

Moderate Dose of Trolox Preventing the Deleterious Effects of Wi-Fi Radiation on Spermatozoa *In vitro* through Reduction of Oxidative Stress Damage

Shang-Shu Ding¹, Ping Sun¹, Zhou Zhang², Xiang Liu², Hong Tian³, Yong-Wei Huo³, Li-Rong Wang³, Yan Han⁴, Jun-Ping Xing¹

¹Department of Urology, School of Medicine, The First Affiliated Hospital, Xi'an Jiaotong University, Xi'an, Shaanxi 710061, China

²Department of Andrology, Shaanxi Maternal and Child Care Service Center, Xi'an, Shaanxi 710061, China

³Research Center of Reproduction Medicine, School of Medicine, Xi'an Jiaotong University, Xi'an, Shaanxi 710061, China

⁴Department of Biochemistry, Institute of Biochemistry and Molecular Medicine, School of Medicine, Xi'an Jiaotong University, Xi'an, Shaanxi 710061, China

Abstract

Background: The worsening of semen quality, due to the application of Wi-Fi, can be ameliorated by Vitamin E. This study aimed to demonstrate whether a moderate dose of trolox, a new Vitamin E, inhibits oxidative damage on sperms *in vitro* after exposure to Wi-Fi radiation.

Methods: Each of the twenty qualified semen, gathered from June to October 2014 in eugenics clinic, was separated into four aliquots, including sham, Wi-Fi-exposed, Wi-Fi plus 5 mmol/L trolox, and Wi-Fi plus 10 mmol/L trolox groups. At 0 min, all baseline parameters of the 20 samples were measured in sequence. Reactive oxygen species, glutathione, and superoxide dismutase were evaluated in the four aliquots at 45 and 90 min, as were sperm DNA fragments, sperm mitochondrial potential, relative amplification of sperm mitochondrial DNA, sperm vitality, and progressive and immotility sperm. The parameters were analyzed by one-way analysis of variance and Tukey's posttest.

Results: Among Wi-Fi plus 5 mmol/L trolox, Wi-Fi-exposed and Wi-Fi plus 10 mmol/L trolox groups, reactive oxygen species levels (45 min: 3.80 ± 0.41 RLU $\cdot 10^{-6}\cdot ml^{-1}$ vs. 7.50 ± 0.35 RLU $\cdot 10^{-6}\cdot ml^{-1}$ vs. 6.70 ± 0.47 RLU $\cdot 10^{-6}\cdot ml^{-1}$, $P < 0.001$; 90 min: 5.40 ± 0.21 RLU $\cdot 10^{-6}\cdot ml^{-1}$ vs. 10.10 ± 0.31 RLU $\cdot 10^{-6}\cdot ml^{-1}$ vs. 7.00 ± 0.42 RLU $\cdot 10^{-6}\cdot ml^{-1}$, $P < 0.001$, respectively), percentages of tail DNA (45 min: $16.8 \pm 2.0\%$ vs. $31.9 \pm 2.5\%$ vs. $61.3 \pm 1.6\%$, $P < 0.001$; 90 min: $19.7 \pm 1.5\%$ vs. $73.7 \pm 1.3\%$ vs. $73.1 \pm 1.1\%$, $P < 0.001$, respectively), 8-hydroxy-2'-deoxyguanosine (45 min: 51.89 ± 1.46 pg/ml vs. 104.89 ± 2.19 pg/ml vs. 106.11 ± 1.81 pg/ml, $P = 0.012$; 90 min: 79.96 ± 1.73 pg/ml vs. 141.73 ± 2.90 pg/ml vs. 139.06 ± 2.79 pg/ml; $P < 0.001$), and percentages of immotility sperm (45 min: $27.7 \pm 2.7\%$ vs. $41.7 \pm 2.2\%$ vs. $41.7 \pm 2.5\%$; 90 min: $29.9 \pm 3.3\%$ vs. $58.9 \pm 4.0\%$ vs. $63.1 \pm 4.0\%$; all $P < 0.001$) were lowest, and glutathione peroxidase (45 min: 60.50 ± 1.54 U/ml vs. 37.09 ± 1.77 U/ml vs. 28.18 ± 1.06 U/ml; 90 min: 44.61 ± 1.23 U/ml vs. 16.86 ± 0.93 U/ml vs. 29.94 ± 1.56 U/ml; all $P < 0.001$), percentages of head DNA (45 min: $83.2 \pm 2.0\%$ vs. $68.2 \pm 2.5\%$ vs. $38.8 \pm 1.6\%$; 90 min: $80.3 \pm 1.5\%$ vs. $26.3 \pm 1.3\%$ vs. $26.9 \pm 1.1\%$; all $P < 0.001$), percentages of sperm vitality (45 min: $89.5 \pm 1.6\%$ vs. $70.7 \pm 3.1\%$ vs. $57.7 \pm 2.4\%$; 90 min: $80.8 \pm 2.2\%$ vs. $40.4 \pm 4.0\%$ vs. $34.7 \pm 3.9\%$; all $P < 0.001$), and progressive sperm (45 min: $69.3 \pm 2.7\%$ vs. $55.8 \pm 2.2\%$ vs. $55.4 \pm 2.5\%$; 90 min: $67.2 \pm 3.3\%$ vs. $38.2 \pm 4.0\%$ vs. $33.9 \pm 4.0\%$; all $P < 0.001$) were highest in Wi-Fi plus 5 mmol/L trolox group at 45 and 90 min, respectively. Other parameters were not affected, while the sham group maintained the baseline.

Conclusion: This study found that 5 mmol/L trolox protected the Wi-Fi-exposed semen *in vitro* from the damage of electromagnetic radiation-induced oxidative stress.

Key words: Electromagnetic Radiation; Oxidative Stress Damage; Trolox

INTRODUCTION

Wi-Fi electromagnetic radiation at 2.45 GHz is in widespread use worldwide.^[1] Studies on electromagnetic radiation to the male reproductive system have focused on spermatogenesis,^[2] testosterone,^[3] and reactive oxygen species (ROS) in semen.^[4] The complex defense system against electromagnetic radiation on the adverse effects of radiation-induced ROS

Address for correspondence: Dr. Jun-Ping Xing,

Department of Urology, School of Medicine, The First Affiliated Hospital, Xi'an Jiaotong University, No. 277 Yanta West Road, Xi'an, Shaanxi 710061, China

E-Mail: xingjpsx@163.com

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includes enzymatic and nonenzymatic antioxidants, such as Vitamin C,^[5] Vitamin E,^[6] superoxide dismutase (SOD), and glutathione peroxidase (GSH-P_x).^[7] Mailankot *et al.*,^[8] Atasoy *et al.*,^[9] and Esmekaya *et al.*^[10] reported that electromagnetic radiation induced oxidative injury through increasing nitric oxide levels and inhibiting antioxidant defense mechanisms. Many studies on the male reproductive system have demonstrated that electromagnetic radiation had a deleterious effect on sperm count, vitality, motility, and morphology in humans and animals *in vivo*.^[11–15] Moreover, human semen exposed to electromagnetic radiation from mobile phones or Wi-Fi *in vitro* demonstrated oxidative stress, apoptosis, and loss of vitality and motility.^[13,16,17]

The 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) is a water-soluble derivative of Vitamin E and has been considered for antioxidant therapy in the presence of excess ROS in myocardial injury and diabetic retinopathy.^[18–20] In cultured bursal cell and neuronal studies, trolox protected against cell death resulting from oxidative stress.^[21] Trolox has an antioxidant role in reducing apoptosis in mouse thymocytes, rabbit myocytes, and anterior pituitary cells.^[22,23]

Male factors account for half of the infertility in reproductive-age couples, i.e., over 70 million families in developed countries.^[24] It is concerning that semen quality has declined in the past few decades. Electromagnetic radiation from Wi-Fi radiation impairs semen through generation of excess ROS. Experts in male infertility advise the use of antioxidants such as Vitamin C and Vitamin E to protect against the pathological mechanisms of oxidative stress. A significant randomized controlled trial is needed before men can be advised to take Vitamin E to protect against harm from Wi-Fi exposure, as evidence from *in vitro* trials is insufficient. However, some studies have reported a protective role of Vitamin E in the freezing of sperm.^[25]

This study aimed to demonstrate that trolox can act as a protective ROS scavenger for semen exposed to 2.45 GHz Wi-Fi radiation *in vitro*. We hypothesized that increase in 8-hydroxy-2'-deoxyguanosine (8-OHdG) level, DNA fragmentation in spermatozoa, changes in sperm mitochondrial membrane potential, and mitochondrial DNA damage should be preventable to some extent. The improvement in sperm quality might result in improvement in male fertility.

METHODS

Ethical approval

The research protocol was submitted to the Ethics Department of Xi'an Jiaotong University and strictly adhered to ethical regulations and rules. The study was conducted in accordance with the *Declaration of Helsinki* and was approved by the Chinese Ethics Committee for Registration of Clinical Trials (ChiCTR-OCH-14004802). Informed written consent was obtained from all participants prior to their enrolment in this study.

Wireless fidelity device and electromagnetic exposure field evaluation

Under room temperature conditions at 25°C (IVF Thermometer, USA), a smart phone was set to connect to the internet wirelessly, which means the uploading and downloading of data (Wi-Fi frequency, 2.45 GHz, as defined by the Institute of Electrical and Electronics Engineers 802.11b protocol).^[26] The phone was set to an exposure radius calibrated to 1.5 m. The electromagnetic field density was adjusted to achieve a 1.0–2.5 W/kg whole-body average specific absorption rate, and the total exposure was measured using a portable radiofrequency survey system (Lenovo B41, China). A laptop computer was placed 3–5 cm above the exposed samples.

Collection and treatment of semen samples

After obtaining approval, semen samples were collected from twenty volunteers attending the eugenics clinic at the First Affiliated Hospital, Xi'an Jiaotong University and Shaanxi Maternal and Child Care Service Center from June 2014 to October 2014. Samples were collected in a sterile container by masturbation after abstinence of 3–4 days. The normal pH semen samples, which contained over 60% progressive sperm, over 80% sperm viability, and over $60 \times 10^6/\text{ml}$ sperm concentration, were elected. Specimens with an evidence of infection (white blood cells in semen more than 5/high-power field) and a semen volume <4 ml were excluded. The qualified individuals involved were selected by the criteria mentioned above. The main demographic data of the volunteers who enrolled in this study embraced the age (23–33 years), body mass index (19.9–25.3 kg/m²), the educational years (12–22 years), and income per month (RMB 3150–3910 Yuan/month). Besides, the fertility history of them was gathered as well. Ten of them had no child in 1-year marriage and four of them had no child over 1 year, while six of them had one child.

Liquefied semen from each sample was diluted in the ratio of 1:1 with semen culture medium (Ham's F10; Biowest, France) and human serum albumin (State medical permit No. 10820115, China) and centrifuged at 300 ×g for 5 min. The supernatant was removed and the pellet was carefully suspended with 6 ml semen culture medium and incubated at 37°C. After 30 min, the top 5 ml of the supernatant, which had many motile sperms, was prepared for testing. The sperm concentration of each aliquot was adjusted to $10 \times 10^6/\text{ml}$ with semen culture medium. At 0 min, all baseline parameters of the twenty samples were measured in sequence (the total volume was about 500 μl). The remainder of each sample was equally divided into four aliquots. Aliquot A (sham group) was added to a vehicle (2 ml of 1.0 mol/L phosphate-buffered saline [PBS]) and set in a nonelectromagnetic field. Aliquot B (Wi-Fi-exposed group) was exposed to 2.45 GHz Wi-Fi electromagnetic radiation and mixed with 2 ml of 1.0 mol/L PBS. Aliquot C (Wi-Fi plus 5 mmol/L trolox group) and aliquot D (Wi-Fi plus 10 mmol/L trolox group) were treated with 2 ml of 5 mmol/L or 10 mmol/L trolox (Sigma Chemical Co., USA), respectively,

and exposed to the condition of 2.45 GHz Wireless fidelity device. The same parameters of these four aliquots were measured at 45 and 90 min, respectively.

Semen reactive oxygen species, total glutathione peroxidase, and superoxide dismutase measurements

At 0, 45, and 90 min, 200 μl of each fraction was mixed with 5 μl of 5 mmol/L luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) and dissolved in dimethyl sulfoxide (Sigma), and the level of ROS in each sample was measured using an FLx800TM analyzer (Biotek, USA). In 15 min, the results were collected as $\text{RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$. The SOD and GSH-Px levels in each fraction were measured using test kits (Cat. No. A001-3; Jiancheng, Nanjing, China).

8-Hydroxy-2'-deoxyguanosine measurements

Approximately 150 μl of each fraction was placed in an Eppendorf tube. A kit (QIAGEN, Germany) was used to extract the total DNA in spermatozoa and then quantified using a nanodrop spectrophotometer (Thermo 2000, Thermo Fisher Scientific, USA). The concentrated DNA in each aliquot was suspended in double-distilled water to 1–3 $\mu\text{g}/\text{L}$ in a volume of 100 μl working solution. Single-stranded DNA was obtained by incubation at 95°C for 5 min. Then, 1 ml of 30 mmol/L sodium acetate solution (pH 5.2) and 1 μl of 5 $\mu\text{g}/\text{L}$ nuclease P1 solution were mixed with the working solution. The mixed solution was incubated at 37°C for 30 min. After adding 1 ml of 1.39 U/ml alkaline phosphatase, the prepared DNA specimen was obtained after incubation for 1 h at 37°C. The amount of 8-OHdG in the DNA specimen was detected using an enzyme-linked immunosorbent assay kit (Cell Biolabs, Inc., San Diego, CA, USA).

Comet assays

Frosted glass slides were precoated with 200 μl 0.5% (w/v) normal-melting-point agarose (Biowest, Spain) in PBS under coverslips for 15 min to allow solidification. The concentrations of samples were adjusted to $6 \times 10^6/\text{ml}$ using PBS, and the coverslips were removed. Then, 10- μl adjusted samples were placed into Eppendorf tubes with 75 μl 0.5% (w/v) low-melting-point agarose (Biowest), and then pipetted onto the top of the normal-melting-point agarose gel. The coverslips were placed again and removed 15 min later, and the slides were immersed in fresh 4°C lysis solution containing 2.5 mol/L sodium chloride, 100 mmol/L sodium ethylenediaminetetraacetic acid (EDTA; Sigma), 10 mmol/L Tris-HCL (Sigma), and 1% TritonX-100 (pH 10.0; Sigma) for 60 min at 4°C. The slides were taken out and returned to the incubation solution with 1.25 ml dithiothreitol (Sigma) for 30 min at 4°C. Then, the slides were removed, drained, and placed in a horizontal electrophoresis tank with fresh alkaline electrophoresis solution consisting of 0.3 mol/L sodium hydroxide and 1 mmol/L EDTA (Sigma), with a 300-mA current, and then flooded with neutralization buffer (0.4 mol/L Tris; Sigma) for 5 min. Subsequently, the drained slides were placed in 50 μl of 200 $\mu\text{g}/\text{ml}$ ethidium bromide solution (Sigma) for 2 h and coverslips were placed.

The images on the slides were captured using a $\times 40$ Olympus fluorescence microscope equipped with a 515–560 excitation filter and 50 comets per slide were analyzed using Comet Assay Software Project (CASP) software (CASP 6.0, University of Wroław, Poland).

Sperm vitality and motility measurements

At 0, 45, and 90 min, 5–10 μl of each sample was dripped onto a slide, and indices of viability, progressive sperm motility percentage, and sperm immotility percentage of each aliquot were analyzed with a QingHuaTongFang computer-assisted sperm analysis system (CASAS-QH-III GK-9900). The entire procedure followed the World Health Organization guidelines, 5th edition. All assays were performed by the same laboratory technicians.

Sperm mitochondrial potential tests

A 5–10 μl sample of each fraction was diluted in 1–2 $\times 10^6/\text{ml}$ PBS and 3 mmol/L JC-1 (Beyotime, China). Semen samples which were treated with 100 μl of 8 mol/L H_2O_2 were considered positive samples and the 0-min fraction were considered negative ones. All specimens were placed in 0.5-ml Eppendorf tubes and incubated with JC-1 at 37°C for 20 min (protected from light). The Eppendorf tubes were loaded in a cytometer (Guava easyCyte™; 8HT, USA) and analyzed for fluorescence (FL1-H: green; FL2-H: orange or red). The measurements were replicated three times. The data for FL2-H (+)/FL1-H (–), which represent intact mitochondrial membranes, were collected and compared.

Measurement of mitochondrial DNA injuries

The total DNA in about 150 μl of each aliquot was extracted using a DNA kit (QIAGEN) and quantified using a nanodrop spectrophotometer (Thermo Fisher Scientific). For detection of cytochrome c oxidase II (CO_2), template DNA was diluted to 1 ng/ μl and divided into two aliquots; one served as the original template and the other was preheated at 95°C for 6 min and cooled down at 10°C. The 5-ng/ μl template DNA was used to quantify a nuclear marker (β -actin). A 5-point standard ranging from 0.064 ng/ μl to 40.000 ng/ μl was set using the sample at 0-min in order to verify the efficiency of CO_2 , with preheating of CO_2 and β -actin. The primers for CO_2 (forward: 5'-CCCCACATTAGGCTTAAAAACAGAT-3'; reverse: 5'-TATACCCCGGTTCGTGTAGCGGT-3') and β -actin (forward: 5'-TCACCCACACTGTGCCCA TCTACGA-3'; reverse: 5'-CAGCGGAACCGCTCATTG CCAATGG-3') were diluted to 10 $\mu\text{mol}/\text{L}$ for the working solution. In each reaction tube, 15 $\mu\text{l} \times 2$ of IQ™ SYBR Green Supermix (BIO-RAD, USA), 1 ml of 10 $\mu\text{mol}/\text{L}$ forward primer, 1 ml of 10 $\mu\text{mol}/\text{L}$ reverse primer, and the prepared DNA templates were mixed well. For the Bio-Rad MyIQ™ Single-Color Real-Time Polymerase Chain Reaction (PCR) Detection System with iCycler™ thermocycler base (iQ5 Real-Time PCR; Bio-Rad, USA), the PCR program was set as follows: cycle 1 (1 \times), 95°C for 1.5 min; cycle 2 (30 \times), step 1 at 95°C for 20 s, step 2 at 61°C for 30 s; cycle 3 (1 \times), 95°C for 1 min; cycle 4 (1 \times), 55°C

for 1 min; and cycle 5 (40×), 55°C for 10 s, with an increase of 1.0°C after each repeat for collection of melt-curve data for the expression of CO₂ and β-actin (real-time data collection at cycle 2, step 2). The data were analyzed with the Relative Expression Software Tool (REST software REST 2009, <http://www.REST.de.com>, QIAGEN and Technical University, Munich, Germany).^[26]

Statistical analysis

Statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). All data were shown as mean ± standard deviation (SD). The parameters were analyzed by the analysis of variance (ANOVA) and Tukey's posttest among various groups. A *P* < 0.05 was considered statistically significant.

RESULTS

Results for reactive oxygen species, glutathione peroxidase, and superoxide dismutase

There were no significant differences in ROS at 0, 45, and 90 min between the sham and Wi-Fi plus 5 mmol/L trolox groups. In the Wi-Fi-exposed group, the levels of ROS were $3.70 \pm 0.42 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$ at 0 min, $7.50 \pm 0.35 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$ at 45 min, and $10.10 \pm 0.31 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$

at 90 min, which increased significantly along with longer exposure time ($F = 79.264, P < 0.001$). In the Wi-Fi plus 10 mmol/L trolox group, the ROS levels at 45 min ($6.70 \pm 0.47 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$) and 90 min ($7.00 \pm 0.42 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$) increased significantly, compared with the ROS level at 0 min ($3.70 \pm 0.42 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$, all $P < 0.001$). The Wi-Fi plus 5 mmol/L trolox group showed significantly reduced ROS levels at 45 min ($3.80 \pm 0.41 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$ vs. $7.50 \pm 0.35 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$, $F = 16.208, P < 0.001$) and 90 min ($5.40 \pm 0.21 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$ vs. $10.10 \pm 0.31 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$ vs. $7.00 \pm 0.42 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$, $F = 54.920, P < 0.001$), compared with the Wi-Fi-exposed group and Wi-Fi plus 10 mmol/L trolox group [Figure 1a and 1b].

In the sham group, the differences in GSH-Px among 0, 45, and 90 min were not significant. In the Wi-Fi-exposed group, the ANOVA revealed that the GSH-Px levels decreased significantly at 0, 45, and 90 min ($51.47 \pm 0.65 \text{ U/ml}$ vs. $37.09 \pm 1.77 \text{ U/ml}$ vs. $16.86 \pm 0.93 \text{ U/ml}$, $F = 205.520, P < 0.001$). In the Wi-Fi plus 5 mmol/L trolox group, the ANOVA showed that the GSH-Px levels significantly differed at three time points ($51.47 \pm 0.65 \text{ U/ml}$ vs. $60.50 \pm 1.54 \text{ U/ml}$ vs. $44.61 \pm 1.23 \text{ U/ml}$, $F = 44.070, P < 0.001$). However, in the Wi-Fi plus 10 mmol/L trolox group, the ANOVA revealed that

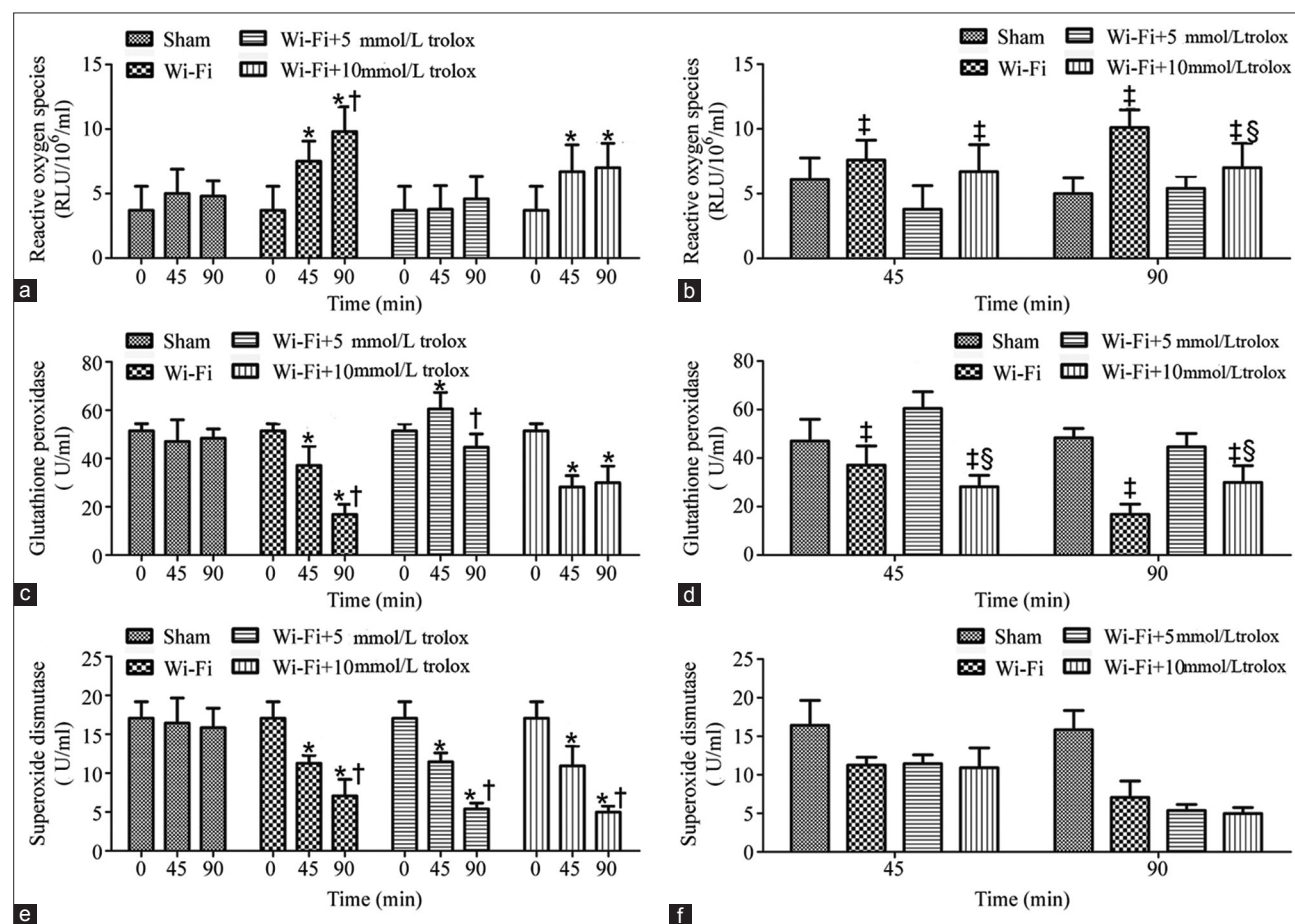


Figure 1: Assay of reactive oxygen species (a and b), glutathione peroxidase (c and d), and superoxide dismutase (e and f) at the time points of 0, 45, and 90 min in four groups. **P* < 0.05 vs. 0 min in each group. †*P* < 0.05 vs. 45 min in each group. ‡*P* < 0.05 vs. Wi-Fi plus 5 mmol/L trolox group at 45 or 90 min. §*P* < 0.05 vs. Wi-Fi-exposed group at 45 or 90 min.

the GSH-Px levels decreased significantly at 45 min (28.18 ± 1.06 U/ml) and 90 min (29.94 ± 1.56 U/ml), compared with 0 min (51.47 ± 0.65 U/ml, all $P < 0.001$). At both 45 and 90 min, the GSH-Px levels in the Wi-Fi plus 5 mmol/L trolox group were highest (45 min: 60.50 ± 1.54 U/ml vs. 37.09 ± 1.77 U/ml vs. 28.18 ± 1.06 U/ml, $F = 71.887$, $P < 0.001$; 90 min: 44.61 ± 1.23 U/ml vs. 16.86 ± 0.93 U/ml vs. 29.94 ± 1.56 U/ml, $F = 150.551$, $P < 0.001$, respectively) among Wi-Fi-exposed and Wi-Fi plus 5 mmol/L and 10 mmol/L trolox groups [Figure 1c and 1d].

The SOD levels were similar at 0, 45, and 90 min in the sham group. When the exposure time increased, the SOD levels decreased significantly in the Wi-Fi-exposed group (17.05 ± 0.48 U/ml vs. 11.25 ± 0.23 U/ml vs. 7.07 ± 0.48 U/ml, $F = 148.567$, $P < 0.001$), Wi-Fi plus 5 mmol/L trolox group (17.05 ± 0.48 U/ml vs. 11.45 ± 0.25 U/ml vs. 5.38 ± 0.17 U/ml, $F = 317.777$, $P < 0.001$), and Wi-Fi plus 10 mmol/L trolox group (17.05 ± 0.48 U/ml vs. 10.92 ± 0.57 U/ml vs. 4.99 ± 0.17 U/ml, $F = 186.750$, $P < 0.001$) at the three time points. At 45 or 90 min, the SOD levels did not show significant differences among the Wi-Fi-exposed, Wi-Fi plus 5 mmol/L trolox, and Wi-Fi plus 10 mmol/L trolox groups [Figure 1e and 1f].

Comet Assay Software Project analyses and the levels of 8-hydroxy-2'-deoxyguanosine

The images of comet assays of spermatozoa in four groups at 0, 45 and 90 min were shown in Figure 2a. Along with the prolonged time, the numbers of spermatozoa DNA with longer tail increased obviously in Wi-Fi-exposed, Wi-Fi plus 5 mmol/L and Wi-Fi plus 10 mmol/L trolox groups, except for sham group. At 45 and 90 min, the expression of comet assays of the spermatozoa DNA in Wi-Fi plus 5 mmol/L trolox group showed less long-tailed DNA than the other three groups. The percentages of head DNA and tail DNA and olive tail moment were used in the CASP analyses. The percentages of head DNA showed a significant decreasing tendency at 0, 45, and 90 min in the Wi-Fi-exposed ($86.2 \pm 0.8\%$ vs. $68.2 \pm 2.5\%$ vs. $26.3 \pm 1.3\%$, $F = 335.229$, $P < 0.001$) and Wi-Fi plus 10 mmol/L groups ($86.2 \pm 0.8\%$ vs. $38.8 \pm 1.6\%$ vs. $26.9 \pm 1.1\%$, $F = 675.348$, $P < 0.001$; Figure 2b), but the percentages of tail DNA showed a significant increasing tendency in the Wi-Fi-exposed and Wi-Fi plus 10 mmol/L groups ($13.8 \pm 0.8\%$ vs. $31.9 \pm 2.5\%$ vs. $73.7 \pm 1.3\%$, $F = 335.229$, $P < 0.001$; $13.8 \pm 0.8\%$ vs. $61.3 \pm 1.6\%$ vs. $73.1 \pm 1.1\%$, $F = 675.348$, $P < 0.001$, respectively; Figure 2d). There were no differences in the percentages of head DNA and tail DNA among three time points in the sham and Wi-Fi plus 5 mmol/L groups (all $P > 0.05$). Moreover, among the Wi-Fi plus 5 mmol/L trolox, Wi-Fi-exposed, and Wi-Fi plus 10 mmol/L trolox groups, the percentage of head DNA was highest (45 min: $83.2 \pm 2.0\%$ vs. $68.2 \pm 2.5\%$ vs. $38.8 \pm 1.6\%$, $F = 118.488$, $P < 0.001$; 90 min: $80.3 \pm 1.5\%$ vs. $26.3 \pm 1.3\%$ vs. $26.9 \pm 1.1\%$, $F = 479.075$, $P < 0.001$, respectively), but the percentage of tail DNA was lowest (45 min: $16.8 \pm 2.0\%$ vs. $31.9 \pm 2.5\%$ vs. $61.3 \pm 1.6\%$, $F = 118.488$, $P < 0.001$; 90 min:

$19.7 \pm 1.5\%$ vs. $73.7 \pm 1.3\%$ vs. $73.1 \pm 1.1\%$, $F = 479.075$, $P < 0.001$, respectively) at 45 and 90 min in the Wi-Fi plus 5 mmol/L trolox group [Figure 2c and 2e]. In Wi-Fi-exposed group, the olive tail moments uplifted significantly at 0, 45 and 90 min (27.57 ± 1.95 vs. 34.04 ± 0.74 vs. 77.04 ± 1.15 , $F = 382.701$, $P < 0.001$). In Wi-Fi plus 10 mmol/L trolox group, the olive tail moments manifested highest at 90 min (27.57 ± 1.95 vs. 26.78 ± 1.80 vs. 76.41 ± 0.99 , $F = 303.080$, $P < 0.001$; Figure 2f). At 90 min, the ANOVA of the olive tail moments showed the significant difference (77.04 ± 1.15 vs. 34.04 ± 0.74 vs. 76.41 ± 0.99 , $F = 266.987$, $P < 0.001$) among the Wi-Fi-exposed, Wi-Fi plus 5 mmol/L, and Wi-Fi plus 10 mmol/L trolox samples [Figure 2g].

The amounts of 8-OHdG increased significantly at 0, 45, and 90 min in the Wi-Fi-exposed (50.17 ± 1.44 pg/ml vs. 104.89 ± 2.19 pg/ml vs. 141.72 ± 2.90 pg/ml, $F = 416.740$, $P < 0.001$) and Wi-Fi plus 10 mmol/L trolox groups (50.17 ± 1.44 pg/ml vs. 106.11 ± 1.81 pg/ml vs. 139.06 ± 2.79 pg/ml, $F = 460.510$, $P < 0.001$). In Wi-Fi plus 5 mmol/L trolox group, the amount of 8-OHdG at 90 min (79.96 ± 1.73 pg/ml) increased significantly, compared with those of 0 and 45 min (50.17 ± 1.44 pg/ml and 51.89 ± 1.46 pg/ml, respectively, all $P < 0.001$). The amounts of 8-OHdG in the Wi-Fi plus 5 mmol/L group was lowest at 45 min ($F = 3.919$; $P = 0.012$) and 90 min ($F = 399.614$; $P < 0.001$) among the Wi-Fi, Wi-Fi plus 5 mmol/L, and Wi-Fi plus 10 mmol/L trolox groups [Figure 2h and 2i].

Sperm mitochondrial potential tests and mitochondrial DNA injuries

The sperm particles were isolated with flow cytometry [Figure 3a]. The FL2-H (+)/FL1-H (-) rate (the upper left quadrant) was considered as the result of quality sperm mitochondrial in the study. The planar scatter plot results in the positive (H_2O_2 treated) group presented the lowest rate of FL2-H (+)/FL1-H (-) and highest FL2-H (-)/FL1-H (+) rate (the lower right quadrant; Figure 3b). The scatter images of group in sham, Wi-Fi-exposed, Wi-Fi plus 5 mmol/L trolox, and Wi-Fi plus 10 mmol/L trolox groups at 0, 45, and 90 min are shown in Figure 3c. The proportion of the upper left quadrant cells in sham group did not show the evident changes at the three time points. However, compared with the proportion of the upper left quadrant cells at 0 min, it revealed the obvious declines in Wi-Fi-exposed, Wi-Fi plus 5 mmol/L, and Wi-Fi plus 10 mmol/L trolox groups at 45 and 90 min. Besides, in addition with trolox, the cell distribution in the upper left quadrant expressed the low level without significant differences among Wi-Fi-exposed, Wi-Fi plus 5 mmol/L, and Wi-Fi plus 10 mmol/L trolox groups at 45 and 90 min.

Except for sham group, it showed that the rates of FL2-H (+)/FL1-H (-) decreased significantly in Wi-Fi-exposed ($38.3 \pm 1.3\%$ vs. $29.0 \pm 1.4\%$ vs. $18.6 \pm 0.5\%$, $F = 75.000$, $P < 0.001$), Wi-Fi plus 5 mmol/L ($38.3 \pm 1.3\%$ vs. $28.5 \pm 1.2\%$ vs. $18.3 \pm 0.4\%$, $F = 92.541$, $P < 0.001$), and Wi-Fi plus 10 mmol/L trolox groups ($38.3 \pm 1.3\%$ vs.

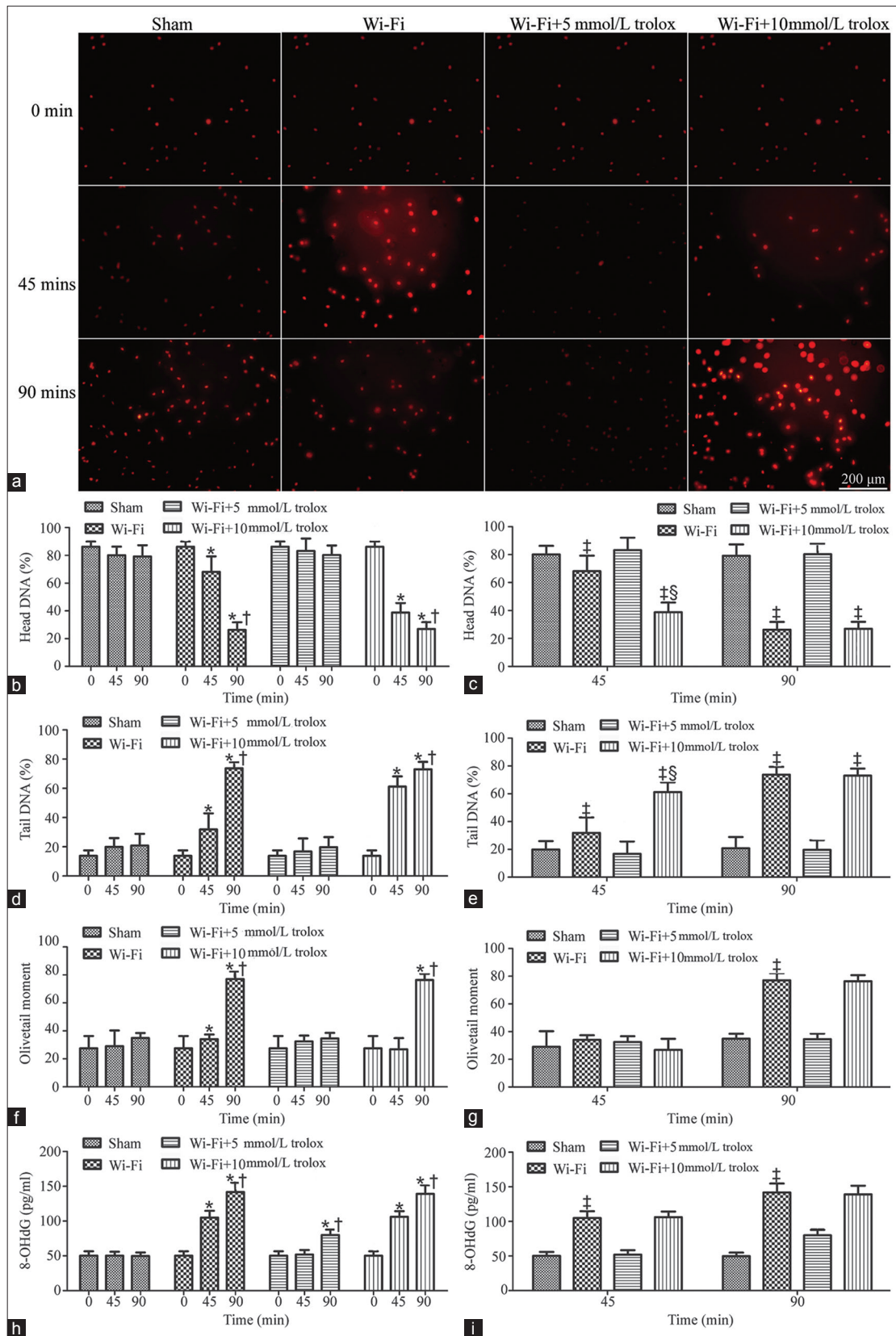


Figure 2: CASP analyses and the amounts of 8-OHdG at the time points of 0, 45, and 90 min in four aliquots. (a) Images under green color fluorescence (515–560 excitation filters; original magnification, $\times 200$) at the time points of 0, 45, and 90 min in sham, Wi-Fi, Wi-Fi plus 5 mmol/L trolox, and Wi-Fi plus 10 mmol/L trolox groups. Head DNA (%), tail DNA (%), olive tail moment, and the amounts of 8-OHdG (h and i) at the time points of 0, 45, and 90 min in sham, Wi-Fi-exposed, Wi-Fi plus 5 mmol/L trolox, and Wi-Fi plus 10 mmol/L trolox groups. * $P < 0.05$ versus 0 min in each group. † $P < 0.05$ versus 45 min in each group. ‡ $P < 0.05$ versus Wi-Fi plus 5 mmol/L trolox group at 45 or 90 min. § $P < 0.05$ versus Wi-Fi-exposed group at 45 or 90 min. CASP: Comet Assay Software Project; 8-OHdG: 8-Hydroxy-2'-deoxyguanosine.

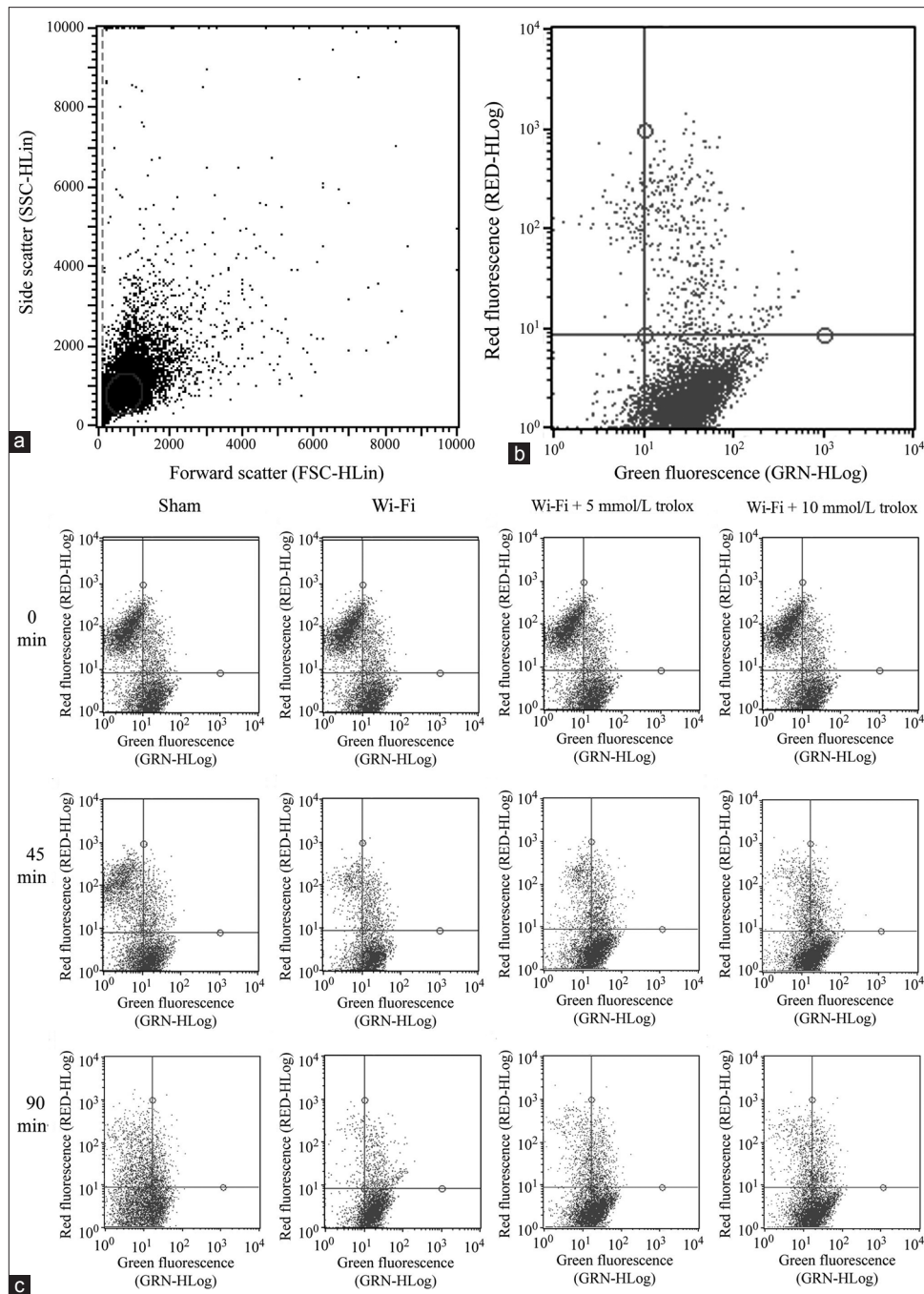


Figure 3: The sperm mitochondrial potential tests at the time points of 0, 45, and 90 min in four aliquots. (a) The image of the option of the particles of sperm concerned. (b) The planar scatter plot of flow cytometry results of H_2O_2 -induced group. (c) The planar scatter plot of flow cytometry results of 0, 45, and 90 min in sham, Wi-Fi exposed, Wi-Fi plus 5 mmol/L trolox, and Wi-Fi plus 10 mmol/L trolox groups.

$28.7 \pm 1.2\%$ vs. $18.2 \pm 0.5\%$, $F = 96.293$, $P < 0.001$) along with longer exposure time [Figure 4a and 4b]. The relative amplification of preheat sperm mitochondrial DNA, which was always higher than nontreatment ones, was considered the symbol of the damage of mitochondrial DNA. While the more damages happened to the sperm mitochondrial DNA, the relative amplifications of sperm mitochondrial CO_2 DNA showed higher values. The relative amplification of mitochondrial CO_2 DNA increased significantly in Wi-Fi exposed (2.68 ± 0.16 vs. 3.19 ± 0.18 vs. 4.03 ± 0.10 , $F = 20.080$, $P < 0.001$), Wi-Fi plus 5 mmol/L (2.68 ± 0.16 vs.

3.50 ± 0.21 vs. 4.00 ± 0.14 , $F = 14.043$, $P < 0.001$), and Wi-Fi plus 10 mmol/L trolox groups (2.68 ± 0.16 vs. 3.56 ± 0.19 vs. 4.24 ± 0.14 , $F = 22.359$, $P < 0.001$) at the three time points. At 45 and 90 min, there was no significant difference in sperm mitochondrial potential and relative amplification of mitochondrial CO_2 DNA among Wi-Fi-exposed, Wi-Fi plus 5 mmol/L, and Wi-Fi plus 10 mmol/L trolox groups [Figure 4c–4e].

Sperm vitality and motility measurements

Along with the increased exposed time (0, 45, and 90 min),

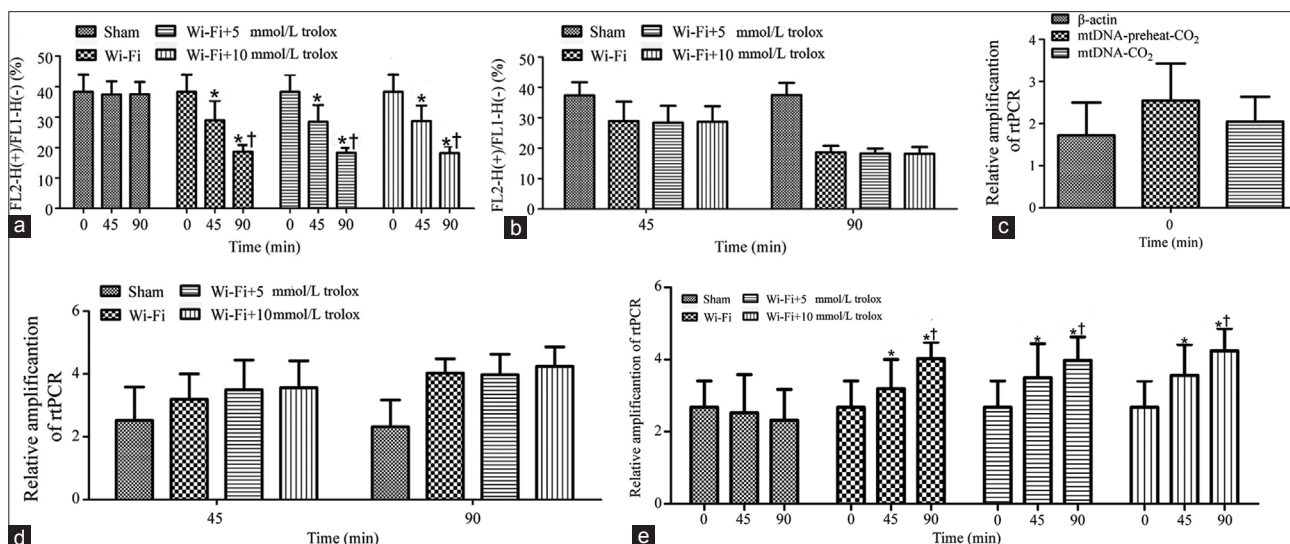


Figure 4: The comparison of the proportion of FL2-H (+)/FL1-H (-) and the relative amplification of mitochondrial DNA-CO₂ at the time points of 0, 45, and 90 min in four aliquots. (a and b) The proportion of FL2-H (+)/FL1-H (-) at the time points of 0, 45, and 90 min in sham, Wi-Fi, Wi-Fi plus 5 mmol/L trolox, and Wi-Fi plus 10 mmol/L trolox groups. (c-e) Relative amplification of sperm mitochondrial DNA-CO₂ (cytochrome c oxidase II) at the time points of 0, 45, and 90 min in sham, Wi-Fi, Wi-Fi plus 5 mmol/L trolox, and Wi-Fi plus 10 mmol/L trolox groups. **P* < 0.05 versus 0 min in each group. †*P* < 0.05 vs. 45 min in each group.

the percentages of sperm vitality reduced significantly in Wi-Fi exposed ($86.4 \pm 2.1\%$ vs. $70.7 \pm 3.1\%$ vs. $40.4 \pm 4.0\%$, $F = 55.242$, $P < 0.001$) and Wi-Fi plus 10 mmol/L trolox groups ($86.4 \pm 2.1\%$ vs. $57.7 \pm 2.4\%$ vs. $34.7 \pm 3.9\%$, $F = 79.132$, $P < 0.001$). As same as the vitality, the percentages of progressive sperm decreased significantly in Wi-Fi exposed ($68.8 \pm 4.0\%$ vs. $55.8 \pm 2.2\%$ vs. $38.2 \pm 4.0\%$, $F = 19.415$, $P < 0.001$) and Wi-Fi plus 10 mmol/L trolox groups ($68.8 \pm 4.0\%$ vs. $55.4 \pm 2.5\%$ vs. $33.9 \pm 4.0\%$, $F = 24.512$, $P < 0.001$) at the three time points. On the contrary, the percentages of immotility sperm increased dramatically in the Wi-Fi-exposed ($28.2 \pm 4.0\%$ vs. $41.7 \pm 2.2\%$ vs. $58.9 \pm 4.0\%$, $F = 19.371$, $P < 0.001$) and Wi-Fi plus 10 mmol/L trolox groups ($28.2 \pm 4.0\%$ vs. $41.7 \pm 2.5\%$ vs. $63.1 \pm 4.0\%$, $F = 24.512$, $P < 0.001$) at the three time points.

Among the Wi-Fi plus 5 mmol/L trolox, Wi-Fi-exposed, and Wi-Fi plus 10 mmol/L trolox groups, at 45 and 90 min, the percentages of sperm vitality (45 min: $89.5 \pm 1.6\%$ vs. $70.7 \pm 3.1\%$ vs. $57.7 \pm 2.4\%$, $F = 35.541$, $P < 0.001$; 90 min: $80.8 \pm 2.2\%$ vs. $40.4 \pm 4.0\%$ vs. $34.7 \pm 3.9\%$, $F = 73.107$, $P < 0.001$) and progressive sperm (45 min: $69.3 \pm 2.7\%$ vs. $55.8 \pm 2.2\%$ vs. $55.4 \pm 2.5\%$, $F = 6.049$, $P = 0.001$; 90 min: $67.2 \pm 3.3\%$ vs. $38.2 \pm 4.0\%$ vs. $33.9 \pm 4.0\%$, $F = 23.681$, $P < 0.001$, respectively) in the Wi-Fi plus 5 mmol/L trolox group were highest, and the percentages of immotility sperm (45 min: $27.7 \pm 2.7\%$ vs. $41.7 \pm 2.2\%$ vs. $41.7 \pm 2.5\%$, $F = 6.241$, $P < 0.001$; 90 min: $29.9 \pm 3.3\%$ vs. $58.9 \pm 4.0\%$ vs. $63.1 \pm 4.0\%$, $F = 23.681$, $P < 0.001$, respectively) in the Wi-Fi plus 5 mmol/L trolox group were lowest [Figure 5].

DISCUSSION

Compared with the sham group, the oxidative stress, induced by the increase in ROS and the decrease in GSH-PX and

SOD, resulted in an increase in 8-OHdG and sperm DNA fragments, more damage to sperm mitochondrial DNA, and a decline in sperm mitochondrial potential in the Wi-Fi-exposed group. The parameters of semen quality showed the decreased vitality and progressive sperm percentages, accompanied by an increase in immotility sperm percentage under Wi-Fi exposure. The oxidative condition between ROS and antioxidants under Wi-Fi exposure was considered as the main biological effect on the organisms. La Vignera *et al.*^[27] reported that electromagnetic radiation-induced injury led to increased production of ROS through a variety of mechanisms. With regard to antioxidant consumption, electromagnetic radiation might overwhelm protective systems and result in cell injury and apoptosis.^[28] Atasoy *et al.*^[9] investigated the harmful effects of 2.45 GHz electromagnetic radiation on antioxidant and oxidant levels in rat sperm and found an increase in lipid peroxidation and pathological degeneration and a decrease in GSH-Px antioxidant enzymes in the rat testis. In male reproductive system, many evidences about damages from Wi-Fi exposure emerged. Research by Avendaño *et al.*^[16] demonstrated that 2.45 GHz Wi-Fi exposure led to a decrease in progressive sperm and an increase in DNA fragmentation in human sperm *in vitro*.

At 45 and 90 min, the 5 mmol/L trolox prevented oxidation and oxidative damage more effectively than 10 mmol/L trolox. To summarize, 5 mmol/L trolox could improve sperm quality after Wi-Fi exposure through the inhibition of oxidative stress *in vitro*. In the Wi-Fi plus 5 mmol/L trolox group, the sperm quality improved apparently because of less oxidative injury, with less 8-OHdG and fewer sperm DNA fragments. However, there was no benefit for sperm mitochondrial function. The same results were seen with the addition of 10 mmol/L trolox. Trolox had a direct role

in reversing ROS damage in a study of Tompkins *et al.*^[29] Rimbach *et al.*^[30] suggested that trolox can be regenerated by ascorbate, resulting in the formation of an ascorbyl radical in different doses. The 5 mmol/L trolox significantly enhanced SOD and GSH-Px activities and decreased the ROS levels in melamine-treated NRK-52e cells.^[31] A 4 mmol/L trolox reduced oxidative stress induced by lipopolysaccharide on duodenal contractility.^[32] The 50 $\mu\text{mol/L}$ trolox protected ovarian tissue of capuchin monkey from endoplasmic reticulum-derived cytoplasmic vacuolization.^[33] Addition of 40 $\mu\text{mol/L}$ trolox in the cryopreservation media improved the postthawed human semen quality.^[34] The 60 $\mu\text{mol/L}$ and 120 $\mu\text{mol/L}$ presented greater structural integrity, including membrane and mitochondria and kinematics for ram spermatozoa after cryopreservation.^[35] Addition of 60 nmol/ml trolox to skim milk preserved the plasma membrane and mitochondrial sheath integrity in goat spermatozoa after cryopreservation. Peña *et al.*^[36] used 100 mmol/L and 200 mmol/L trolox to prevent oxidative damage in boar semen and showed improved motility and vitality of spermatozoa. In addition, following the demonstration of inhibition of oxidation by tocopherol, much evidence has shown that Vitamin E, or α -tocopherol, is a crucial antioxidant in the lipid phase of cell metabolism and protects cells against

electromagnetic radiation-induced free radicals.^[37] Based on the GSH-Px and SOD assays, the trolox had no role in salvaging the antioxidant SOD, which is consistent with the findings of another study.^[38]

However, it is still unclear whether trolox can actually prevent oxidation. For example, increased trolox intake did not improve semen quality in humans. Different doses were studied by Kessopoulou (600 mg/d), Giovenco (300 mg/d), and Suleiman (200 mg/d). In an *in vitro* study, Gao and Cutler^[39] reported that the net hydroxyl radical-absorbing capacity of trolox increased at a concentration of 0.1–20.0 $\mu\text{mol/L}$, but decreased at higher concentrations (20–400 $\mu\text{mol/L}$), and even acted as an oxidation stimulator at concentrations of more than 1000 $\mu\text{mol/L}$. Furthermore, Dyatlov *et al.*^[40] found that trolox acted as a toxic pro-oxidant and was transformed to an α -tocopheroxyl radical as cellular functions deteriorated when oxidative stress was induced by Cu^{2+} or Fe^{3+} . Ko *et al.*^[41] reported that trolox exerts a metal-independent, peroxy radical-induced oxidation effect prior to functioning as an antioxidant. In some cases, trolox enhanced curcumin cytotoxicity through induction of oxidative stress.^[42]

Our study had several limitations. First, the mechanisms that adversely affect human semen *in vitro* are complex. In

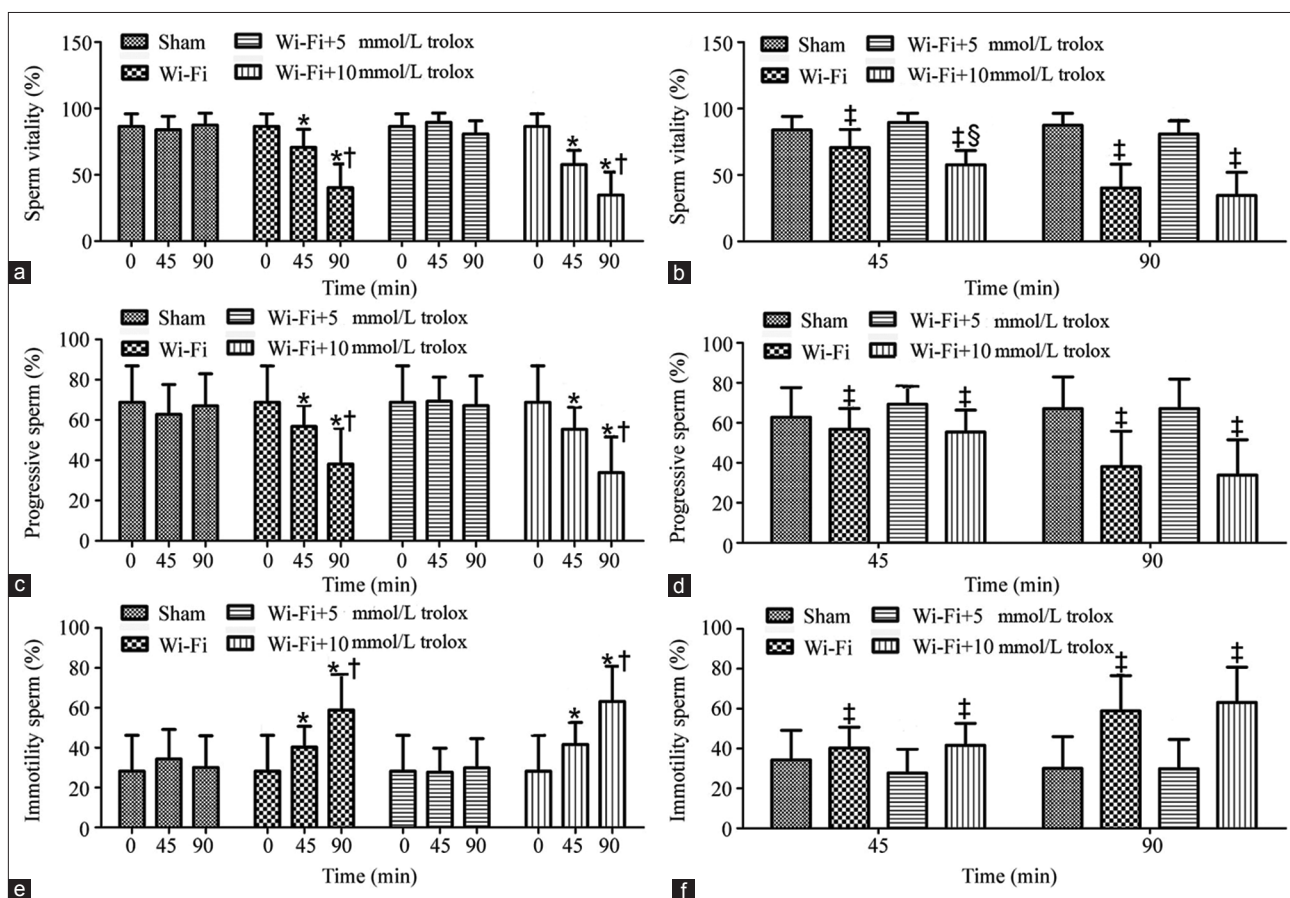


Figure 5: Sperm vitality and motility measurements at the time points of 0, 45, and 90 min in four aliquots. Sperm vitality (% a and b), progressive sperm (% c and d), and immotility sperm (% e and f) at the time points of 0, 45, and 90 min in sham, Wi-Fi, Wi-Fi plus 5 mmol/L trolox, and Wi-Fi plus 10 mmol/L trolox groups. * $P < 0.05$ vs. 0 min in each group. † $P < 0.05$ vs. 45 min in each group. ‡ $P < 0.05$ vs. Wi-Fi plus 5 mmol/L trolox group at 45 or 90 min. § $P < 0.05$ vs. Wi-Fi-exposed group at 45 or 90 min.

addition, precise exposure to 2.45 GHz Wi-Fi was not verified in the present research, due to lack of suitable equipment. Last, supplementation with trolox *in vitro* is not exactly comparable to the use of trolox *in vivo*. The current results supported a protective mechanism for a moderate dose of trolox on human sperm exposed to Wi-Fi electromagnetic radiation.

In conclusion, the study showed that 2.45 GHz Wi-Fi could impair human sperm *in vitro* through the effects of oxidative stress on sperm DNA and mitochondria. Trolox could mitigate the harmful effects of 2.45 GHz Wi-Fi electromagnetic radiation by inhibiting oxidative stress damage to sperm DNA and mitochondria. The results of this study would offer supportive evidence for clinical use of trolox to prevent the adverse effects of Wi-Fi exposure on the male reproductive system.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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中等剂量的 trolox 通过减少氧化应激降低 Wi-Fi 电磁辐射对体外精子的损伤

摘要

背景: 维生素E (生育酚) 可以改善Wi-Fi辐射对精液质量的不良影响。本研究旨在研究中等剂量的 trolox 是否能够通过抑制氧化损伤保护暴露在Wi-Fi电磁辐射的体外精子。

方法: 2014年6月到10月间, 在优生门诊收集20份合格精液标本, 将每一份标本分为假暴露组, Wi-Fi 暴露组, Wi-Fi加5 mmol/L trolox组和Wi-Fi加10 mmol/L trolox组。评估20份精液标本的基线参数。分别在45 和90分钟评估活性氧, 谷胱甘肽过氧化物酶和超氧化物歧化酶的含量, 精子DNA碎片, 精子线粒体膜电位, 精子线粒体DNA相对扩增情况, 以及精子活率, 前向运动精子和不动精子比率。以上参数通过单因素方差分析和事后Tukey检验进行比较分析。

结果: 在Wi-Fi 加5 mmol/L trolox组、Wi-Fi暴露组和Wi-Fi加10 mmol/L trolox组中, Wi-Fi 加5 mmol/L trolox组在45和90分钟的, 活性氧水平 (45分钟: $3.80 \pm 0.41 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$ vs. $7.50 \pm 0.35 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$ vs. $6.70 \pm 0.47 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$, $P < 0.001$; 90分钟: $5.40 \pm 0.21 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$ vs. $10.10 \pm 0.31 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$ vs. $7.00 \pm 0.42 \text{ RLU} \cdot 10^{-6} \cdot \text{ml}^{-1}$, $P < 0.001$), 尾部DNA百分比 (45分钟: $16.8 \pm 2.0\%$ vs. $31.9 \pm 2.5\%$ vs. $61.3 \pm 1.6\%$, $P < 0.001$; 90分钟: $19.7 \pm 1.5\%$ vs. $73.7 \pm 1.3\%$ vs. $73.1 \pm 1.1\%$, $P < 0.001$), 8羟化脱氧鸟苷含量 (45分钟: $51.89 \pm 1.46 \text{ pg/ml}$ vs. $104.89 \pm 2.19 \text{ pg/ml}$ vs. $106.11 \pm 1.81 \text{ pg/ml}$, $P = 0.012$; 90分钟: $79.96 \pm 1.73 \text{ pg/ml}$ vs. $141.73 \pm 2.90 \text{ pg/ml}$ vs. $139.06 \pm 2.79 \text{ pg/ml}$; $P < 0.001$) 和不动精子比率 (45分钟: $27.7 \pm 2.7\%$ vs. $41.7 \pm 2.2\%$ vs. $41.7 \pm 2.5\%$; 90分钟: $29.9 \pm 3.3\%$ vs. $58.9 \pm 4.0\%$ vs. $63.1 \pm 4.0\%$; all $P < 0.001$) 均最低; 谷胱甘肽过氧化物酶 (45分钟: $60.50 \pm 1.54 \text{ U/ml}$ vs. $37.09 \pm 1.77 \text{ U/ml}$ vs. $28.18 \pm 1.06 \text{ U/ml}$; 90分钟: $44.61 \pm 1.23 \text{ U/ml}$ vs. $16.86 \pm 0.93 \text{ U/ml}$ vs. $29.94 \pm 1.56 \text{ U/ml}$; $P < 0.001$), 头部DNA百分比 (45分钟: $83.2 \pm 2.0\%$ vs. $68.2 \pm 2.5\%$ vs. $38.8 \pm 1.6\%$; 90分钟: $80.3 \pm 1.5\%$ vs. $26.3 \pm 1.3\%$ vs. $26.9 \pm 1.1\%$; $P < 0.001$), 精子活率 (45分钟: $89.5 \pm 1.6\%$ vs. $70.7 \pm 3.1\%$ vs. $57.7 \pm 2.4\%$; 90分钟: $80.8 \pm 2.2\%$ vs. $40.4 \pm 4.0\%$ vs. $34.7 \pm 3.9\%$; $P < 0.001$) 和前向运动精子比率 (45分钟: $69.3 \pm 2.7\%$ vs. $55.8 \pm 2.2\%$ vs. $55.4 \pm 2.5\%$; 90分钟: $67.2 \pm 3.3\%$ vs. $38.2 \pm 4.0\%$ vs. $33.9 \pm 4.0\%$; $P < 0.001$) 均最高。其他的参数没有明显变化, 假暴露组的参数保持基线水平不变。

结论: 5 mmol/L trolox能够保护体外Wi-Fi暴露的精液免受电磁辐射引起的氧化应激损害。