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Effects of 5G radiofrequency electromagnetic radiation on indicators of vitality and DNA integrity of *in vitro* exposed boar semen

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ABSTRACT

The effects of radiofrequency electromagnetic radiation (RF-EMR) on semen quality have been in the spotlight in recent years, though research results to date have been contradictory. The effects of RF-EMR amongst others depend upon frequency, and there is currently no literature concerning the influence of 5G frequencies on both DNA integrity and spermatozoa vitality in males. The aim of this study was to investigate the effect of 5G RF-EMR on sperm membrane integrity, mitochondrial potential, and DNA integrity of *in vitro* exposed semen of breeding boars. The study included semen samples of eight breeding boars of the Pietren breed and four breeding boars of the German Landrace breed, from 1.5 to 3.5 years in age. Freshly diluted semen of each boar was divided into a control (n = 12) and experimental group (n = 12). The samples of the experimental group were exposed for 2 hours to continuous RF-EMR at a single frequency (700 MHz, 2500 MHz and 3500 MHz) and an electromagnetic field strength of 10 V/m using a transverse gigahertz electromagnetic cell. Sperm DNA fragmentation was assessed using a Halomax® kit and sperm membrane integrity and mitochondrial potential was assessed using a PI/SYBR-14 LIVE/DEAD viability kit with JC-1. A significantly higher proportion of spermatozoa with DNA fragmentation was found in exposed semen samples for all frequencies compared to the control group. The highest DNA damage was recorded in semen samples exposed to 5G RF-EMR at 2500 MHz ($p < 0.01$) and 3500 MHz ($p < 0.05$) *vs.* control semen samples. A significantly higher proportion of spermatozoa with damaged cell membrane and good mitochondrial potential was recorded in semen samples exposed with 3500 MHz. *In vitro* exposure of breading boar semen to 5G RF-EMR significantly increases the proportion of DNA fragmentation. The harmful effect of 5G RF-EMR on the proportion of spermatozoa with damaged DNA was frequency dependent. The 3500 MHz frequency displayed the most harmful effects due to significant impacts on DNA integrity and spermatozoa vitality indicators.

1. Introduction

The presence of different frequencies from radiofrequency electromagnetic radiation (RF-EMR) of anthropogenic origin, *i.e.*, radio communication systems, mobile phones, microwave ovens, and wireless communication system in the environment is known as electromagnetic smog or electrosmog [1]. Public concern about the impact of RF-EMR on health is controversial [2]. 5G technology is considered a potential health risk which requires further investigation and research regarding the possible health effects of electromagnetic field exposure [3]. Although the energy of RF-EMR is not sufficient to break chemical bonds, the results of numerous studies have shown that exposure to this energy at the frequency of 4G and earlier technology can cause various biological effects in different cell models. Previous studies on the effects of RF-EMF at frequencies from 900 MHz to 2.4 GHz on human and animal sperm mainly investigated sperm motility, vitality and DNA damage under short-term conditions [4,5]. For example, several (*in vitro*) studies showed that sperm motility, sperm viability and sperm-fertilizing ability were decresed as well as DNA fragmentation was enhanced in the human sperm after exposure at frequency of 850 MHz, 900 MHz and 2.45 GHz for 1 h exposure [6–8].

Male fertility can be reduced by a range of environmental factors. Parameters that indicate reduced fertility can appear as a decreased sperm count, motility disorder or structural disorder. These changed sperm fertility parameters can be induced by biochemical factors, stress, ionising radiation, or electromagnetic waves [9]. Most research to date has focused on the harmful impact of RF-EMR on the male reproductive system, especially humans [10], while data on the effects of RF-EMR, especially the 5G assigned frequency, on the reproductive system of male domestic animals are scarce. In recent decades, pigs have become a common choice as animal models in biomedical research, due to their great similarity in physiological processes, *i.e.*, cellular mechanisms, with humans [11]. In addition, the pig genome is three times more similar to the human genome than the mouse genome, making pigs a more suitable model than rodents [12]. In this study, boar spermatozoa was selected as the model because of the high volume and sperm count that boar ejaculate (approx. 250 mL), enabling a large number of analyses [13]. Research on boar semen could also have practical applications, as large number of sows are fertilised with a single ejaculate, and electrosmog is a potential threat not only to human health but also to the health of domestic and wild animals.

Currently known data indicate that exposure to RF-EMR induces DNA damage and accumulation of reactive oxygen species (ROS) or oxidative stress due to increased levels of free radicals or superoxide anions [14]. An increase in superoxide anion concentration can cause a decrease in spermatozoa motility [15]. Some studies have shown that

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the RF-EMR will reduce the motility and vitality of spermatozoa cells, concurrently stimulating the formation of a DNA base adduct, which ultimately results in DNA fragmentation [14]. Formation of a biomarker for oxidative damage of DNA and DNA fragmentation are most likely co-correlated and ultimately linked to cell death [16].

Electron leakage from spermatozoa mitochondria is thought to be the main source of ROS in these cells [17]. Oxidative damage is induced when the production of ROS by the spermatozoa mitochondria exceeds the gamete's limited endogenous antioxidant defences, which are rapidly overwhelmed. Loss of fertilizing potential and vitality is due to lipid peroxidation in the spermatozoa cell membrane [18]. ROS will also induce DNA damage in the spermatozoa nucleus and mitochondria and will do so at levels of oxidative stress that are still compatible with the retention of motility and a capacity for fertilisation [16,18,19]. However, the potentially harmful effects of RF-EMRs at frequencies higher than 2500 MHz have not been previously investigated on quality and/or indicators of spermatozoa vitality of human or animal semen. Also, there are no data on the effects of RF-EMR on structural stability of spermatozoa chromatin *in vitro* exposed semen of breeding boars.

The hypothesis of the research is that there is a harmful effect of 5G RF-EMR on vitality and chromatin structural stability of spermatozoa and that the level of the effect depends on the frequency of radiation. Therefore, the aim of this study was to investigate the effects of 5G RF-EMR at three frequencies (700 MHz, 2500 MHz and 3500 MHz) on spermatozoa vitality indicators and DNA integrity on *in vitro* exposed semen of breeding boars.

2. Material and methods

2.1. Boar management and sampling

The study was performed on semen samples of eight breeding boars of the Pietren breed and four breeding boars of the German Landrace breed, aged from 1.5 to 3.5 years. Semen was collected using manual fixation of the penis at a commercial centre for artificial insemination in Varaždin, Croatia (46°18'15″N, 16°20'16″E). Boars were kept in separate pens of approximately 12 m^2 (4 × 3 m). All pens were illuminated with natural light and the floors were covered with straw litter. Boars were fed daily with approximately 2 kg of mixture for boars divided into two meals: the first meal in the morning before the jump (around 6:00 a.m.) and the second around 2:00 p.m. Boars had unlimited access to water.

Semen was collected once per week (during the three weeks study period), by the same person in the presence of a phantom. Ejaculates were assessed for sperm concentration using a photometer (Accucell, IMV Technology, Normandy, France). Initial score of total motility (%), gross motility and sperm morphology (Spermac, Minitube, Germany) was assessed using an Olympus BX 50F (Olympus, Tokyo, Japan). Standard evaluation of semen ensured that all samples were of commercial standard. Average volume (±SD) of the boar sperm samples was 354.5 ± 126.3 mL, concentration (±SD) was 358.7 ± 169.4 million spermatozoa per mL, total sperm concentration (±SD) was 113.5 ± 34.6 billion. After sperm evaluation, semen samples were diluted with a Cronos diluent (Medi-Nova, Reggio Emilia, Italy) to obtain a concentration of 30 million spermatozoa/mL.

To simplify the study, exposure of semen 2 h after sampling was carried out in three one-week periods. In each of the three one-week periods, semen samples were collected from 12 breeding boars, and the freshly diluted semen of each boar was divided into a control and an experimental group (n = 12 semen samples/group).

2.2. Exposure and analyses

The semen samples of the experimental group (n = 12, test samples) were exposed to continuous RF-EMR at only one frequency within one week as follows: in the first week the experimental group was exposed to RF-EMR at a frequency of 700 MHz, in the second week at a frequency of 2500 MHz and in the third week at a frequency of 3500 MHz. All frequencies used the same field strength of 10 V/m for 2 h with the gigahertz transverse electromagnetic (GTEM)-Cell made at the Department of Communication and Space Technologies, Faculty of Electrical Engineering and Computing, University of Zagreb (Fig. 1). In addition to the GTEM-Cell, an HP 8657A signal generator and an RFGA0101-05 linear amplifier was used to obtain 10 V/m electromagnetic field strengths. The amplifier was connected to a computer and the desired frequencies were set using the Windfreak Technologies SynthNV programme (Windfreak Technologies, LLC., New Port Richey). Inside the cell, during each exposure, a digital thermometer was placed to measure the temperature due to exclusion of the thermal effect (Fig. 1). The average temperature inside the cell during semen exposure was 19.1 °C (range 18.7–19.5 °C. The control group (n = 12 semen samples/frequency) was subjected to the same conditions (time and temperature), placed in a metal container (imitation for GTEM-Cell), for 2 h without exposure (sham exposure).

2.2.1. Assessment of spermatozoa cell membrane integrity and mitochondrial potential using PI/SYBR-14 LIVE/DEAD with JC-1

Assessment of spermatozoa cell membrane integrity and mitochondrial potential of spermatozoa was determined by triple staining using PI/SYBR-14 LIVE/DEAD Sperm Viability Kit with 5,5′,6,6′- tetrachlor −1,1′,3,3' - tetraethyl - benzimidazolyl - carbocyanine iodide (JC-1, Molecular Probes, Leiden, Netherland) according to De Pauw et al. [20] with modifications. Control and test semen samples in the amount of 50 μL were incubated with 3 μL PI (2.99 mM), 2 μL SYBR-14 (0.5 mM) and 2 μL JC-1 (1.53 mM) diluted in 150 μL TALP at 37 °C for 15 min in the dark. The combination of these two fluorescent colours makes it possible to distinguish membrane-intact and membrane-damaged spermatozoa in the same preparations. JC-1 is a cationic dye that has the ability to differentially label high- and low-potential of the inner mitochondrial membrane (IMM). Spermatozoa membrane integrity and mitochondrial membrane potential were determined by evaluating at least 200 spermatozoa for each sample using an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a 450–490 nm filter of a 100 W mercury lamp and examined at 450× magnification.

The analysed SYBR-14/PI and JC-1 samples showed four types of fluorescent spermatozoa cells: those with a red head and green mitochondria (damaged spermatozoa cell membrane with low mitochondrial potential), those with green heads and orange mitochondria (intact cell membrane and high mitochondrial membrane potential), those with a green head and green mitochondria (intact cell membrane with low mitochondrial potential), and finally those with a red head and orange mitochondria (damaged cell membrane and high mitochondrial membrane potential).

In addition, the analyses were performed immediately after exposure of the samples and the samples were sufficiently diluted to ensure visibility of samples. The tests were performed by an expert in the field with many years of experience who did not know which sample was being tested (blind sample).

2.2.2. Assessment of DNA integrity/chromatin structural stability

DNA damage was assessed using the Halomax DNA Fragmentation Test by Halotech DNA (Madrid, Spain). The samples were prepared as follows: the agarose Eppendorf tube (ACS) was placed into the float and melted using a water bath at 95–100 °C for 5 min. Then, the ACS was placed with the float inside the beaker and heated at maximum power for 1.5 min. At the same time, lysis solution was kept at room temperature (22 °C) during the whole process. Furthermore, 25 μL of the semen sample was pipetted into an empty test tube, with the addition of 50 μL agarose, the test tube was closed and gently rotated, then 1.5–2 μL of the sample mixture was pipetted out the test tube and dripped onto the marked field of the slide, then covered with a cover. The slide was refrigerated at 4 °C for 5 min to solidify the agarose. After 5 min, the specimen was taken out of the refrigerator and the cover was removed.

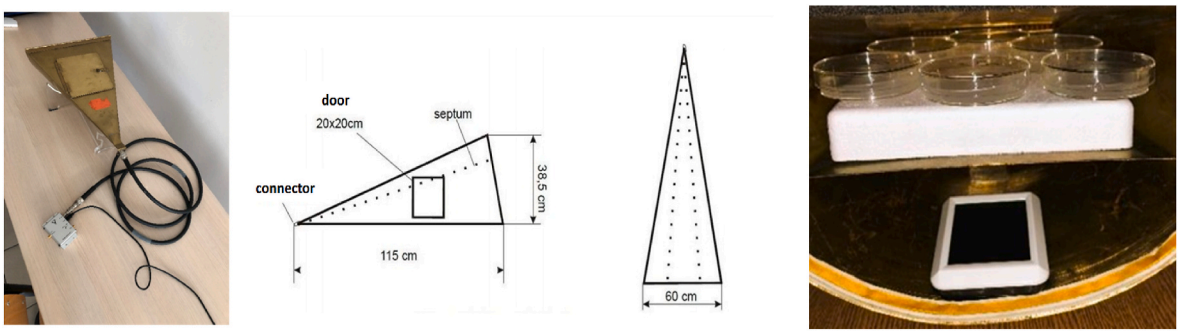


Fig. 1. The gigahertz transverse electromagnetic cell (GTEM) and petri dishes inside a GTEM with a digital thermometer.

The slide was then transferred to a Petri dish measuring 9.5 × 9.5 cm and covered with lysing liquid at room temperature. The prepared sample was incubated for 5 min and then washed with redistilled water for 5 min. Rinsing followed in two steps: 2 min with 70 % alcohol, and 2 min with 100 % alcohol. The slide was dried and stored in a dark place for one month, then the samples were stained with FLUORED fluorescent dye (REF HT-FR100, Halotech). The Halotech kit consists of reagent A: red fluorochrome (200 μL) and reagent B: medium and fixation (200 μL). During staining, reagents A and B were mixed in a 1:1 ratio, 3 μL of the mixture was pipetted onto the prepared sample and covered, and the sample was examined under a fluorescent microscope by evaluating at least 200 spermatozoa. Spermatozoa with fragmented DNA showed a large, patchy halo around chromatin dispersion, while those with unfragmented DNA had no halo.

2.3. Statistical analyses

Statistical data analysis was performed using the statistical program SAS 9.4 (Statistical Analysis Software 2002–2012 by SAS Institute Inc., Cary, USA). Descriptive statistics were performed using SAS modules PROC MEANS, PROC UNIVARIATE and PROC FREQ. Data distribution was tested using the TRANSREG procedure. In cases where the normal distribution of data and homogeneity of variances was disturbed, Box-Cox data transformation was performed. After the analysis, transformed data were returned to their original values and presented as such in tables and graphs. When transforming data, the logarithmic transformation to base 10, followed by the second root and the square, was most often used.

The general linear mixed model (PROC GLIMMIX) was used for the analysis of quantitative indicators. The statistical model included a fixed effect of radiation frequency and group, and animal age as a continuous variable. A constant term with the subject radiation frequency was also included in the model as a random effect. Proportions were analysed using the PROC GLIMMIX module with binomial distribution and logit link function. The structure of the model was similar to the previous model, only the residual deviation was included in the random effect. The mean values were calculated by the method of least squares (LSM - least squares means) using the LSMEANS command and the PDIFF and CL options. To compare mean values, the Tukey-Kramer method of multiple comparisons was used at the level of statistical significance $p < 0.05$. Comparison of differences between groups within radiation frequency was done using the SLICE option. Pearson's or Spearman's correlation coefficient was calculated between different indicators using the CORR module of SAS (PROC CORR). The data are presented in the graphs as mean values the 95 % confidence interval.

3. Results

3.1. Spermatozoa DNA integrity and spermatozoa vitality indicators of boar semen

The results of sperm DNA integrity and spermatozoa vitality indicators are shown in Fig. 2 and Fig. 3. In the figures comparisons between samples exposed to 5G RF-EMR at 700 MHz, 2500 MHz or 3500 MHz and corresponding control samples (non-exposed) as well as between the total of all control and exposed samples regardless of frequency are shown.

In total, all exposed samples (all frequencies) in comparison with all control semen samples had a statistically significantly higher proportion of spermatozoa with DNA damage (6.0 ± 0.9 % *vs* 2.0 ± 0.5 %; $p < 0.01$). The largest increase in the proportion of spermatozoa with DNA damage (Fig. 2, A) was recorded in the test samples exposed at 2500 MHz (9.5 ± 1.8 % *vs* 1.7 ± 0.8 %; $p < 0.01$), while a slightly smaller increase was observed in samples exposed at 3500 MHz (6.8 ± 1.6 % *vs* 2.6 ± 1.0 %; $p < 0.05$). The sum of spermatozoa counts with damaged cell membrane and/or poor mitochondrial potential (Fig. 2, B) did not show a statistically significant difference in any of the exposed groups (all 5G frequencies tested).

An increase in the proportion of spermatozoa with a damaged cell membrane but good mitochondrial potential was observed in test samples exposed at 3500 MHz (16.7 ± 2.3 % *vs* 8.1 ± 1.7 %) (Fig. 3).

4. Discussion

To date, many *in vivo* and *in vitro* studies in humans and animals have shown that RF-EMR exposure can alter homeostasis, endocrine function, reproductive function and foetal development [21,22]. Furthermore, the results of numerous studies have indicated an association between cell phone use and a decrease in the number and motility of spermatozoa, spermatozoa with normal morphology and viability [14,23–25]. In addition, the ROS generated by RF-EMR can have deleterious effects on spermatozoa DNA and other biomolecules such as proteins and lipids, which in turn leads to male infertility [26,27]. One of the first studies was conducted by Diem et al. [28], in which the non-thermal effect of RF-EMR at 1800 MHz with different mobile phone modulations on *in vitro* exposed human diploid fibroblasts and transformed rat granulosa cells (GFSH-R17) was determined. They found that different mobile phone modulations cause single-strand and double-strand DNA breaks in human diploid fibroblasts and in rat granulosa cells in culture.

Since the possible harmful effects of RF-EMR at frequencies above 2500 MHz on humans and animals reproduction have not been investigated so far, in this study semen samples were exposed to a 5G frequency (2500 MHz, 3500 MHz and 700 MHz) to investigate the effects on DNA damage of spermatozoa.

The greatest increase in the proportion of spermatozoa with DNA damage in this study was recorded in the test samples exposed at 2500 MHz. The results corroborate those of Avendaño et al. [8]. They exposed

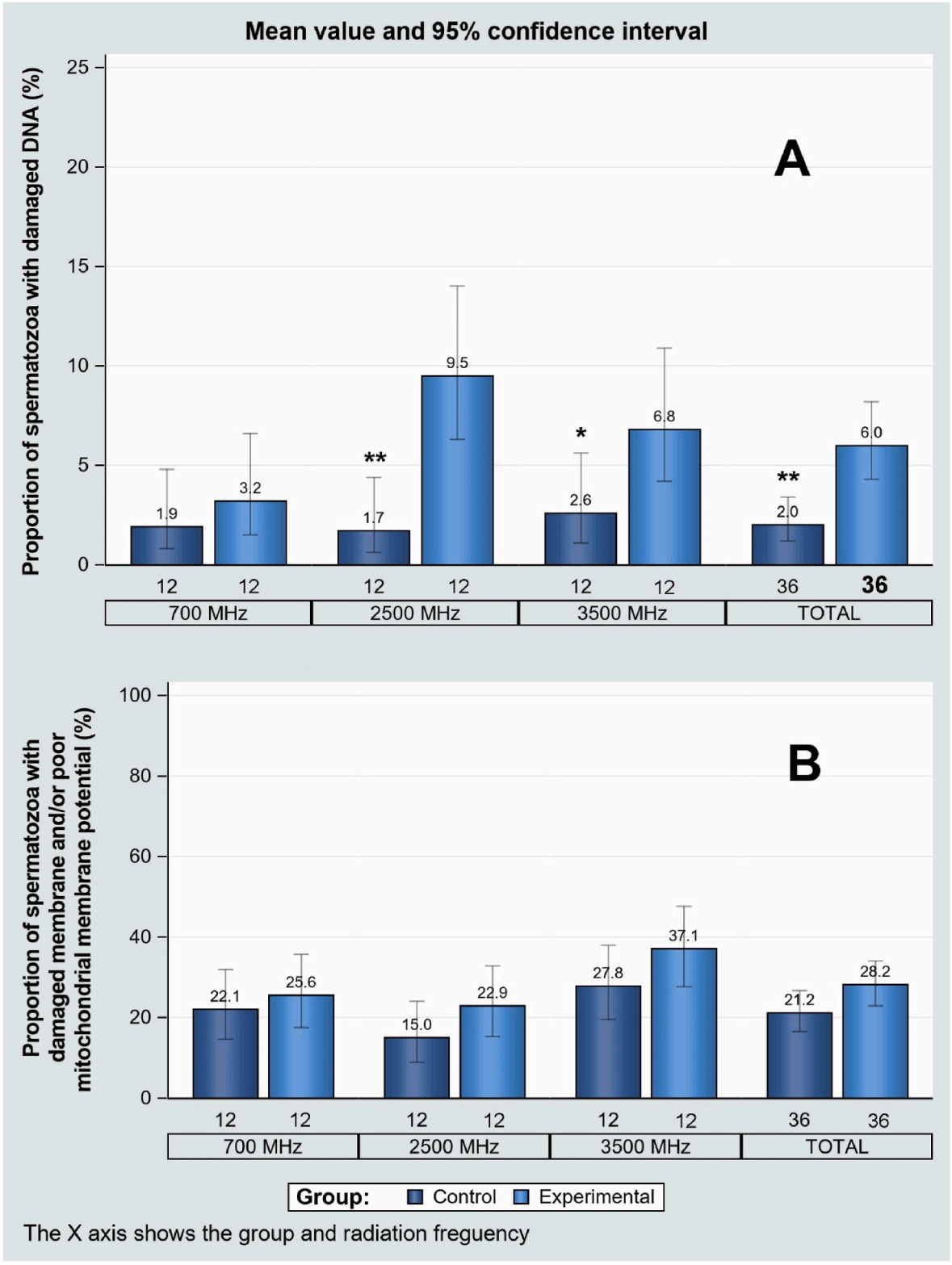


Fig. 2. **A,** Percentage of DNA damaged spermatozoa. **B,** percentage of spermatozoa with damaged membrane and/or poor mitochondrial potential. Mean values and 95 % confidence intervals of three independent experimental groups (in each experimental group 12 semen samples were exposed to continuous RF-EMR at a single frequency of 700 MHz, 2500 MHz and 3500 MHz) and control groups (unexposed samples). The last two columns correspond to the mean values and 95 % confidence intervals of all control or exposed samples (N = 36). Statistically significant differences between the control and experimental group are marked with * $p < 0.05$, ** $p < 0.01$.

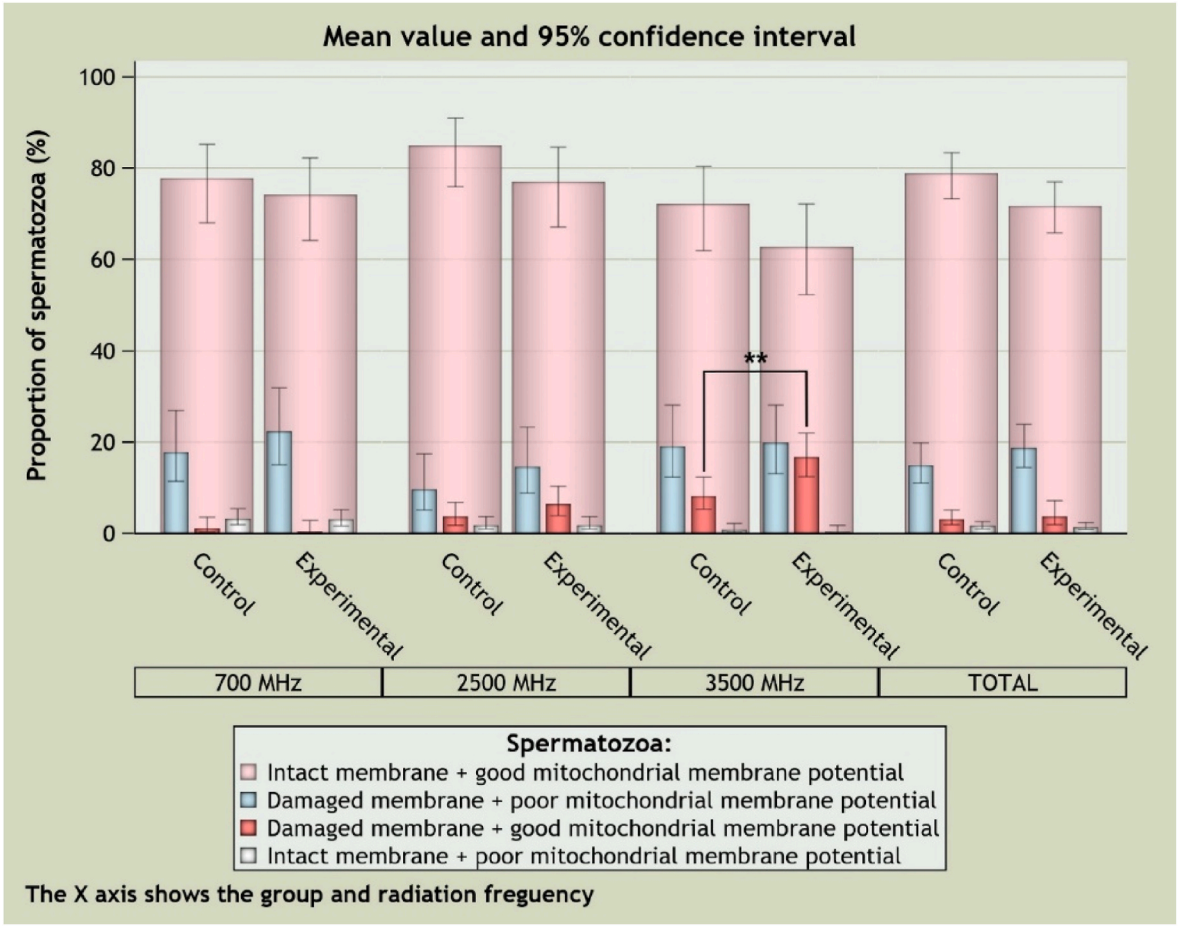


Fig. 3. Percentage of intact or damaged membrane spermatozoa with good or poor mitochondrial potential (mean values and 95 % confidence interval) of three independent experimental groups (in each experimental group 12 semen samples were exposed to continuous RF-EMR at a single frequency of 700 MHz, 2500 MHz and 3500 MHz) and control group (non-exposed samples). The last two columns correspond to the means and 95 % confidence intervals of all the control or exposed samples (N = 36). Asterisks marks the statistically significant difference between control and experimental group (**p < 0.01).

male semen samples to a frequency of 2500 MHz, though for 4 hours, and found significant DNA damage in the exposed spermatozoa. Similar results were reported in *in vitro* studies by De Iuliis et al. [14], Zalata et al. [27] and Gorpinchenko et al. [29] on human male semen samples, and in an *in vivo* study on rats conducted by Kumar et al. [30], which found DNA fragmentation following exposure of semen to RF-EMR with mobile phone and wireless at 2500 MHz. A significant increase in the proportion of spermatozoa with DNA damage was also recorded in experimental group exposed at 3500 MHz compared to control (non-exposed) semen samples. In addition, all exposed semen samples from the experimental groups (regardless of the frequency of exposure) showed a statistically significantly higher proportion of spermatozoa with DNA damage compared to the control semen samples. It is known that RF-EMR can cause DNA damage, alterations in gene expression and changes in cell proliferation. These results showed that frequency affects certain spermatozoa indicators of vitality and DNA integrity, which is consistent with previous findings that the effect of RF-EMR depends on radiation frequency [5]. In addition, Kesari et al. [5] found that the mechanism of action of RF-EMR on spermatozoa is DNA damage leading to abnormal spermatozoa heads and mitochondrial damage, which ultimately leads to apoptosis.On the other hand, the results of a recent study indicate that spermatozoa motility gradually decreases with increasing duration of mobile phone use. However, there were no significant differences in the DNA fragmentation index ($p = 0.579$) [34]. In a review, Shulte et al. [31] report that there is evidence that sperm from infertile men have more DNA damage than sperm from fertile men. It has been hypothesized that exposure to RF-EMR is one of the etiological factors associated with sperm DNA fragmentation [26,27,32].

In the present study, no statistically significant difference was recorded in the proportion of spermatozoa with DNA damage in test semen samples exposed at 700 MHz compared to control (non-exposed) semen samples, which could indicate that 700 MHz is not a frequency that causes significant DNA damage to boar spermatozoa, *i.e.,* oxidative stress when the sample is exposed for 2 hours. The available literature primarily outlines the effects of RF-EMR with a frequency of 2450 MHz on semen samples, and thus these results are difficult to compare and discuss. Wongkasem [33] found that the absorbance in all human organs and tissues is significantly higher as frequency of RF-EMR increases.

It is known that ROS produced as a result of tissue/cell exposure to RF-EMR can cause damage to proteins or ion channels in cell membranes, thus causing cell membrane permeability and, consequently, the reduction and cessation of cell functions [34]. Also, since apoptosis and cell viability are characterised by cell membrane permeability, the reproductive potential of spermatozoa under the influence of oxidative stress can be determined based on membrane permeability [35]. In the present study, despite an increase in the proportion of spermatozoa with a damaged cell membrane and/or weak mitochondrial potential in all test (exposed) samples, the differences to control samples were not statistically significant. Only a significantly higher proportion of spermatozoa with a damaged cell membrane and good mitochondrial potential was found in samples exposed at 3500 MHz. One possible explanation is that different cell organels react differently to different frequencies.

5. Conclusions

It can be concluded that the frequencies of 5G used in this study increase the proportion of spermatozoa with damaged DNA in the *in vitro* exposed sperm of breeding boars. The proportion of spermatozoa with damaged DNA was frequency-dependent. The 3500 MHz frequency showed the most detrimental effects due to significant effects on spermatozoa DNA integrity and vitality indicators. Our results suggest that the effects of 5G wireless communication technologies on sperm quality *in vitro* and *in vivo* need to be further researched, including the different physical properties of RF-EMR in livestock. The results also show that it is necessary to provide and implement protective measures against this type of radiation during the technological process of semen processing in artificial insemination ceters and breeding boar farms.

CRediT authorship contribution statement

Ivan Butković: Investigation, Funding acquisition, Formal analysis. **Silvijo Vince:** Visualization, Supervision, Conceptualization. **Martina Lojkić:** Writing – original draft, Methodology. **Ivan Folnožić:** Writing – original draft, Supervision, Investigation, Conceptualization. **Suzana Milinović Tur:** Supervision, Data curation. **Marinko Vilić:** Writing – original draft, Conceptualization. **Krešimir Malarić:** Supervision, Methodology. **Velimir Berta:** Investigation. **Marko Samardžija:** Writing – original draft, Funding acquisition. **Mario Kreszinger:** Investigation. **Ivona Žura Žaja:** Writing – original draft, Methodology, Conceptualization.

Declaration of competing interest

The authors declare no potential conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2024.09.025.

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