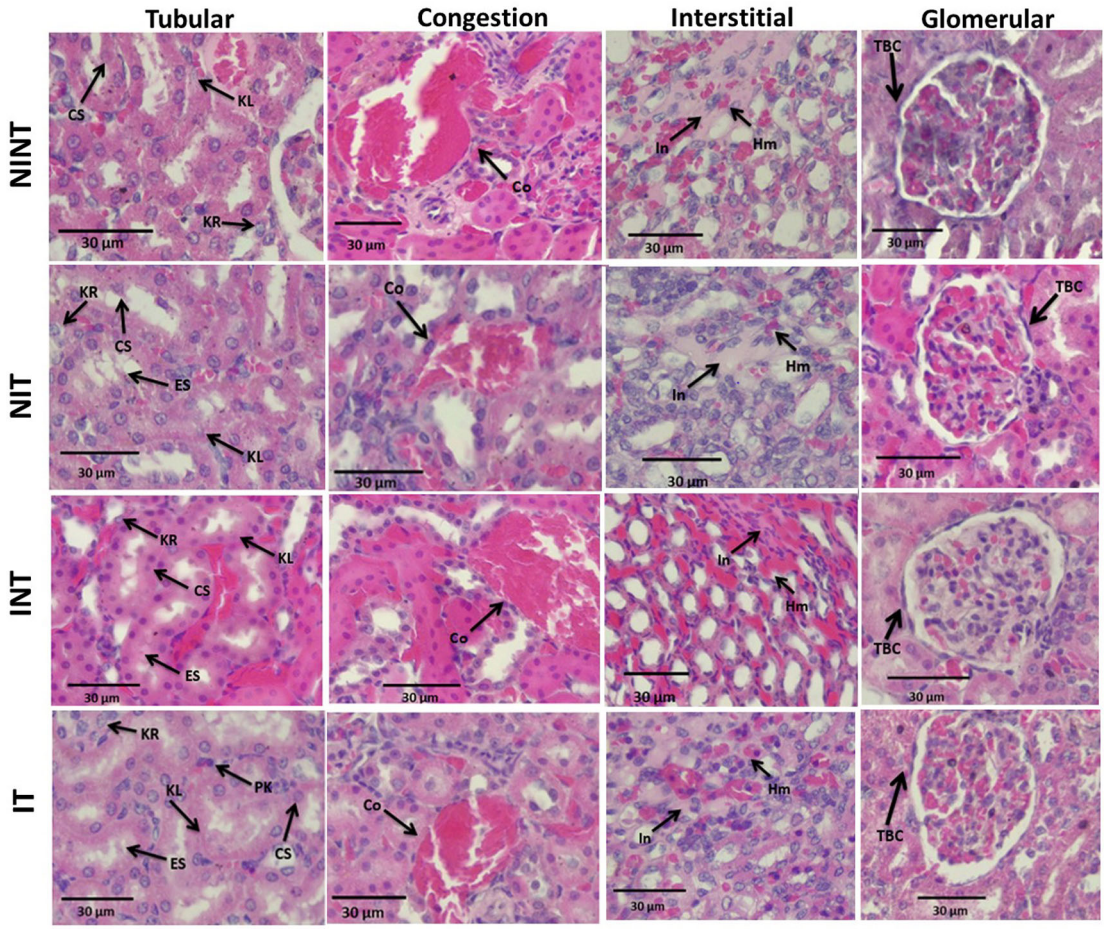

Figure 2. Histological features of tubular, interstitial, glomerular damages, and congestion in rat kidney sections stained with H&E. KL=Karyolysis, KR=karyorrexhis, PK=pyknosis, CS=cloudy swelling, ES=epithelial sloughing, Co=congestion, In=inflammation, Hm=haemorrhage, TBC=thickening of Bowman's capsule, NINT=non-induction and non-therapy group, NIT=non-induction and therapy group, INT=induction and non-therapy group, and IT=induction and therapy group.

level. Since there was no significant difference in the scores of congestion among groups and no fibrosis was found, congestion in all groups was still considered normal.

Discussion

In the present study, the safety of the non-contact intermediate frequency electric fields was revealed in the results of the histopathological analysis of kidney and liver in mammary tumour-induced rats, as discussed below.

The thickening of Bowman's capsule as the main damage to the glomerulus (Figure 2) may be a result of glomerular hyperfiltration,[25] DMBA-induced nephrotoxicity,[26] and exposure to electric fields.[27] Since significant glomerular damage was observed in non-therapy (INT) and therapy (IT) groups, both DMBA administration and non-contact electric field exposure affected the thickening of Bowman's capsule. Sharma and Paliwal[28] reported that the kidney is one of the main target organs of DMBA (nephrotoxicity) and that epithelial cells of Bowman's capsule and proximal convoluted tubule appear to be more susceptible to DMBA. Whereas the electric fields affected this damage by changing the transmembrane potential and the distribution of ion channels and dipoles following changes in the membrane structure.[29] Although both DMBA administration and non-contact electric field exposure affected the thickening of Bowman's capsule, the individual toxicity effect of electric field exposure was lower than DMBA administration, as shown in Figure 3. Therefore, DMBA administration would have a greater risk of inducing renal impairment. In our other study using an electric field frequency of 150 kHz with the same intensity (50-60 V/m), thickening of Bowman's capsule was also found, but DMBA administration and exposure to electric fields did not significantly affect the damage.[30]

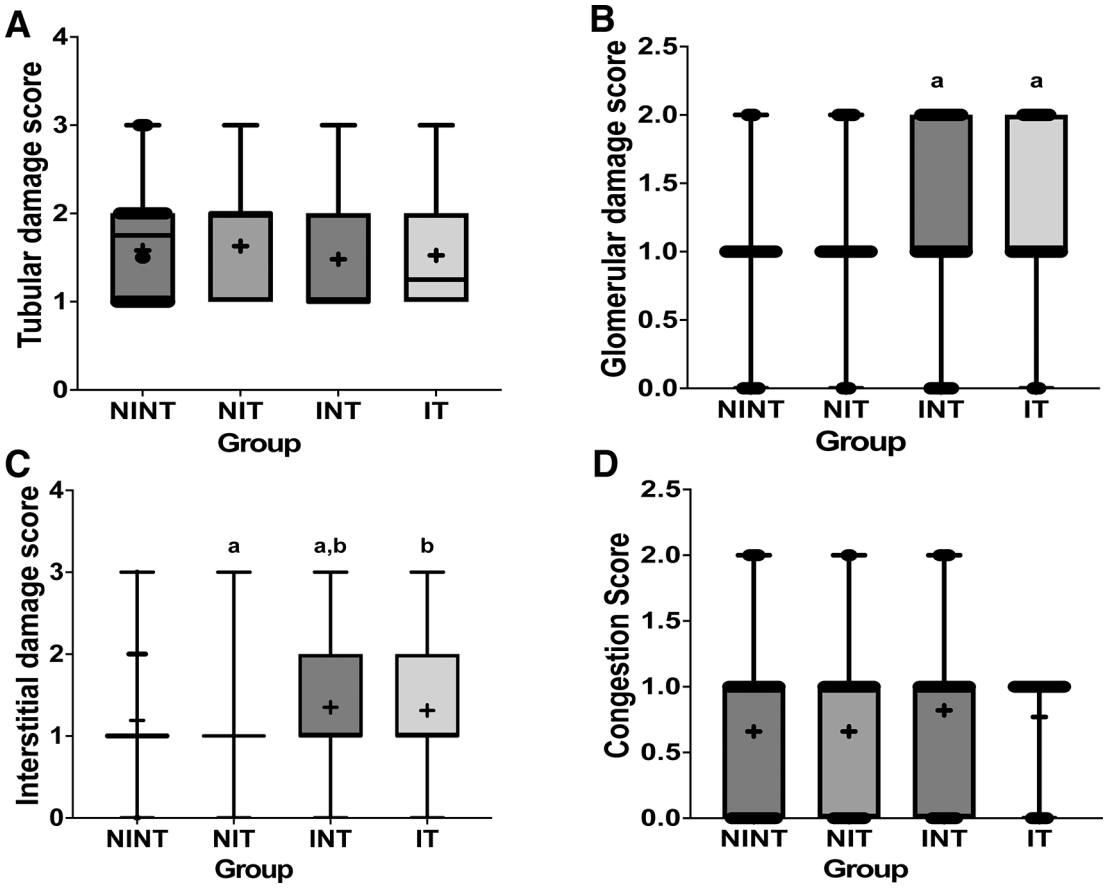

Figure 3. Scoring of tubular, interstitial, glomerular damages, and congestion in rat kidney sections. (A) Tubular damage, (B) interstitial damage, (C) glomerular damage, and (D) number of congestions.

The nephrotoxic effect of DMBA did not only occur in the glomerulus, but also in the tubules. In addition, DMBA caused substantive nephrotoxicity which is characterized by renal tubular necrosis including karyolysis, karyorrexhis and pyknosis,[31] as shown in Figure 2. Moreover, DMBA created obvious reversible histological changes in the tubules, such as epithelial sloughing and cloudy swelling, as illustrated in Figure 2. Epithelial sloughing represented the progressive tubular disintegration,[32] and cloudy swelling may lead to cell necrosis.[4] However, since the score of each injury to the renal tubules was not significantly different among groups, the nephrotoxic effect of DMBA and the exposure to non-contact electric fields were not harmful to the renal tubules. In our other study using an electric field frequency of 150 kHz with the same intensity (50-60 V/m), DMBA induction significantly damaged renal tubules. This suggests a nephrotoxic effect of DMBA to the renal tubules. In contrast, exposure to this electric field frequency resulted in a decrease in tubular damage in the normal kidneys (NIT group). Moreover, the frequency of this electric field may be able to compensate for tissue repair from the damage caused by DMBA.[30] Therefore, exposure to intermediate frequency and low intensity non-contact electric fields was not harmful to the renal tubules. It can even improve the condition of damaged renal tubules.

In the renal interstitial tissue, the nephrotoxic effect of DMBA significantly caused inflammation and haemorrhage, as shown in Figure 3. This inflammation can be affected by oxidative stress and can lead to impaired kidney function, including endothelial dysfunction, atherosclerosis, and glomerular injury.[33] Oxidative stress activates transcription factors including NF-kB, which activates expression of inflammatory response gene.[34] In addition, Kandeel *et al.*[35] reported that oxidative stress may alter kidney structure and function due to the effects of reactive oxygen species (ROS) on mesangial and endothelial cells. Oxidative injury happens when ROS, including O_2, H_2O_2 and -OH, ruin the antioxidant defence system of the cells.[36] These ROS can be produced due to DMBA administration[37] and can spread from their site of production to other sites inside the cell or even prolong the injury outside the cell.[38] Moreover, de Oliveira *et al.*[39] revealed that DMBA administration to develop tumours in animal models also causes haemorrhage. In our other study using an electric field frequency of 150 kHz with the same intensity (50-60 V/m), interstitial injury was not significantly induced by DMBA induction or exposure to electric fields.[30] In another study using electromagnetic field

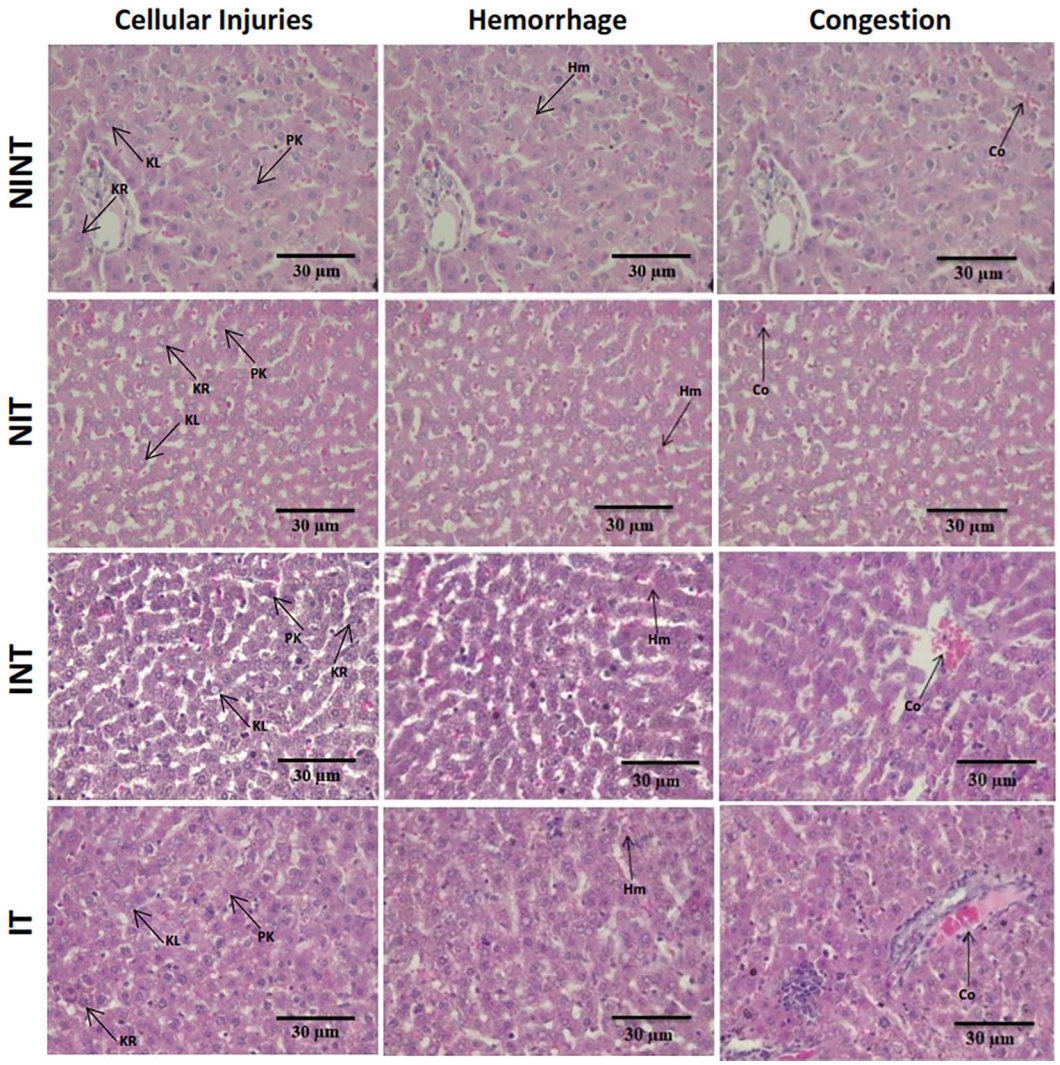

Figure 4. Histological features of haemorrhage, congestion, and cellular damage in rat liver sections stained with H&E. Hr=Haemorrhage, Cg=congestion, Pn=pyknosis, Kr=karyorrhexis, Kl=karyolysis, Cs=cell swelling, Fc=fatty change, NINT=non-induction and non-therapy group, NIT=non-induction and therapy group, INT=induction and non-therapy group, and IT=induction and therapy group.

exposure to 150 kHz in healthy SD rats, showed normal kidney morphology, including normal-appearing glomeruli, tubules, and interstitium.[40] Therefore, exposure to non-contact intermediate frequency electric fields was also not harmful to the renal interstitial tissue. In fact, exposure to this electric field decreased the number of inflammations and haemorrhages in the placebo (NIT) group, as shown in Figure 3.

Almost the same as in the kidney histology, there was no significant damage to the liver after exposure to intermediate frequency non-contact electric fields (Figure 5). The results in the non-therapy (INT) group with the highest scores of hepatocellular damage and haemorrhage indicated that DMBA as a carcinogenic substance can increase the presence of intercellular haemorrhage in the hepatic tissue.[24] Duarte *et al.*[24] reported mild hepatotoxicity in the liver, including the presence of a DMBA-induced pyknotic phase of hepatocyte nuclei. However, haemorrhage in the hepatic tissue has not shown symptoms of acute haemorrhage, such as cellular hypoxia, decreased tissue perfusion, organ damage, and death.[41] The results in the therapy (IT) group with a lower hepatocellular damage score compared to the non-therapy (INT) group suggested that exposure to non-contact electric fields had a lower damaging effect than DMBA administration. In addition, since the vascular congestion score was still in a normal condition and not at a chronic level, exposure to non-contact electric fields was not harmful. In our other study using an electric field frequency of 150 kHz with the same intensity (50-60 V/m), hepatocellular damage and congestion were not significantly induced by DMBA induction or exposure to electric fields. Even exposure to this electric field can significantly reduce haemorrhage in the liver of both

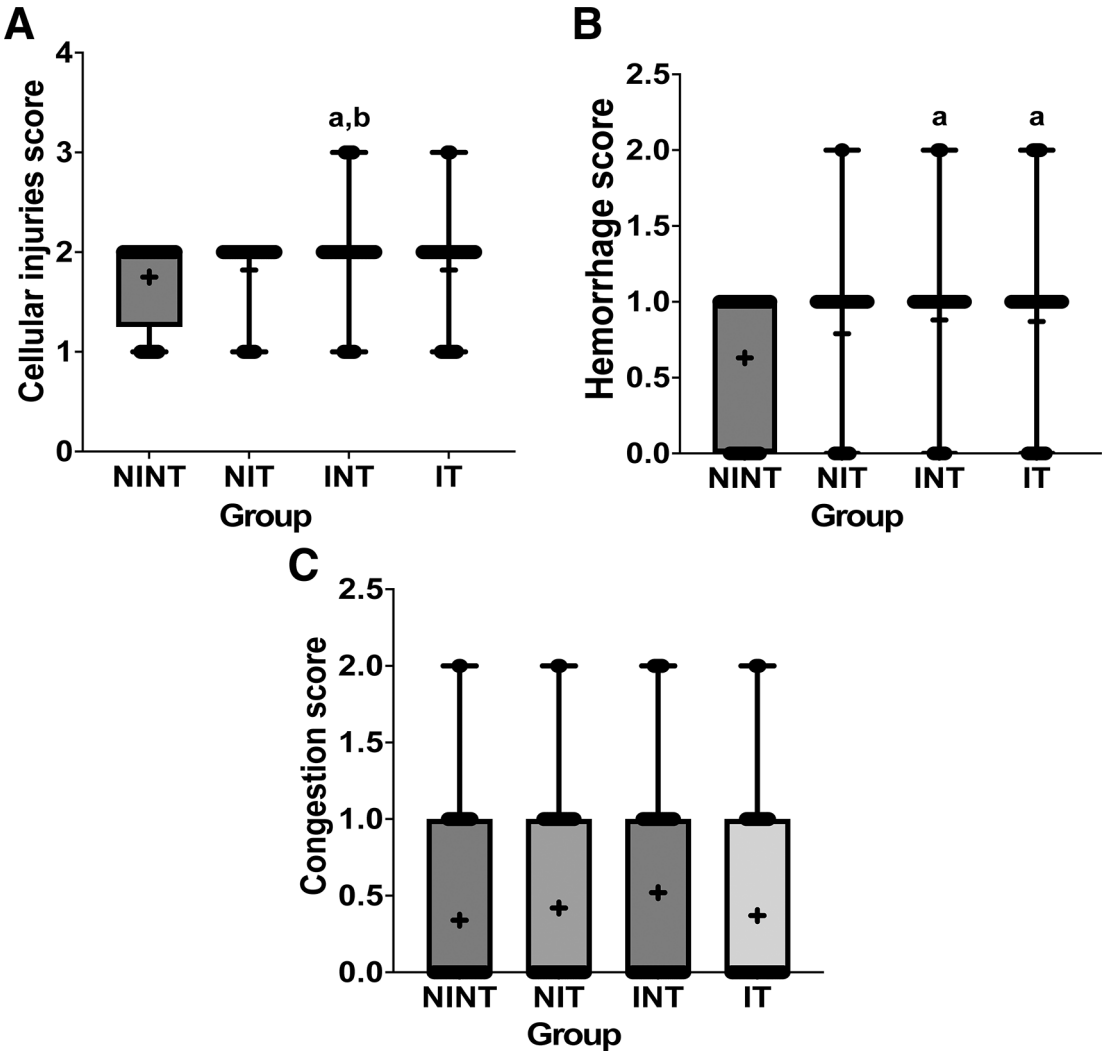

Figure 5. Scoring of cellular damage, haemorrhage, and congestion in rat liver sections. (A) Cellular damage, (B) haemorrhage, and (C) number of congestions.

healthy rats (NIT group) and tumour-induced rats (IT group).[30] In another study using exposure to a 150 kHz electromagnetic field, showed mild inflammatory changes with lymphocytic infiltration and haemorrhages in the liver of healthy rats indicating possible liver damage or infection. However, the liver damage that occurred was insufficient to cause clinical and functional manifestations because the lesions were mild enough without significant changes in liver enzyme levels.[40] Therefore, exposure to intermediate frequency non-contact electric fields was not harmful to the livers of the animals.

Damage to the kidneys and liver of the rats in the control group (NINT) cannot be predicted because rats with symptoms of illness had been excluded and rats were also randomly selected for each group. The thickening of Bowman's capsule in the NINT group can occur naturally due to aging, or due to ischemia.[30,42] Injury to normal renal tubules can occur because of the high rate of reabsorption by the renal tubules.[30] For damage to the renal interstitial tissue, a score below 2 indicates that there is little inflammation or hemorrhage. Inflammation is part of the activation of the immune system in response to acute or chronic kidney injury which can be caused by pathogens that enter the rat's body.[43] For damage to the liver in the NINT group, if we look at the hemorrhagic and congestion scores which are below 1, this indicates that there is little or no damage to the liver. For a cellular damage score below 2, this indicates reversible damage with less than 15% necrosis. Liver hepatocytes have many vital functions, so they can proliferate extensively, which allows efficient regeneration of the liver for reversible damage.[44] In addition, the liver itself is a very vulnerable organ due to its size and is the organ most frequently injured after abdominal trauma.[45]

For the results of this study, we only reported the effect of the intermediate frequency non-contact electric field on the histological structure of the kidney and liver, not yet on their function. Renal function parameters such as creatinine and bilirubin, and liver function parameters such as aspartate aminotransferase (AST) and alanine transaminase (ALT) taken from blood serum samples will be reported together with the hematological profile of the rat blood. Based on the evidence of the efficacy and safety of ECCT on normal tissues and organs,[8,9,30] including kidney and liver as reported in this study, we will conduct a phase I clinical trial of ECCT for healthy volunteers using an intermediate frequency (100 kHz) electric field as used in this study. Moreover, since this electric field exposure can reduce the number of inflammations and haemorrhages in the kidneys, this therapy can be used to treat kidney injuries or related diseases.[46]

Conclusions

Exposure to a non-contact electric field with intermediate frequency had a variety of effects on kidney and liver tissues. Together with the effects of DMBA administration, exposure to this electric field can cause thickening of Bowman's capsule in the glomerulus of the kidney, however, in other kidney tissues and in the liver, exposure to this electric field was safe. In addition, individual exposure to this electric fields did not cause significant thickening of the Bowman's capsule. It can even decrease the number of inflammations and haemorrhages in the organs.

Ethical approval

This research was carried out at the LPPT UGM and at the Animal Structure and Development Laboratory of the Faculty of Biology, UGM. LPPT UGM has been awarded ISO/IEC 17025:2000 accreditation for competence in testing and calibration.[11] Experimental protocol in this research was performed following approval by the Ethical Clearance Committee of LPPT UGM with ethical clearance number: 00015/4/LPPT/IV/2017, that has been previously reported.[9] The Ethical Clearance Committee stated that this research met the ethical requirements for the study on experimental animals and that the Ethical Clearance Committee had the right to conduct monitoring during the research.

Data availability

Underlying data

Open Science Framework: Kidney and liver histology in tumour-induced rats exposed to non-contact electric fields, https://doi.org/10.17605/OSF.IO/54BYF.[47]

This project contains the following underlying data:

- Kidney and liver histological images
- Kidney scoring and statistical analysis
- Liver scoring and statistical analysis
- Kidney and liver charts

Extended data

Open Science Framework: Kidney and liver histology in tumour-induced rats exposed to non-contact electric fields, https://doi.org/10.17605/OSF.IO/54BYF.[47]

This project contains the following extended data:

- Ethical clearance document

Reporting guidelines

Open Science Framework: ARRIVE checklist for 'Kidney and liver histology in tumour-induced rats exposed to non-contact electric fields', https://doi.org/10.17605/OSF.IO/54BYF.[47]

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Many thanks to Sugianto and Fadlil for making the device performed in this study.

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Open Peer Review

Current Peer Review Status: ✔ ✘

Version 2

Reviewer Report 02 June 2023

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✘

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The revised version of the manuscript, "Kidney and liver histology in tumour-induced rats exposed to non-contact electric fields", by Alamsyah *et al*., is an improvement over the first version, with many of the reviewers' suggestions implemented. However, Major Concern #1 from my first review of the manuscript does not appear to have been taken into account; despite the authors' claim that a new statistical analysis was performed, the Data Analysis section still states that "*scoring results were then analysed statistically to determine significant differences among groups (p<0.05) using the* ***Kruskal-Wallis test followed by the Mann-Whitney test***". As highlighted in my first review of the manuscript, this is an incorrect choice of statistical analysis methodology. This is problematic regarding both the interpretation of the data and the subsequent conclusions drawn from the study. Two alternative non-parametric methods (i.e., do not require normally distributed data) that can be used when a measure is affected by two (or more factors) and are thus applicable to the results reported by the authors in the manuscript were suggested; however, neither one appears to have been implemented in the revisions.

The alternating "*non-contact electric fields*" therapy studied by the authors is nearly identical to the relatively recently FDA-approved alternating electric field therapy—often referred to in the literature as tumor-treating fields—which (instead) typically uses electrodes placed on the skin and has shown remarkable efficacy as a non-invasive anticancer treatment modality. Consequently, the most important aspects of the study reported by the authors (as far as I can tell) are how the experimental group that received both tumor induction and EF therapy compares to the other experimental/treatment groups and the control group, as well as how the EF-only group and the control group compare (which is interesting for establishing effects of the treatment on healthy/non-tumoral cells). There are four groups reported in the manuscript, control (NINT), EF only (NIT), tumor induction only (INT), and the combined tumor induction and EF therapy group (IT). The groups thus **differ** very clearly **by two independent variables** (comprising two levels each): EF treatment (yes/no) and tumor induction (yes/no). The Kruskal–Wallis test **cannot** be used to compare these groups; the same is true of the Mann–Whitney test, which **assumes that the groups differ by only one independent variable**. To phrase it differently, the Kruskal–Wallis test is a **one-way test**; comparing the control to the IT group, for example, involves comparing groups

that differ in **two ways**. A simultaneous comparison between the IT group and the three other groups must be performed in order to correctly establish the influence of the two different independent variables on the dependent variable measured (i.e., the histopathological scoring results) and to determine the main effect of each independent variable as well as to determine if there is any interaction between the two independent variables.

"[The] *selection of [an] appropriate statistical method is [a] very important step in [the] analysis of biomedical data. A wrong selection of the statistical method not only creates some serious problem[s] during the interpretation of the findings but also affects the conclusion[(s)] of the study*"[1]. Consequently, the key results and conclusions stated in the manuscript might be erroneous (i.e., false claims of significance/insignificance). Thus, the status of my review of the manuscript remains "not approved". The problem is that without correctly applying an appropriate statistical analysis method that suits the data collected and the results obtained, the actual findings and conclusions of the study are unclear. Therefore, until this major concern is resolved, I do not believe that the manuscript and the reported results should be indexed or cited in order to prevent the propagation of potentially misleading results and conclusions.

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Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Electromagnetic interactions with biological systems; non-invasive therapies; microtubules; biophysics; high energy physics; particle physics.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Version 1

Reviewer Report 02 March 2023

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The manuscript, "Kidney and liver histology in tumour-induced rats exposed to non-contact

electric fields", by Alamsyah *et al*., reports new results on the effects of 100 kHz low-intensity non-contact electric fields on the histological profiles of kidney and liver samples taken from Sprague Dawley rats exposed to such fields (with and without tumour induction). Alternating electric fields with these particular parameters (low intensity and intermediate frequency) have become topical in oncology, especially over the last decade, due to the earlier discovery that these fields (also known as TTFields) greatly hinder the division of cancer cells. Several successful clinical trials on treating different types of cancer with TTFields have led to the subsequent FDA-approval of TTFields therapy for various cancers, such as glioblastoma. Consequently, many *in vivo* and *in vitro* studies on the efficacy of alternating electric field therapy have been conducted; however, safety studies are lacking in number and should grow in tandem with the efficacy studies. Thus, the study reported in the manuscript by Alamsyah *et al.* is both interesting and valuable to the scientific community (and a cancer research audience in particular); however, substantial major issues with the manuscript make the conclusions and, ultimately, the value and impact of the study uncertain. While I do believe the study has potential, the authors must revise the manuscript substantially before it should be indexed. Particularly, the literature review with regards to the Introduction and Discussion sections needs to be revisited and refocused; the statistical analysis used to determine the significance of the results does not apply, and a new analysis is required; and the very limited and overly general conclusions that do not appear to follow from the results and discussion presented in the manuscript must be stated more clearly to accurately reflect the results of the study. A complete list of my comments and concerns that must be addressed, both general and specific, are provided below.

General Comments:

1. There are many problems with the writing regarding grammar, punctuation, missing spaces, article usage problems, misspelled words, incorrect verb forms, faulty tense sequences, incomplete sentences, and several other sentences that simply do not make sense. In aggregate, these errors severely hinder the readability and clarity of the manuscript. I strongly recommend that the authors consider sending the manuscript to a professional English editing service, or at the very least, use one of the numerous free grammar and writing tools available online to assist with correcting many of the writing issues.

2. The manuscript mixes both U.S. and U.K. spellings, e.g., "*tumor*" and "*tumour*" are both used, as are "*acclimatization*" and "*randomisation*" (z vs. s). Please maintain one choice consistently in your writing.

3. The manuscript lacks coherence and does not communicate a cohesive story. Here are some examples of inconsistencies that negatively affect the cohesion:

   - In the "Results" part of the Abstract, it reads, "*The damages in the kidney and liver caused by the* ***exposure*** *were* ***not significant****.",* but then in the Discussion section, the following is stated "*Since the* ***significant damages of the glomerulus were observed in the kidneys*** *of the* ***placebo (NIT)****…*", followed by, "*both DMBA administration and* ***exposure to non-contact electric fields affected the thickening of Bowman's capsule***", which certainly sounds like there were significant damages observed in the kidney samples taken from the group that only received exposure to the electric fields (NIT).

   - The need for studying the effects of electric fields-based cancer therapies on healthy

tissues was motivated in the Introduction by pointing out that there might be interactions between the kidney/liver and **electrostatic** waves; however, the electric fields-based cancer therapies referenced and discussed in the manuscript (and that are actually used in a clinical setting) do not employ static fields, but rather alternating electric fields.

- Several of the references cited in the Discussion section that are discussed with regards to the interpretation of the results studied **vastly different** parameters for the electric fields than those employed in the experiments reported in the manuscript and that are typically used to treat cancers with alternating electric field therapy (e.g., 575 V/cm in one of the studies cited vs. the low-intensity of only a few V/cm maximum for alternating electric field therapy).

4. The manuscript often mentions the **significance** of results/effects; however, none of these statements include the supporting quantitative results that suggest the differences obtained are, in fact, significant (or not). Consequently, the level of significance associated with these statements is also not clear to the reader (without reading through the data files provided alongside the manuscript). Please revise **all** these statements to include the quantitative results of the statistical analyses that support the stated significances. For example, the following sentence on page 5, "*The main damage found in the kidney glomerular was the thickening of the Bowman capsule whose scores were **significant** in all treatment groups (1.12±0.56 for NIT, 1.16±0.74 for INT, and 1.24±0.59 for IT groups) compared to the control (NINT) group (0.88±0.56).",* should be revised to include the corresponding *H*-values (or *p*-values, etc.) that supports the statement that these differences between scores were significant ("*in all treatment groups compared to the control group*"). This makes it both clear to the reader that the differences between groups are indeed significant based on the statistical analysis results obtained and provides the reader with the quantitative results, so they also get a sense of **how significant** (or insignificant) each particular result is without having to dig through spreadsheets of data while reading the article.

5. The quality of the plots provided in Figures 2 and 4 is quite low. Additionally, the dots used to represent the data are very small and difficult to distinguish (when reading the article on a tablet at 100% magnification). Please consider reproducing these plots at a higher quality (600 DPI or greater) and with the points used to represent the data enlarged and spread out more clearly, so the individual points are distinguishable without needing to zoom in (which also leads to substantial blurring due to the low quality of the plots).

Specific Comments:

Major concerns:

1. The data analysis section states that the scoring results were analyzed statistically using the Kruskal–Wallis test; however, the scoring results were obtained for four different groups that **differed independently in two ways** (i.e., there were **two independent variables** involved—tumour induction (or not) and treatment (or not)). Consequently, the Kruskal–Wallis test, which is only applicable in the one-factor case, cannot be used. Due to this problem, the actual statistical significance of the obtained differences between the various groups studied is not clear, and the soundness of the conclusions drawn is uncertain. If you do require a non-parametric rank-based method, then consider the Scheirer–Ray–Hare extension of the Kruskal–Wallis test, which can be used in the case where a measure may be affected by two or more factors. Alternately, the (two-factor) aligned rank transform analysis of variance (ART-ANOVA) may also be suitable.

2. Moreover, the conclusions do not appear to be clearly supported by the results. This concern is not only due to the previous major comment noted but also due to the following:

   - The first conclusion drawn is that "*The non-contact electric fields were **not harmful** to the renal and liver structure of tumour-induced rats";* however, one of the outcomes described in the Results and Discussion sections was that the non-contact low-intensity electric fields significantly "*affected the thickening of Bowman's capsule*" (NINT versus NIT), which is apparently indicative of renal damage. Consequently, I am not sure that this general conclusion is completely supported by the results of this study.

   - The second conclusion drawn is that non-contact electric fields "*may optimise/increase the renal function in normal rats*". What results obtained in this study demonstrate an optimization and/or increase in renal function in normal rats exposed to low-intensity non-contact electric fields? The only discussion in the manuscript regarding renal function appears to be that provided on page 8; however, the discussion there mentions renal function impairment as a possible result of DMBA-induced inflammation and does not discuss electric fields. At the end of this paragraph, non-contact electric fields are mentioned: "*non-contact electric fields decreased the number of inflammations and haemorrhages in the placebo (NIT) group, as shown in Figure 2*"; however, the statistical significance of this difference is not stated. Is the decrease being mentioned here actually meaningful statistically? And if so, how statistically significant was this decrease, and how exactly does this marginal decrease in observed inflammations and haemorrhages connect to the conclusion that renal function has been **optimized** due to treatment with non-contact electric fields? As far as I can tell, no tests or metrics that could be used to accurately evaluate any changes in kidney and/or liver function and that would support this particular conclusion were included in the experiments and analyses reported in the manuscript.

3. Why were damages also exhibited in the control (NINT) group? Naively, I would not have expected this outcome, and it is not explained or discussed in the manuscript.

Due to these major concerns, I do not believe that the conclusions stated in the manuscript are clearly supported by the results.

Minor concerns:

1. More details regarding both the electric field used in the experiments and how it was applied should be provided for clarity and reproducibility of the study:

   - How were the electric fields generated? What device was used (make, model, etc.)?

   - Were the electric fields static or alternating?

   - What was the duty cycle?

   - Given that the rats were exposed to non-contact electric fields, I assume that the electrodes were not fixed to the skin of the rats and that they received whole-body exposure to the electric fields. Is this true? Additionally, how many electrodes were used? where were they placed/attached? and with what orientation? In other words, how many electric fields were there, were they uniform or non-uniform, and with what

directionality(ies)?

Please provide this information in the manuscript.

2. While the exposures were being performed, were there any other sources of electromagnetic radiation in the room that could be considered a source of interference?

3. On page 3, the last sentence in the Introduction states, "*According to our knowledge, this is the first study investigating the abnormalities in the kidney and liver under exposure to 100 kHz intermediate frequency and low-intensity non-contact electric fields*", which does appear to technically be true; however, a very similar[1] study was recently published that reports the results of analyzing histological profiles of vital organs of Sprague Dawley rats exposed to 150 kHz low-intensity non-contact electromagnetic radiation. It would be valuable and interesting to revise the discussion in the manuscript to compare and contrast your results and conclusions obtained versus those reported in this very similar study in the literature.

4. On page 3, a value of 18 V_{pp} is noted after stating that the electric fields used in the study are **low intensity**. The electric field intensity has units of electric potential per distance (V/m in SI units), not simply volts, which is the unit of electric potential. Please revise accordingly (including at the bottom of page 3, where the same issue occurs again).

5. On page 3, it is stated that "*Although **non-contact electric fields-based therapy has the potential to treat cancer**, the safety of this kind of therapy when treating healthy tissues should be investigated. This is because injuries may occur after exposure to electric fields due to the dielectric property of the kidney and liver, which may interact with **electrostatic waves**. Therefore, it is important to investigate the abnormalities in the kidney and liver under exposure to **electric fields during cancer treatment**.*" The second sentence quoted here does not connect to the previous or subsequent sentences; the electric fields used in cancer treatment that this article alludes to, so-called TTFields, are **alternating** electric fields (**time-varying**), whereas ***electrostatic* always refers to time-invariant electric fields**. Please revise.

6. Additionally, on page 3, the sentence "*the proliferation of cancer cells was successfully inhibited under exposure to intermediate frequency and low-intensity electric fields*" should be revised to read "low-intensity alternating electric fields". This statement would also benefit from more specificity, i.e., what types of cancer cells was this inhibition demonstrated for in the studies cited here and with what parameters for the electric fields employed (frequency, intensity, duration of exposure)?

7. Should the *y*-axis label for Figure 2d read "Congestion score" instead? (To maintain consistency with the other subfigures presented in Figure 2 and with Figure 4c.)

8. In Figure 4, the values of mean ± SD noted in each of the subfigures use inconsistent numbers of significant digits. Specifically, the values of the uncertainties are reported with greater precision than the mean values themselves, which indicates that the errors are known more precisely than the values and is very unusual.

9. Figures 2 and 4 include a footnote partially describing the meaning of the labels "a", "b", etc.

as indicating significance; however, the difference between each of these labels is not clear. The exact meaning of "a", "b", etc. should be clarified in the notes provided under Figures 2 and 4. Additionally, while it is clear that, for example, in Figure 2a, the NINT group has the label "a" to denote that it is significant, it is not clear what it is being compared to, i.e., significant compared to which group(s)? Lastly, please change "*different words a, b, c*" to read "different letters" or "different labels".

10. In the second paragraph of the Discussion on page 7, it is mentioned that "*some biological effects of exposure to electric fields (0.6 and 340 kV/m) were revealed in humans and vertebrates, but no histological abnormalities were found in the organs, including the kidneys.*" Comparing with this study does not make much sense for the following reasons:

    - The particular study cited here evaluated the biological effects of **static electric fields**, which are not the same as the **alternating** electric fields exploited in cancer therapy, and that the manuscript purportedly studies the effects of.

    - The parameters stated (0.6 and 340 kV/m) corresponding to this particular study that is being referenced are **NOT** consistent with the **low-intensity** electric fields used in cancer therapy (approx. 1–3 V/cm) and, again, that the manuscript appears to be studying.

11. The abbreviation ECCT is not defined anywhere in the manuscript.

12. The first sentence in the last paragraph of the Discussion section (page 9) does not make sense. Specifically, what does "*Based on the evidence for the efficacy and safety of normal tissues and organs…*" mean? I believe that rather than describing evidence on the "efficacy and safety of a normal tissue", what is trying to be said here is something like, "Based on the evidence for the efficacy and safety of non-contact low-intensity electric fields regarding normal tissues and organs…", or something along these lines. Please rephrase this sentence accordingly.

13. The statement regarding the role of the funders, "*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*", should also mention the role of the funders regarding the interpretation of the data/results.

References

1. Sundaram V, Mohammed S, Cockburn BN, Srinivasan MR, et al.: Effects of Intermediate Frequency (150 kHz) Electromagnetic Radiation on the Vital Organs of Female Sprague Dawley Rats.*Biology (Basel)*. 2023; **12** (2). PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?
No

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Electromagnetic interactions with biological systems; microtubules; biophysics; high energy physics; particle physics.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 23 Mar 2023

Firman Alamsyah

We thank you for all the comments and suggestions and we will revise our paper. Below are our answers to the comments and suggestions provided in order.

General comments
1. We have used a professional English editing service (PaperTrue) before submitting this article. We will do one more proofreading.

2. We will use UK spelling in our revised article.

3. We will revise the abstract so that it is coherent with the contents of the paper. We will replace some of the references in the Discussion section. Previously, we did not find any references in accordance with the study conducted.

4. In the Data Analysis section and in the Figures, we have mentioned a $p<0.05$ value for a significant difference among groups. We will rewrite it for each data in the Results section.

5. We will fix Figures 2 and 4.

Specific comments
Major concerns
1. We will evaluate the statistical tests performed.

2. We will revise the Conclusions section to suit the Results and Discussion sections.

3. Damage to the kidneys and liver of the rats in the control group (NINT) cannot be predicted because we have excluded rats with symptoms of illness as stated in the article. Rats were also randomly selected for each group as stated also in article.

For damage to the kidney in the NINT group, if we look at the interstitial tissue, a score below 2 indicates that there is little inflammation or hemorrhage. Inflammation is part of the activation of the immune system in response to acute or chronic kidney injury which can be caused by pathogens that enter the rat's body (Imig & Ryan, 2013).

For damage to the liver in the NINT group, if we look at the hemorrhagic and congestion scores which are below 1, this indicates that there is little or no damage to the liver. For a cellular damage score below 2, this indicates reversible damage with less than 15% necrosis. Liver hepatocytes have many vital functions, so they can proliferate extensively, which allows efficient regeneration of the liver for reversible damage (Chen et al., 2020). In addition, the liver itself is a very vulnerable organ due to its size and is the organ most frequently injured after abdominal trauma (Bilgic et al., 2014).

Minor concerns
1. We will provide more detailed information about the electric fields used in the experiment in our revised article.

2. The experiment was carried out in a special room which only contained experimental animal cages.

3. We will revise the Discussion section using the appropriate references.

4. We will include electric fields intensity data in our revised article.

5. We will revise this section (page 3).

6. We will revise this section (page 3).

7. We will revise Figure 2d.

8. We will revise Figure 4.

9. We will revise Figures 2 and 4.

10. We will revise the Discussion section using the appropriate references.

11. We will write what ECCT stands for in our revised article.

12. We will rephrase this sentence. There may be phrases lost in the editing process.

13. We will add the statement regarding the role of the funders in the interpretation of the data/results.

References
Bilgiç I, Gelecek S, Akgün AE, *et al*.: Evaluation of liver injury in a tertiary hospital: a retrospective study. *Ulus Travma Acil Cerrahi Derg*. 2014; 20(5): 359-365.

Chen F, Jimenez RJ, Sharma K, *et al*.: Broad Distribution of Hepatocyte Proliferation in Liver Homeostasis and Regeneration. *Cell Stem Cell*. 2020; 26(1):27-33

Imig JD, Ryan MJ: Immune and Inflammatory Role in Renal Disease. *Compr. Physiol*. 2013; 3(2): 957-976.

Competing Interests: No competing interests were disclosed.

Reviewer Report 27 February 2023

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Editorial Note from F1000Research – 06/03/2023:

This report has been updated after the reviewer informed the editorial team that they had further comments after their initial review was published. The changes are due to some factors which were not assessed in the initial review and were picked up when the reviewer read the article again. This update has not changed the 'Approved' status that the reviewer originally assigned.

This study touches on the effect of a new technology being introduced in oncology and answers some of the questions regarding the safety issue of the device. This information is critical to further utilize this device in the general public.

Some additional information from the authors could elevate the write-up:

1. Why do the authors choose the voltage 100kHz and 18v, specifically? Were the preliminary results referred to published? Are there any other articles supporting the usage of this voltage and frequency?

2. Is there any statistical analysis done? This will help strengthen the conclusion.

3. Why was there damage in the kidney interstitial tissue and liver damage in the NINT group?

In addition, some of the articles referred to use different voltage and frequency levels; therefore, it

couldn't be a direct comparison of this study. Such correlation needed to be taken with caution.

Overall, this study tries to answer the safety aspect of this non-contact electrical field therapy and highlights some promising changes.

First of all, I maintain that the paper is quite impressive and constructive with a novel idea. However, I would suggest a few additional points:

1. Figures 1 and 3 are very small, with many icons. Please enhance them.

2. Figures 2, and 4 suggest changing to the Whisker box plot and being made bigger. It will be better.

3. Utilization of rank order, like Mann Whitney, might help to bring forward the idea better.

4. Conclusion is a little too short and needs to be improved. Adding limitations and further direction might help. This can be done in the discussion.

5. IRB date, number, and place should be shown.

6. In several places, language is unclear, ambiguous, or confusing. It is necessary to use a professional editing service to improve it before indexing.

7. I think the title "Effects of non-contact electric fields on kidney and liver histology in tumour-induced rats." Might be more appropriate.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Interventional oncology, interventional and diagnostic radiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 01 Mar 2023

Firman Alamsyah

I will answer the reviewer's comments sequentially in the order of the comments.

1. We have conducted an *in vitro* study using various frequencies and intensities, but this study has not been published. We used 100 kHz and 18 Vpp for *in vivo* study because this frequency and intensity gave the best results in *in vitro* study, where 28-39% of breast cancer cells died (Alamsyah et al., 2015). In the preliminary *in vivo* study using 9 mice, the frequency of 100 kHz and intensity of 18 Vpp also gave good results, where the tumor size was reduced by more than 67% and the results of histopathological analysis on normal skin and breast tissue, showed no damage (Alamsyah *et al.*, 2015).

2. Yes, we did the statistical analysis and it has been written in the article in the Data Analysis section. Kidney and liver scoring data, as well as statistical analysis can be seen in the links provided in the Data Availability section of the article.

3. Damage to the kidneys and liver of the rats in the control group (NINT) cannot be predicted, because we have excluded rats with symptoms of illness as stated in the article. Rats were also randomly selected for each group as stated also in article.

For damage in the renal interstitial tissue of the NINT group, a score below 2 indicated that there was little inflammation or hemorrhage. Inflammation is part of the activation of the immune system in response to acute or chronic kidney injury which can be caused by pathogens that enter the rat's body (Imig & Ryan, 2013).

For damage in the liver of the NINT group, if we look at the hemorrhagic and congestion scores which are below 1, this indicated that there was little or no damage to the liver. For a cellular damage score below 2, this indicated reversible damage with less than 15% necrosis. Liver hepatocytes have many vital functions, so they can proliferate extensively, which allows efficient regeneration of the liver for reversible damage (Chen *et al.*, 2020). In addition, the liver itself is a very vulnerable organ due to its size and is the organ most frequently injured after abdominal trauma (Bilgic *et al.*, 2014). Rats are active animals and may chase or fight each other in communal cages which can cause trauma to their body (Steimer, 2011).

4. We found no reference to kidney or liver damage at intermediate frequency and low intensity electric fields, especially 100 kHz and 18 Vpp. We have also stated this in the article. In the Introduction section, we have also stated that this is the first study to investigate abnormalities in the kidney and liver under exposure to a intermediate frequency of 100 kHz and a low intensity non-contact electric field.

We will add necessary information to our article from the answers to the reviewer's questions.

Thank you.

References

Alamsyah F, Ajrina IN, Dewi FN, et al.: Antiproliferative Effect of Electric Fields on Breast Tumor Cells In Vitro and In Vivo. Indones. J. Cancer Chemoprev. 2015; 6(3): 71–77.
Bilgiç I, Gelecek S, Akgün AE, *et al*.: Evaluation of liver injury in a tertiary hospital: a retrospective study. *Ulus Travma Acil Cerrahi Derg*. 2014; 20(5): 359-365.
Chen F, Jimenez RJ, Sharma K, *et al*.: Broad Distribution of Hepatocyte Proliferation in Liver Homeostasis and Regeneration. *Cell Stem Cell*. 2020; 26(1):27-33
Imig JD, Ryan MJ: Immune and Inflammatory Role in Renal Disease. *Compr. Physiol*. 2013; 3(2): 957-976.
Steimer T**:** Animal models of anxiety disorders in rats and mice: some conceptual issues. *Trans Res*. 2011; 495-506.

Competing Interests: No competing interests were disclosed.

Author Response 20 Mar 2023

Firman Alamsyah

We thank you for the additional suggestions and we will revise our paper. Below are our answers to the suggestions provided in order.

1. We will enhance Figure 1 and Figure 3 in our revision.

2. We will consider to use the Whisker box plot in Figure 2 and Figure 4.

3. We have conducted Mann-Whitney test (α=0.05) in our statistical analysis.

4. We will improve the conclusions of our study and we will discuss the limitations of this study. We have written further directions of this study at the end of the discussion section.

5. IRB date, number, and place have been written in the ethical approval section.

6. We have used a professional editing service (PaperTrue) before submitting this article. We will do one more proofreading.

7. We will improve the title of this article.

Competing Interests: No competing interests were disclosed.