

Inhibition of mitochondrial calcium uptake by Ru360 enhances the effect of 1800 MHz radio-frequency electromagnetic fields on DNA damage

Chuan Sun^{a,*}, Longtao Zhu^{b,1}, Houbing Qin^{c,1}, Huili Su^a, Jing Zhang^a, Sanying Wang^a, Xiaogang Xu^a, Zhenlei Zhao^a, Genxiang Mao^{a,*}, Jun Chen^{a,*}

^a Zhejiang Provincial Key Lab of Geriatrics & Geriatrics Institute of Zhejiang Province, Department of Geriatrics, Zhejiang Hospital, Hangzhou, China

^b Bioelectromagnetics Laboratory, Zhejiang University School of Medicine, Hangzhou, China

^c Department of Respiratory Medicine, Children's Hospital of Nanjing Medical University, Nanjing, China

ARTICLE INFO

Edited by Dr Fernando Barbosa

Keywords:

Ruthenium 360
Radio-frequency electromagnetic fields
DNA damage
Mitochondria
Calcium

ABSTRACT

Today, the existence of radio-frequency electromagnetic fields (RF-EMF) emitted from cell phones, wireless routers, base stations, and other sources are everywhere around our living environment, and the dose is increasing. RF-EMF have been reported to be cytotoxic and supposed to be a risk factor for various human diseases, thus, more attention is necessary. In recent years, interfere with mitochondrial calcium uptake by using mitochondrial calcium uniporter (MCU) inhibitor were suggested to be potential clinical treatment in mitochondrial calcium overload diseases, like neurodegeneration, ischemia/reperfusion injury, and cancer, but whether this approach increases the health risk of RF-EMF exposure are unknown. To address our concern, we did a preliminary study to determine whether inhibition of MCU will increase the genotoxicity of RF-EMF exposure in cells, and found that short-time (15 min) exposure to 1800 MHz RF-EMF induced significant DNA damage and cell apoptosis in mouse embryonic fibroblasts (MEFs) treated with Ruthenium 360 (Ru360), a specific inhibitor of MCU, but no significant effects on cell cycle, cell proliferation, or cell viability were observed. In conclusion, our results indicated that inhibiting MCU increases the genotoxicity of RF-EMF exposure, and more attention needs to be paid to the possible health impact of RF-EMF exposure under these treatments.

1. Introduction

With the popularization of mobile communication, radio-frequency electromagnetic fields (RF-EMF) emitted from mobile phones, wireless routers, base stations, and other sources was increasing. Studies have shown that RF-EMF exposure have potential health effect including but not limited to carcinogenesis (Balmori, 2022; Pinto et al., 2023), neurodegenerative disease (Wyszkowska and Pritchard, 2022), cardiovascular diseases (Bandara and Weller, 2017) hearing loss (Sharma et al., 2022), circadian rhythm disorder (Singh et al., 2022), sperm quality reduction (Chen et al., 2022; Zhang et al., 2022b), and mental health (Girela-Serrano et al., 2022). Thus, the potential health hazards caused by RF-EMF exposure are getting public attention.

In real living and working environments, various pollutants agents such as marco/nano-particles, chemicals in air, water, food, and sometimes consumed drugs coexists. These agents themselves can induce

biological effects (Akhavan et al., 2012; Akhavan et al., 2015), and also interaction with EMF that could modify the bioeffect of EMF (Akhavan and Ghaderi, 2009; Arslan et al., 2022; Benavides et al., 2023). Thus, it is possible that RF-EMF might induce serious consequences on health when combined with certain agents, more studies are required to address the possible risk of combined exposure.

Mitochondria are one of the most crucial organelles inside the cell that is critical in cellular homeostasis maintain. Although no direct evidence had shown that mitochondria play important role in cellular homeostasis maintaining in cells under RF-EMF exposure, many studies showed that mitochondria function, like reactive oxygen species (ROS) generation (Houston et al., 2018; Houston et al., 2016; Santini et al., 2018), Ca²⁺ regulation (Zhang et al., 2022a), unfolded protein response (Xie et al., 2021), mitochondrial structure (Li et al., 2020), and mitochondria respiration (von Niederhausern et al., 2019), were significantly affect by RF-EMF exposure, indicated mitochondria may be the sensitive

* Corresponding authors.

E-mail addresses: sun_chuan@aliyun.com (C. Sun), maogenxiang@163.com (G. Mao), zjdcxj@126.com (J. Chen).

¹ Co-first author.

target of RF-EMF in cells and which function possibly determine the cellular response to RF-EMF exposure. Coincidentally, the most common diseases correlated with mitochondria dysfunction, like carcinogenesis (Liu et al., 2023), neurodegenerative disease (Collier et al., 2023) and cardiovascular diseases (Liu et al., 2022), are also suggested to be potentially correlated with RF-EMF exposure. Thus, mitochondrial function may be important in cellular homeostasis maintaining in cells and which dysregulation may result in health hazards in bodies under RF-EMF exposure.

In recent years, accumulating body of researches indicated mitochondria as the potential target for therapeutic interventions (Viscomi and Zeviani, 2023). Despite cell respiration and energy production, mitochondria also serve as a key regulator for calcium metabolism, like buffering intracellular calcium ($[Ca^{2+}]_i$) levels by mitochondrial calcium ($[Ca^{2+}]_m$) uptake when cytosolic levels are elevated (De Stefani et al., 2011). $[Ca^{2+}]_m$ uptake is mediated by the highly selective and inwardly rectifying mitochondrial calcium uniporter (MCU) which is essential for signaling and bioenergetic process (De Stefani et al., 2011). But dysregulation of $[Ca^{2+}]_m$ uptake, like mitochondria Ca^{2+} overload, will contribute to apoptotic cell death by inducing cytochrome *c* release, ROS overproduction, mitochondrial swelling, and mitochondrial permeability transition pore (mPTP) opening (Lesnefsky et al., 2017). Over the past two decades, a significant number of studies have shown that dysregulation of $[Ca^{2+}]_m$ levels caused by improper MCU activity manifest a number of serious pathological conditions (Arduino and Perocchi, 2018; Mammucari et al., 2017; Nemani et al., 2018), which was the possible trigger for neurodegeneration, ischemia/reperfusion injury, and cancer (Wang and Zhou, 2020; Wang et al., 2016; Woods and Wilson, 2020). Thus, interfere with $[Ca^{2+}]_m$ uptake by inhibiting MCU has arisen as a potential therapeutic treatment of diseases related to $[Ca^{2+}]_m$ dysregulation (Woods and Wilson, 2020). But whether this approach is safe or will increase the health risk of bodies under a RF-EMF exposure environment have not been investigated.

Carcinogenesis is one of the most concerned possible health impacts of RF-EMF exposure emitted from mobile phone and other sources (Balmori, 2022; Grimes, 2022). Since the major cancer are initiated by cell genome damage (Phillips et al., 2009), the genotoxicity of RF-EMF exposure had received high attention. Most studies investigated the effect of RF-EMF single exposure on DNA damage in vivo and in vitro (Phillips et al., 2009), both negative and positive results had been reported, like Houston et al. reported that 1800 MHz RF-EMF exposure induce oxidative DNA damage in spermatozoa derived from mouse (Houston et al., 2018) but Yang et al. reported that 1800 MHz RF-EMF do not induce DNA damage in cochlear stria marginal cells (Yang et al., 2020). The inconsistent results among different studies on the DNA damage effect suggested that the effect of RF-EMF on DNA damage can be affect by many factors, such as exposure time, dose and model (Sun et al., 2016), cell types (Xu et al., 2013), and methodology (Phillips et al., 2009). Inconsistent results make it hard to draw a conclusion about the genotoxicity of RF-EMF but conveyed an important message that the effects of RF-EMF on DNA damage are highly susceptible to the interference from other factors. However, there were very few studies on the combined effect of RF-EMF with other factors, especially consumed drugs, on DNA damage.

To address our concerns, we did a preliminary in vitro study to determine whether inhibition of MCU will increase the genotoxicity of RF-EMF exposure, and found that Ru360, a specific inhibitor MCU (Hamilton et al., 2018; Liu et al., 2019; Nguyen et al., 2018; Paillard et al., 2018), have significant effect in enhancing DNA damage effect of 1800 MHz RF-EMF exposure in mouse embryonic fibroblasts (MEFs). Thus, according to our data, it is necessary to pay attention to the possible harm induced by RF-EMF exposure under such treatment.

2. Materials and methods

2.1. Cell line and culture

The immortalized MEFs used in this study was established by Chen D. J. group (Lawrence Berkeley National Laboratory, Berkeley, USA) (Burma et al., 2001), we obtained it as a kind gift. The MEFs had been used to investigate the effect of EMF on DNA damage in our previous studies (Sun et al., 2016; Sun et al., 2018). Cells were cultured in Alpha Minimum Essential Medium (α MEM, HyClone, Thermo Scientific, Shanghai, China) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS, HyClone) at 37 °C in a humidified atmosphere containing 5 % CO_2 .

2.2. Chemicals

Ruthenium 360 (Ru360, 557440), Carbonyl Cyanide *m*-Chlorophenylhydrazone (CCCP, C2759), and Adenosine 5'-Triphosphate (ATP, 20–306) were purchased from Sigma-Aldrich (Shanghai, China). Ru360 and CCCP powder were diluted in DMSO at a concentration of 1 mM and 50 mM for storage solution respectively. Oligomycin (HY-N6782) were purchased from MedChemExpress (Shanghai, China).

2.3. Electromagnetic fields exposure system

The RF-EMF exposure system (sXc1800) were built by Foundation for Information Technologies in Society (IT²S Foundation, Zurich, Switzerland) and had been described previously (Xu et al., 2013). The system contains two-resonant R18-waveguide setup, each holds six 35-mm Petri dishes positioned in the H-field maximum of the standing wave. Both the waveguides, one for RF-EMF exposure and another for sham-exposure, are placed in a CO_2 incubator (Hera Cell 240; Thermo Scientific, Waltham, MA, USA). The RF-EMF exposure and temperature in the waveguide chambers were monitored, and the temperature difference between the RF-EMF- and sham-exposed chambers did not exceed 0.1 °C.

2.4. Cell exposure protocol

Cells were sub-cultured into 35-mm Petri dish (1×10^5 /dish) (Nunc, Thermo Scientific, Shanghai, China) for 12 h, and 3 μ M Ru360, 40 μ M CCCP, 2 μ M Oligomycin were added into the cultured medium at 15 min before RF-EMF exposure. For exogenous ATP treatment, 1 μ M ATP were added into the cultured medium immediately before RF-EMF exposure. Then, cells were exposed to 1800 MHz RF-EMF at an average SAR of 4.0 W/kg for 15 min

2.5. Alkaline single-cell gel electrophoresis (comet assay)

The protocol detail can be found in previous study (Xu et al., 2013). Briefly, after exposure, cells in dishes were harvested and re-suspended in culture medium. Then 0.65 % low-melting agarose mixed with cells were added onto 0.65 % normal-melting agarose that had pre-coated on a slice. The slices were soaked in lysis buffer containing 1 % Triton X-100 at 4 °C for 1 h, and then in lysis buffer containing 0.5 mg/mL DNase-free proteinase K (Amresco, OH) at 37 °C for 2 h. Then, the slices were put into ice-cold alkaline electrophoresis solution for 20 min to unwind DNA in cells before electrophoresis. The electrophoresis condition is constant 300 mA for 20 min. After electrophoresis, cells were neutralized in Tris buffer (0.4 M, pH = 7.5) for 15 min. Gel-red were used to stain DNA "comets" and photographed by a fluorescence microscope (Nikon, Tokyo, Japan). About 100 comets for each sample were calculated by using the CASP 1.2.2 software (Krzysztof Konca, Wroclaw, Poland).

2.6. Protein concentration determination

Protein concentrations were determined by using BCA protein assay kit (Leagene, Shanghai, China). Values were read by a microplate reader (Spark, Tecan, Swiss).

2.7. Cellular ATP detection

ATP levels were determined by using an ATP assay kit (S0026, Beyotime). In brief, cells were lysed with ice cold lysis buffer and then centrifuged at 1.2×10^4 g at 4 °C for 5 min. The supernatant was used for ATP level and protein concentration detection. The values were read by the microplate reader (Tecan). ATP levels in per unit protein were calculated and relative values were presented.

2.8. Mitochondrial Ca^{2+} detection

Cells were sub-cultured in 96 well plate. When the confluence was almost 70 %, incubated cells with 2 μM RHOD2/AM (sc-202790, Santa Cruz, Shanghai, China) for 1 h. Then, the cells were washed with pre-warmed Hank's equilibrium salt solution (HBSS, Biosharp, Hefei, China). Cells were cultured in HBSS and the signal of RHOD2/AM were recorded under an inverted fluorescence microscope (Zeiss, Oberkochen, German). The fluorescence images were recorded every 5 s immediately after 20 μM ATP were added to stimulate cellular Ca^{2+} uptake. The average fluorescence density of each cell and background of each image were both measured by Photoshop CS5 Extended (Adobe, San Jose, CA), and the values of Ca^{2+} signal were calculated by subtracting background.

2.9. Cell cycle analysis

After exposure, cells were harvested and resuspended in pre-cooled 70 % ethanol. Then the cells were stored at -20 °C overnight. The cells were stained with 50 $\mu\text{g}/\text{mL}$ propidium iodide (PI, 81845, Sigma-Aldrich) diluted in phosphate-buffered saline containing 50 $\mu\text{g}/\text{mL}$ RNase A (EN0531, Thermo Scientific) at room temperature for 30 min. Then, the cell cycle distribution was analyzed by flow cytometry (Cytoflex, Beckman Coulter, CA, USA), in which about 1×10^4 events per sample were acquired, and the proportions of cells in the G0/G1, S, and G2/M phases were determined.

2.10. Cell viability analysis

After exposure, the cells were detached using 200 μL 0.25 % trypsin-EDTA (2520072, Gibco, Grand Island, NY) and resuspended in 500 μL of culture medium. Then, 2×10^4 cells per well were re-seeded into a 96-well plate. After 1 h of culture, cell viability was determined by using Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan).

2.11. Cell proliferation analysis

The 5-ethynyl-2'-deoxyuridine (EdU) cell proliferation kit (C0071S, Beyotime) was applied to determine cell proliferation. In brief, after exposure, cells were incubated with 10 μM EdU for 30 min and then fixed in 70 % ethanol at -20 °C overnight. The embedded EdU during cell proliferation were labeled and then detected by flow cytometry (Cytoflex, Beckman Coulter).

2.12. Cell apoptosis analysis

After treatment, cells were digested by 0.25 % trypsin-EDTA (Gibco) and resuspended in 1 mL culture medium and then collected into flow tube (20622601, FALCON, Shenzhen, China). The culture medium was removed by centrifuge and cells were incubated with apoptosis analysis

kit agent (A211-02, Vazyme, Nanjing, China) at room temperature for 30 min. The signal of PI and Annexin V were determined via flow cytometry (Cytoflex, Beckman Coulter). PI positive and/or Annexin V positive were considered as apoptotic cells.

2.13. Statistical analysis

All data were presented as mean $\pm$ standard error, and the sample size were marked in the figures or legends. GraphPad Prism 8.0.1 software (GraphPad Software, SD) were used for data analysis, a difference of $P < 0.05$ were considered statistically significant. The statistical methods applied for each data were described in the figure legends.

3. Results

3.1. Short-time exposure to 1800 MHz RF-EMF for 15 min induced significant DNA damage in MEFs treated with Ru360

Alkaline comet assay showed that exposure to 1800 MHz RF-EMF alone at 4.0 W/kg for 15 min did not significantly increase the levels of DNA fragmentation in MEFs compared to sham-exposure group (Fig. 1A). In MEFs treated with Ru360, a specific inhibitor of MCU which had a significant effect in inhibiting $[\text{Ca}^{2+}]_{\text{m}}$ uptake (Fig. 1B), exposure to 1800 MHz RF-EMF at 4.0 W/kg for 15 min significantly induced DNA fragmentation compared to sham-exposure group (Fig. 1C). These results suggest that Ru360 can sensitize cells to 1800 MHz RF-EMF exposure on DNA damage under current conditions. Further, the effect of 1 h-exposure to 1800 MHz RF-EMF were also determined, at this condition, both RF-EMF alone and combined with Ru360 exposure induced significant DNA damage in MEFs compared to sham-exposure group respectively (Supplementary Figure 6).

3.2. Short-time exposure to 1800 MHz RF-EMF for 15 min had no significant effect on cell cycle progression, cell proliferation, or cell viability but promoted apoptosis in MEFs treated with Ru360

To further determine the impact of Ru360 treatment on 1800 MHz RF-EMF bioeffects, we detected cell cycle progression (Fig. 2A), proliferation (Fig. 2B), cell viability (Fig. 2C), and cell apoptosis (Fig. 3A). The results showed that Ru360 do not significant affect the effect of RF-EMF exposure (15 min) on cell cycle progression, cell proliferation, or cell viability compared to sham exposure group respectively (Fig. 2), but significantly increased the apoptosis rate (Fig. 3A), and this effect were still observed under 1 h-exposure condition (Fig. 3B). These results suggested that Ru360 have an effect in sensitizing MEFs to 1800 MHz RF-EMF exposure on cellular apoptosis under current conditions.

3.3. Ru360 enhanced the DNA damage effect of 1800 MHz RF-EMF short-time exposure through inhibiting ATP synthesis

MCU mediated $[\text{Ca}^{2+}]_{\text{m}}$ uptake is essential for ATP production (Wescott et al., 2019). As a specific inhibitor of MCU, Ru360 also had a significant effect in inhibiting ATP production (Bravo et al., 2015) (Fig. 4A). To determine whether ATP supply shortage have effect on enhancing DNA damage effect of RF-EMF, we used oligomycin, a specific inhibitor of ATP synthase, to inhibit ATP synthesis in MEFs (Fig. 4B), and the results showed that short-time exposure to 1800 MHz RF-EMF for 15 min induce significant DNA fragmentation in MEFs treated with oligomycin, and ATP replenishment alleviated this effect compared to sham-exposure group respectively (Fig. 4C). And then, we found that ATP replenishment can also alleviate the DNA damage induced by 1800 MHz RF-EMF in Ru360 treated MEFs (Fig. 4C). These results suggested that ATP synthesis inhibition play an important role in the enhanced DNA damage effect of RF-EMF exposure under Ru360 treatment.

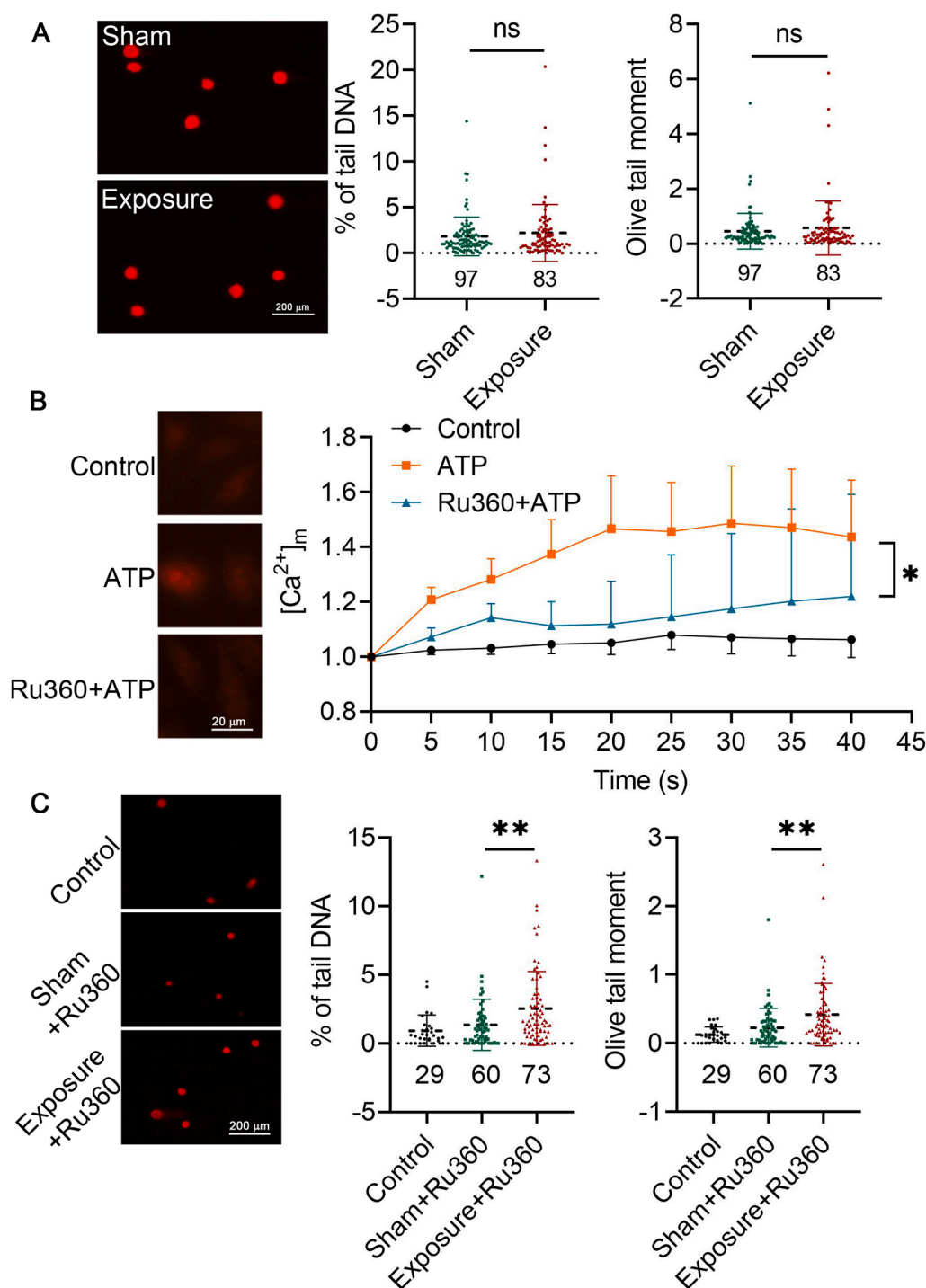


Fig. 1. Ru360 sensitized MEFs to 1800 MHz RF-EMF exposure on DNA damage. (A) DNA fragmentation in MEFs after sham-exposed (Sham) or exposed (Exposure) to 1800 MHz RF-EMF alone for 15 min. Mann-Whitney Rank Sum test were used to compare the difference between Sham and Exposure groups. ^{ns} $P > 0.05$. Scale bar = 200 μ m. (B) Representative fluorescence images and statistical data of mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) signal in MEFs treated with Ru360 and/or ATP or not. Two-way ANOVA analysis were applied to compare the difference between the ATP and Ru360 +ATP treatment groups, $N = 6$. $*P < 0.05$. Scale bar = 20 μ m. (C) Representative images of comet assay and statistical data of DNA fragmentation in MEFs treated with Ru360 and/or ATP or not. Mann-Whitney Rank Sum test were used to compare the difference between each two groups. $**P < 0.01$. Scale bar = 200 μ m.

3.4. DNA damage induced by 1800 MHz RF-EMF short-time exposure were quickly repaired within 1 h after exposure

To further determine the impact of DNA damage induced by 1800 MHz RF-EMF in MEFs treated with Ru360, we detected the dynamics of DNA damage within 1 h after exposure. The results showed that, in MEFs without Ru360 treatment, no significant differences of DNA damage were observed at 0, 30, or 60 min after 1800 MHz RF-EMF exposure compared to sham-exposure respectively (Fig. 5A). In MEFs treated with Ru360, significant DNA damage induced by 1800 MHz RF-EMF exposure were detected at 0 and 30 min but not 60 min after exposure compared to sham-exposure respectively (Fig. 5B). These

results suggested the DNA damage induced by RF-EMF exposure in Ru360 treated MEFs were almost repaired within 1 h after exposure.

4. Discussion

The environmental exposure of RF-EMF emitted from cell phones, wireless routers, base stations, and other sources were increasing fast during the last three decades (Bandara and Weller, 2017), and meanwhile, the increase of incidence rate of certain diseases, like brain tumor (Miller et al., 2021; Salehiniya and Farmanfarma, 2020) and cardiovascular diseases (Bandara and Weller, 2017), that cannot be explained by traditional causes, suggesting a potential correlation with increasing

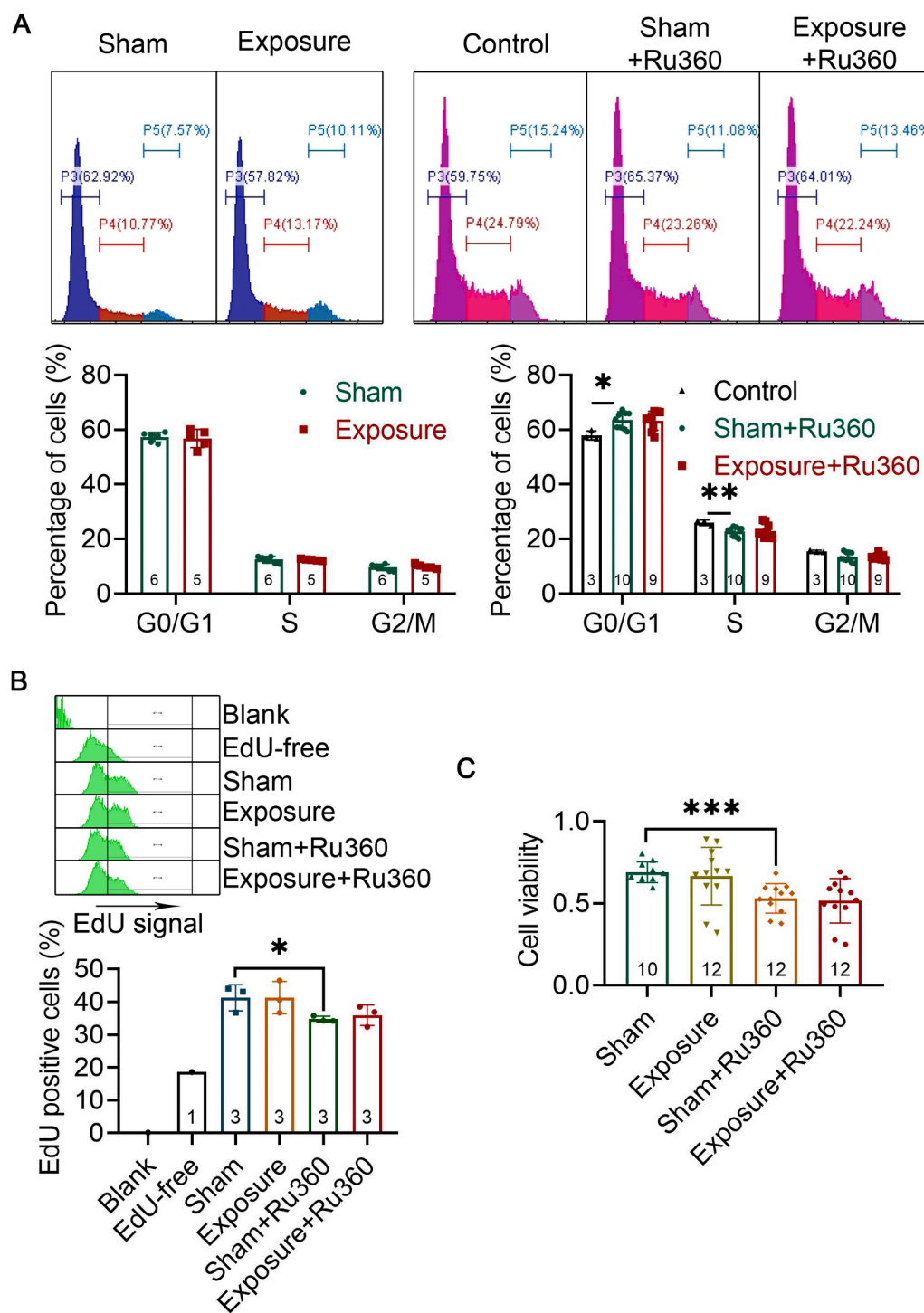


Fig. 2. The effect of 1800 MHz RF-EMF exposure on cellular behaviors in MEFs treated with Ru360. MEFs in different group were exposure to 1800 MHz RF-EMF at 4.0 W/kg for 15 min (A) Cell cycle distribution of MEFs in different groups: sham-exposed to RF-EMF (Sham), exposed to RF-EMF (Exposure), receive no treatment at all (Control), treated with Ru360 and then sham-exposed to RF-EMF (Sham+Ru360), and treated with Ru360 and then exposed to RF-EMF (Exposure+Ru360). One-way ANOVA analysis were applied to compare the difference between different groups. * $P < 0.05$. ** $P < 0.01$. (B) Cell proliferation rate and (C) cell viability of MEFs of Sham, Exposure, Sham+Ru360, and Exposure+Ru360 groups. Students' t -test were applied to compare the difference between each two groups. * $P < 0.05$. *** $P < 0.001$.

RF-EMF exposure. RF-EMF had been reported as an environmental pollutant with cytotoxic effects (Bandara and Weller, 2017), though its potential health effect were still controversy, we cannot ignore it before the potential health impact have been fully understood, more investigations are required.

In real environment, many factors coexist, such as macro/nano-particles, chemicals in air, water and food and sometimes the consumed drugs, combined effect of EMF with other factor have been widely observed (Akhavan and Ghaderi, 2009), such as black carbon particles have an interaction with RF-EMF on cell toxicity (Benavides et al., 2023), and carnosine prevent the damage effect of RF-EMF

exposure on ovarian follicles in rats (Arslan et al., 2022). EMF may also affect the bioeffect of other factors, such as RF-EMF exposure suppressed the ability of cells in resistance to hydrogen peroxide stimuli (Yu et al., 2023), and pre-exposure to RF-EMF triggered adaptation to high dose X-ray radiation in rats (Borzoueisileh et al., 2022), thus, the issue of composite exposure need to be considered which play important role in determine the bioeffects of RF-EMF exposure. The bioeffects of RF-EMF exposure possibly be personalized, different living environment and lifestyle that are known health related factors (Pacey, 2010) may also determine the bioeffects of RF-EMF. In this study, we found that use Ru360 to disturb $[Ca^{2+}]_m$ uptake significantly affect the effects of

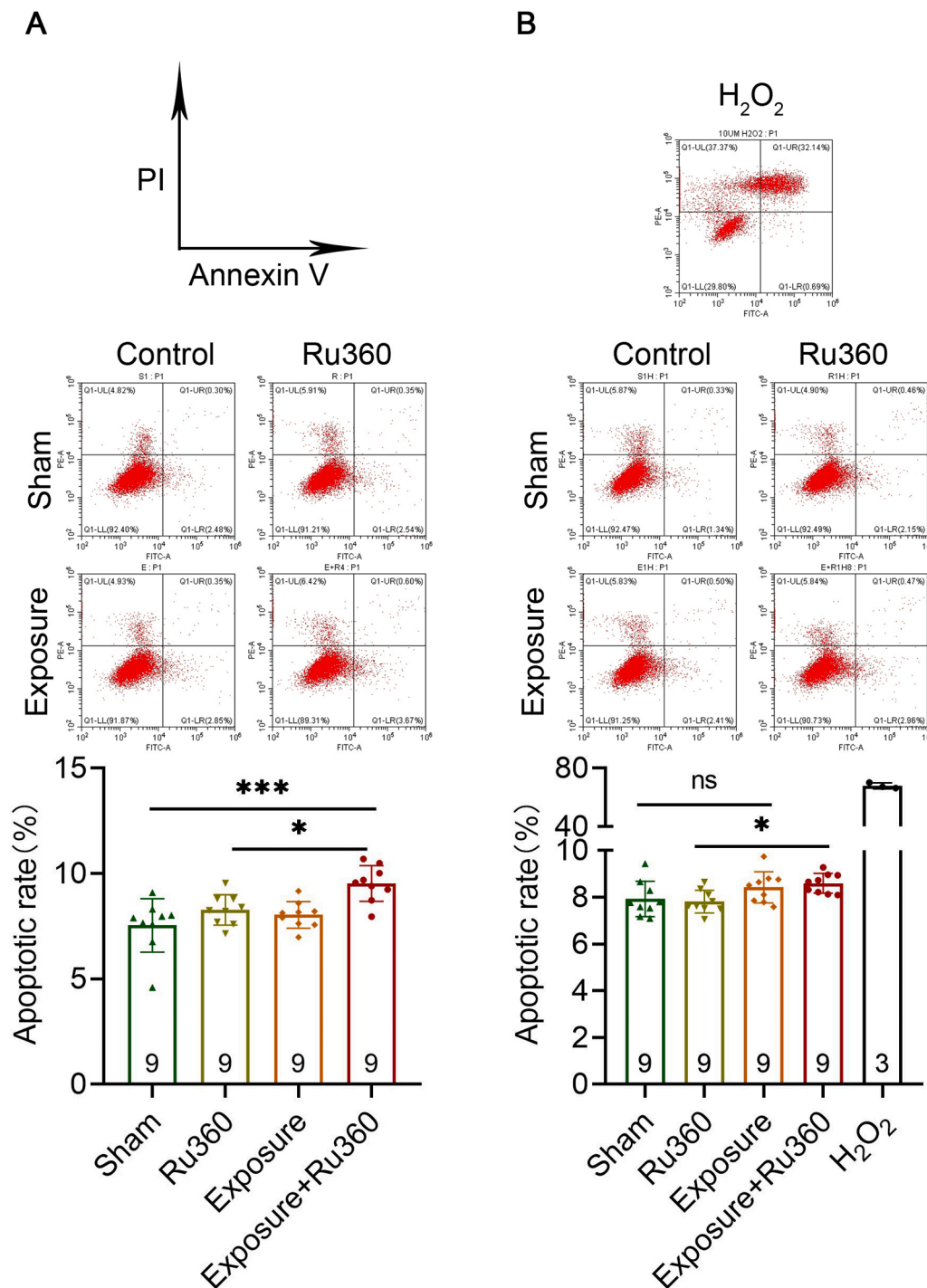


Fig. 3. Ru360 sensitized MEFs to 1800 MHz RF-EMF exposure on apoptosis. MEFs were exposed to 1800 MHz RF-EMF at 4.0 W/kg for 15 min (A) or 1 h (B) and then the cell apoptosis were determined by flow cytometry-H₂O₂: cells treated with 10 μ M hydrogen peroxide solution for 30 min. Sham: cells were shamed exposed to RF-EMF. Exposure: cells were exposed to RF-EMF. Ru360: cells were treated with Ru360. One-way ANOVA analysis were used to compare the difference between each two groups. ^{ns} $P > 0.05$. * $P < 0.05$. *** $P < 0.001$.

RF-EMF on DNA damage suggested the calcium regulation might play a role in the different combined effect of RF-EMF.

Mitochondria are one of the critical sub-cellular organelles and involved in various diseases, such as neurodegenerative disease (Collier et al., 2023), cardiovascular diseases (Liu et al., 2022), and carcinogenesis (Liu et al., 2023), all of which RF-EMF were also frequently reported to be a potential risk factor (Balmori, 2022; Bandara and Weller, 2017; Pinto et al., 2023; Wyszowska and Pritchard, 2022). Although there were no direct evidence about that mitochondria are important in cellular homeostasis maintaining in cells under RF-EMF exposure, many studies reported that mitochondrial structure and function were significantly affected by RF-EMF exposure (Houston et al., 2018; Houston et al.,

2016; Li et al., 2020; Santini et al., 2018; von Niederhausern et al., 2019; Xie et al., 2021; Zhang et al., 2022a), indicating mitochondria are sensitive organelles in cellular response to RF-EMF exposure, and interfere mitochondrial function might change cellular response to RF-EMF which might cause potential health risk under certain conditions.

In recent years, accumulating body of researches indicated that mitochondria were the potential target for therapeutic interventions in mitochondria related diseases (Viscomi and Zeviani, 2023), and interfere with mitochondrial calcium uptake by MCU inhibitor were suggested to be effective in mitochondria calcium overload diseases, like neurodegeneration, ischemia/reperfusion injury, and cancer (Wang and Zhou, 2020; Wang et al., 2016; Woods and Wilson, 2020). However,

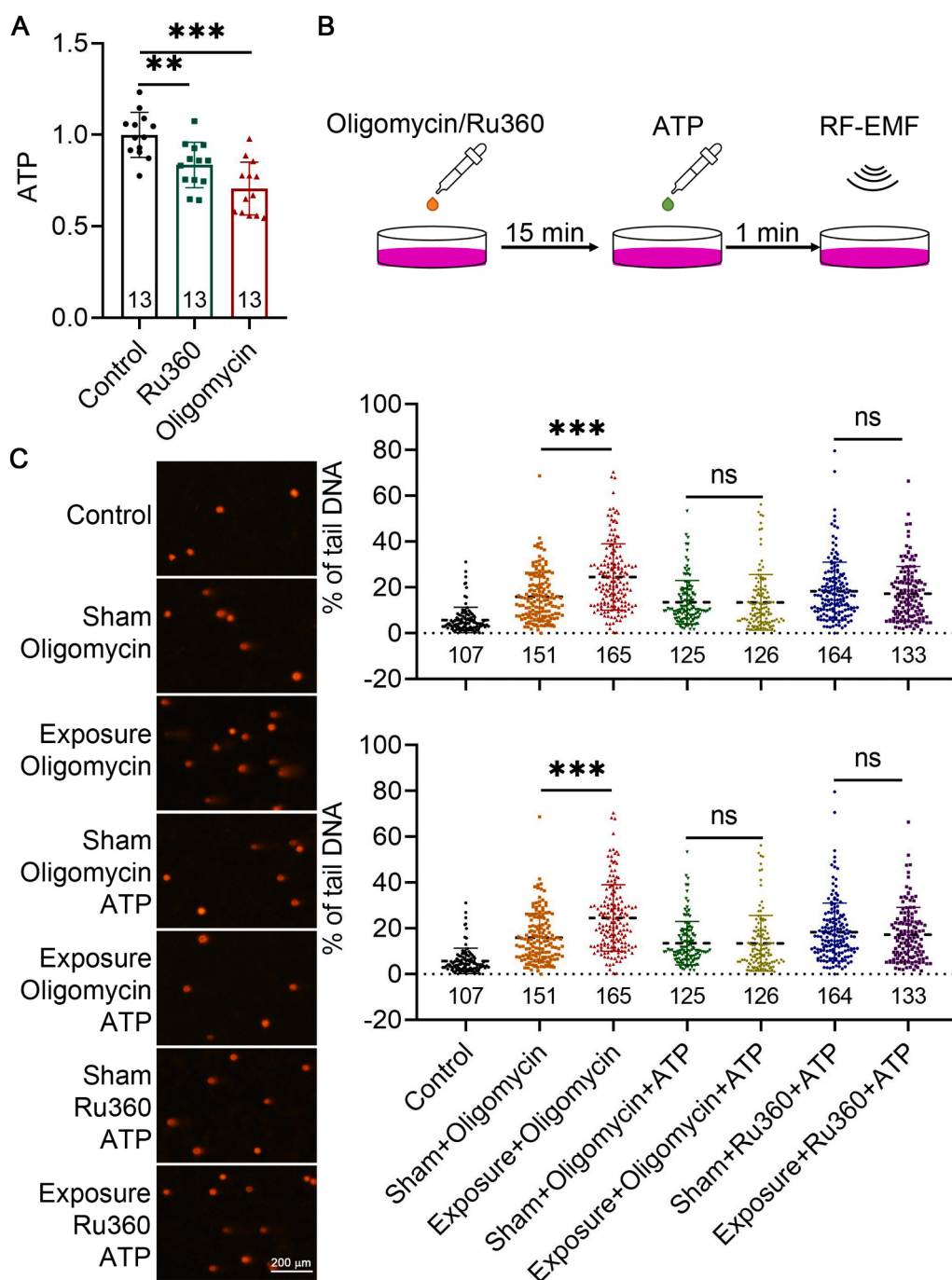


Fig. 4. Ru360 enhanced the DNA damage effect of 1800 MHz RF-EMF exposure through inhibiting ATP synthesis. (A) Relative ATP concentrations in MEFs treated with Ru360 or Oligomycin for 15 min, students' *t*-test were applied to compare the difference between each two groups, $N = 13$. ** $p < 0.01$. *** $p < 0.001$. (B) Schematic diagram of cell treatment process, cells were pretreated with Oligomycin/Ru360 for 15 min and then with ATP or not for 1 min before RF-EMF exposure. (C) DNA fragmentation in MEFs of each group indicated in (B) after exposure or sham-exposure to 1800 MHz RF-EMF for 15 min. Mann-Whitney Rank Sum test were used to compare the difference between each two groups. *** $p < 0.001$. ns $p > 0.05$. Scale bar = 200 μ m.

whether this approach is safe under a RF-EMF exposure environment are unknown. To address this question, we did a preliminary study to determine if MCU inhibitor, Ru360, sensitize cells to RF-EMF exposure on DNA damage, and the results showed that Ru360 significantly increased the DNA damage in MEFs exposed to 1800 MHz RF-EMF at 4.0 W/kg for 15 min, suggested a possible effect of MCU inhibitor on cellular response to RF-EMF exposure. Although the DNA damage were almost repaired within 1 h after exposure, the possible harm on health should not be overlooked.

The effect of RF-EMF exposure on DNA damage has been highly regarded by researchers for the importance of carcinogenic evaluation, but it is still hard to get a definite conclusion from these existed studies due to the inconsistent results among different studies (Jageti, 2022; Kocaman et al., 2018; Panagopoulos, 2019). In this study, inhibiting

MCU by Ru360 significantly changed the effect of 1800 MHz RF-EMF exposure on DNA damage suggested that mitochondria function, especially MCU mediated ions transport, may play a key role in cellular response to RF-EMF exposure. The different regulation of $[Ca^{2+}]_m$ metabolism might cause different results of RF-EMF exposure on DNA damage.

MCU is the center transporter for mitochondrial calcium uptake, to further determine its role in cellular response to RF-EMF on DNA damage, we also used CCCP to interpret mitochondrial function (Supplementary Figure 1A), but this approach did not change the DNA damage effect of RF-EMF exposure in MEFs (Supplementary Figure 1B). Both Ru360 and CCCP have significant effect in interfering mitochondrial function, but the mechanisms are different. CCCP, members of the lipophilic weak acid class, are known as proton shuttling compounds,

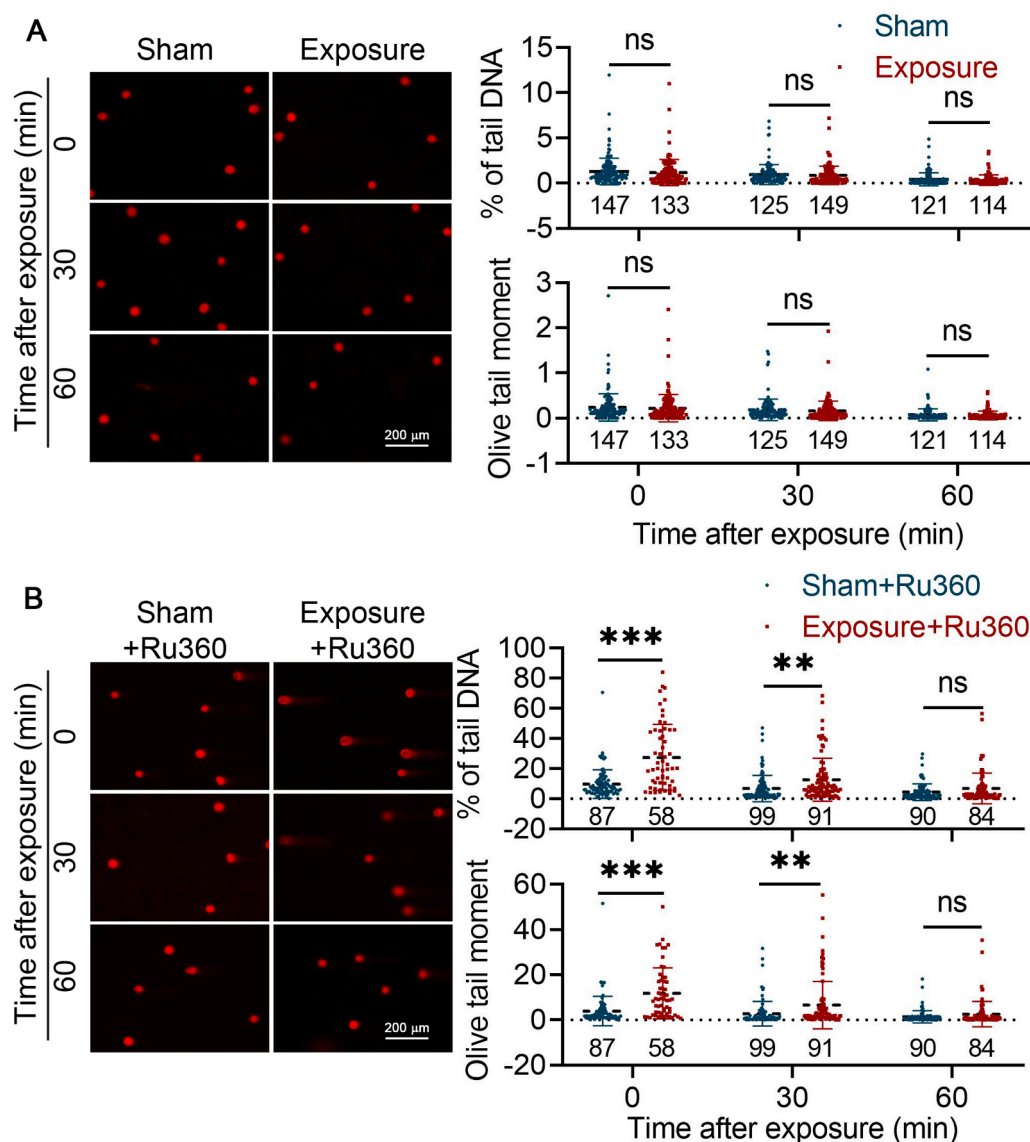


Fig. 5. Dynamics of DNA damage in MEFs after RF-EMF exposure. DNA fragmentation in MEFs without (A) or with (B) Ru360 treatment at 0, 30, and 60 min after RF-EMF exposure. Mann-Whitney Rank Sum test were used to compare the difference between each two groups. $**P < 0.01$. $***P < 0.001$. $ns P > 0.05$. Scale bar = 200 μ m.

can selectively increase the permeability of lipid membranes to protons, dissipating the mitochondrial membrane potential (MMP) (Kane et al., 2018). Ru360, a specific inhibitor of MCU, blocks mitochondrial calcium uptake (Garcia-Rivas Gde et al., 2006; Kirichok et al., 2004). Thus, MCU mediated calcium metabolism, but not all mitochondrial function, is the possible special mechanism for regulating cellular response to RF-EMF exposure. However, our data were not sufficient to illustrate this suppose, and more investigations are required.

We also explored the mechanism of how Ru360 sensitize cells to 1800 MHz RF-EMF exposure on DNA damage, and the data showed that mitochondrial ATP synthases inhibition play an important role in this process (Fig. 4), but the mechanisms of how ATP participate in this process is not clarified. ATP is consumed as a biological energy source by many intracellular reactions which supply is required to maintain cellular homeostasis (Arduino and Perocchi, 2018), such as DNA repair (Kulkarni et al., 2011; Qin et al., 2015). Ru360 induced mitochondrial energy supply shortage (Bravo et al., 2015; Fernandez-Sada et al., 2014) (Fig. 4A) may delayed the initiation of DNA repair system that result in improperly repaired DNA damage in cells under RF-EMF exposure. In this study, we had also tried to determine the DNA damage in MEFs by

detecting γ H2AX, a protein marker of DNA damage (Sharma et al., 2012), but the results seemed to be contract when compared with the comet assay (Supplementary Figure 5). Comet assay directly show the DNA fragmentation, thus, there is no doubt that the DNA damage occurred. Considering histone H2AX phosphorylation (γ H2AX formation) is also one link in DNA damage response, we think γ H2AX decreasing indicated that DNA damage repair might be inhibited by RF-EMF in MEFs treated with Ru360. Another explanation is that γ H2AX is usually identified as a biomarker of DNA double strand breaks (Sharma et al., 2012), while alkaline comet assay detected both DNA single and double strand breaks. In our previous study, we have observed that 1800 MHz RF-EMF only induce DNA single strand breaks in MEFs (Sun et al., 2016). So, whether DNA repair inhibition is the main mechanism await further studies. Despite energy supply, ATP also act as an important messenger to regulate cellular metabolism, like ions metabolism including Ca^{2+} (Nemirovskaya and Sharlo, 2022), Mg^{2+} (Jin et al., 2022), Na^{2+} (Palty et al., 2012), K^{+} (Lv et al., 2022), and others, thus, ATP may supplement the signal interruption when MCU is inhibited. However, more studies are required.

Oxidative stress were suggested to be the most possible mediator in

RF-EMF inducing DNA damage and other bioeffects (Schuermann and Mevisen, 2021), but in this study, we did not observe significant increase of either intracellular or mitochondrial ROS in cells after 1800 MHz RF-EMF exposure in either Ru360 treated or not treated MEFs (Supplementary Figure 2 and Supplementary Figure 3). First, this might be due to the exposure time was short, only 15 min and 1 h, previous study has showed that the increase in ROS production do not occur immediately but with increasing exposure time (>12 h) or cumulative dose (Liu et al., 2014), the ROS changes might not be detected under our experimental conditions. Second, ROS independent mechanisms may be existed, RF-EMF exposure might interfere other cellular progress, like cell cycle progression and DNA replication, which are associated with the endogenous DNA damage levels. But our data showed that cell cycle progression (Fig. 2A) or DNA replication (Fig. 2B) were not significantly affected in Ru360 treated MEFs by 1800 MHz RF-EMF exposure. So, further studies are required to illustrate the mechanism.

Though Ru360 had been demonstrated to be a specific inhibitor of MCU, we still need to consider the mechanisms other than calcium signal regulation that determine cellular response to RF-EMF exposure. Cause heat is the main mechanism of RF-EMF to induce biological effect (Cosman and Cosman, 2005), though in our exposure system, the temperature increase during exposure were strictly limited to < 0.1 °C (Schuderer et al., 2004), thermal effect should be considered in studies on high power RF-EMF exposure. Other mechanisms, like glucose metabolism (Volkow et al., 2011) and hormone secretion (Alkayyali et al., 2021), that had been reported to be affected by RF-EMF exposure, are possibly involved in the cellular response to RF-EMF exposure.

In this study, we only investigate the effect of short time exposure to RF-EMF. On the one hand, it is convenient to study in this way, and on the other hand, we believe that the bioeffect occurred at the initial exposure stage are also meaningful to the outcomes of long-term exposure. Usually, the longer the exposure time, the more obvious the effect, but in electromagnetic fields, things become complex. In our previous study, we had investigated the effect of 1800 MHz RF-EMF exposure on DNA damage in MEFs, the exposure time were extended from 1 to 36 h (Sun et al., 2016). DNA damage effect of 1800 MHz RF-EMF exposure on MEFs had been observed but was not linearly dependent on the exposure time, by contrast, due to the homeostasis mechanism (like DNA repair), the DNA damage induced by RF-EMF exposure at short-time (1 h) exposure were repaired when the exposure time extended to 12 h or more. And similar effect is also exist in other chemical stimulating factors when the dose is low (Sun et al., 2016). This time-dependent nonlinear or bimodal bioeffect of RF-EMF had also been reported by other studies (Phillips et al., 2009), suggested that biological system have strong adaptability to long-term mild RF-EMF exposure. Thus, in our opinion, electromagnetic fields, like mobile phone radiation, long-term exposure might exhibit no more significant direct biological effect compared to short initial exposure, biological effect induced at initial exposure stage may already determine the long-term outcome, like DNA damage induced by RF-EMF short time exposure, might cause gene mutation if incorrectly repaired and result carcinogenesis after a long period of time. Thus, we believe that the biological changes induced by electromagnetic magnetic fields at the initial exposure stage is also meaningful to understand the effect of long-term exposure. However, more evidence is needed to make our point.

Whether MCU inhibition treatment increases the potential health risk of patient under a RF-EMF exposure environment is a very complex issue, the data provided in this study was not insufficient to answer this question. The first, we only observed the short-time exposure effects, the long-time effect is not known. The second, we only focused on the DNA damage effect, whether there are other impacts of RF-EMF exposure on Ru360 treated cells were not explored. The third, this is an in vitro study, the effects need to be verified by in vivo studies and even clinical trials. The last, the mechanism of how inhibiting MCU changed the effects of RF-EMF exposure are still unclear. Thus, as mentioned above, in the future, more investigations are required to study the potential health

risk of RF-EMF exposure on bodies that receive MCU inhibition treatment, and this study are mainly aimed to draw peoples' attention to this aspect.

In conclusion, we found that inhibiting MCU by Ru360 have an effect in enhancing the DNA damage effect of 1800 MHz RF-EMF exposure on MEFs, thus, potential health impact of MCU inhibiting treatment in a RF-EMF exposure environment need to be noted.

Foundation

This work was supported by National Natural Science Foundation of China (31700734, 81701393), Medical Science and Technology Program of Zhejiang Province (2021KY005, 2023KY432), Chinese Traditional Medicine Science and Technology Projects of Zhejiang Province (2020ZQ002).

CRediT authorship contribution statement

Chuan sun: Conceptualization, Project administration, Methodology, Investigation, Writing – original draft. **Longtao Zhu:** Methodology, Investigation, Data curation, Visualization. **Houbing Qin:** Resources, Writing – review & editing, Formal analysis, Validation. **Huili Su:** Resources. **Jing Zhang:** Resources. **Sanying Wang:** Resources. **Xiaogang Xu:** Resources. **Zhenlei Zhao:** Resources. **Genxiang Mao:** Funding acquisition, Resources. **Jun Chen:** Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgements

We thank Professor Guangdi Chen of Zhejiang University School of Medicine and his group for their support in this study.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115472.

References

- Akhavan, O., et al., 2012. Size-dependent genotoxicity of graphene nanoplatelets in human stem cells. *Biomaterials* 33, 8017–8025.
- Akhavan, O., et al., 2015. Dose-dependent effects of nanoscale graphene oxide on reproduction capability of mammals. *Carbon* 95, 309–317.
- Akhavan, O., Ghaderi, E., 2009. Enhancement of antibacterial properties of Ag nanorods by electric field. *Sci. Technol. Adv. Mater.* 10, 015003.
- Alkayyali, T., et al., 2021. An exploration of the effects of radiofrequency radiation emitted by mobile phones and extremely low frequency radiation on thyroid hormones and thyroid gland histopathology. *Cureus* 13, e17329.
- Arduino, D.M., Perocchi, F., 2018. Pharmacological modulation of mitochondrial calcium homeostasis. *J. Physiol.* 596, 2717–2733.
- Arslan, A., et al., 2022. Effect of carnosine on ovarian follicle in rats exposed to electromagnetic field. *Eur. J. Anat.* 26, 659–668.
- Balmori, A., 2022. Evidence for a health risk by RF on humans living around mobile phone base stations: From radiofrequency sickness to cancer. *Environ. Res.* 214, 113851.
- Bandara, P., Weller, S., 2017. Cardiovascular disease: Time to identify emerging environmental risk factors. *Eur. J. Prev. Cardiol.* 24, 1819–1823.
- Benavides, R.A.S., et al., 2023. The HL-60 human promyelocytic cell line constitutes an effective in vitro model for evaluating toxicity, oxidative stress and necrosis/apoptosis after exposure to black carbon particles and 2.45 GHz radio frequency. *Sci. Total Environ.* 867, 161475.

- Borzoueisileh, S., et al., 2022. Pre-exposure to radiofrequency electromagnetic fields and induction of radioadaptive response in rats irradiated with high doses of X-rays. *J. Biomed. Phys. Eng.* 12, 505–512.
- Bravo, A., et al., 2015. Effect of mitochondrial calcium uniporter blocking on human spermatozoa. *Andrologia* 47, 662–668.
- Burma, S., et al., 2001. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J. Biol. Chem.* 276, 42462–42467.
- Chen, H.G., et al., 2022. Association between electronic device usage and sperm quality parameters in healthy men screened as potential sperm donors. *Environ. Pollut.* 312, 120089.
- Collier, J.J., et al., 2023. Mitochondrial signalling and homeostasis: from cell biology to neurological disease. *Trends Neurosci.* 46, 137–152.
- Cosman, E.R., Cosman, Jr, Sr, E.R., 2005. Electric and thermal field effects in tissue around radiofrequency electrodes. *Pain. Med.* 6, 405–424.
- De Stefani, D., et al., 2011. A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. *Nature* 476, 336–340.
- Fernandez-Sada, E., et al., 2014. Cardiac responses to beta-adrenoceptor stimulation is partly dependent on mitochondrial calcium uniporter activity. *Br. J. Pharmacol.* 171, 4207–4221.
- Garcia-Rivas Gde, J., et al., 2006. Ru360, a specific mitochondrial calcium uptake inhibitor, improves cardiac post-ischaemic functional recovery in rats in vivo. *Br. J. Pharmacol.* 149, 829–837.
- Girela-Serrano, B.M., et al., 2022. Impact of mobile phones and wireless devices use on children and adolescents' mental health: a systematic review. *Eur. Child Adolesc. Psychiatry* 1–31.
- Grimes, D.R., 2022. Radiofrequency radiation and cancer: a review. *JAMA Oncol.* 8, 456–461.
- Hamilton, J., et al., 2018. Deletion of mitochondrial calcium uniporter incompletely inhibits calcium uptake and induction of the permeability transition pore in brain mitochondria. *J. Biol. Chem.* 293, 15652–15663.
- Houston, B.J., et al., 2016. The effects of radiofrequency electromagnetic radiation on sperm function. *Reproduction* 152, R263–R276.
- Houston, B.J., et al., 2018. Probing the origins of 1,800 MHz radio frequency electromagnetic radiation induced damage in mouse immortalized germ cells and spermatozoa in vitro. *Front. Public Health* 6, 270.
- Jageta, G.C., 2022. Genotoxic effects of electromagnetic field radiations from mobile phones. *Environ. Res.* 113321.
- Jin, F., et al., 2022. Recent advances in the structural biology of Mg(2+) channels and transporters. *J. Mol. Biol.* 434, 167729.
- Kane, M.S., et al., 2018. Current mechanistic insights into the CCCP-induced cell survival response. *Biochem Pharmacol.* 148, 100–110.
- Kirichok, Y., et al., 2004. The mitochondrial calcium uniporter is a highly selective ion channel. *Nature* 427, 360–364.
- Kocaman, A., et al., 2018. Genotoxic and carcinogenic effects of non-ionizing electromagnetic fields. *Environ. Res.* 163, 71–79.
- Kulkarni, R., et al., 2011. Expression of DNA repair and apoptosis genes in mitochondrial mutant and normal cells following exposure to ionizing radiation. *Environ. Mol. Mutagen* 52, 229–237.
- Lesnfsky, E.J., et al., 2017. Mitochondrial dysfunction and myocardial ischemia-reperfusion: implications for novel therapies. *Annu. Rev. Pharmacol. Toxicol.* 57, 535–565.
- Li, D.Y., et al., 2020. Apoptotic effect of 1800 MHz electromagnetic radiation on NIH/3T3 cells. *Int. J. Environ. Res. Public Health* 17.
- Liu, K., et al., 2014. The protective effect of autophagy on mouse spermatocyte derived cells exposure to 1800MHz radiofrequency electromagnetic radiation. *Toxicol. Lett.* 228, 216–224.
- Liu, Y., et al., 2022. Mitochondrial dysfunction and therapeutic perspectives in cardiovascular diseases. *Int. J. Mol. Sci.* 23.
- Liu, Y., et al., 2023. An overview: the diversified role of mitochondria in cancer metabolism. *Int. J. Biol. Sci.* 19, 897–915.
- Liu, Z.J., et al., 2019. High glucose enhances bupivacaine-induced neurotoxicity via MCU-mediated oxidative stress in SH-SY5Y cells. *Oxid. Med. Cell. Longev.* 2019, 7192798.
- Lv, J., et al., 2022. ATP-sensitive potassium channels: a double-edged sword in neurodegenerative diseases. *Ageing Res. Rev.* 80, 101676.
- Mammucari, C., et al., 2017. Structure, activity regulation, and role of the mitochondrial calcium uniporter in health and disease. *Front. Oncol.* 7, 139.
- Miller, K.D., et al., 2021. Brain and other central nervous system tumor statistics, 2021. *CA Cancer J. Clin.* 71, 381–406.
- Nemani, N., et al., 2018. Molecular regulation of MCU: Implications in physiology and disease. *Cell Calcium* 74, 86–93.
- Nemirovskaya, T.L., Sharlo, K.A., 2022. Roles of ATP and SERCA in the Regulation of Calcium Turnover in Unloaded Skeletal Muscles: Current View and Future Directions. *Int. J. Mol. Sci.* 23.
- Nguyen, N.X., et al., 2018. Cryo-EM structure of a fungal mitochondrial calcium uniporter. *Nature* 559, 570–574.
- von Niederhausern, N., et al., 2019. Effects of radiofrequency electromagnetic field exposure on neuronal differentiation and mitochondrial function in SH-SY5Y cells. *Toxicol. Vitro* 61, 104609.
- Pacey, A.A., 2010. Environmental and lifestyle factors associated with sperm DNA damage. *Hum. Fertil.* 13, 189–193.
- Paillard, M., et al., 2018. MICU1 interacts with the D-ring of the MCU pore to control its Ca(2+) flux and sensitivity to Ru360. *Mol. Cell* 72, 778–785 e3.
- Palty, R., et al., 2012. Molecular identity and functional properties of the mitochondrial Na+/Ca2+ exchanger. *J. Biol. Chem.* 287, 31650–31657.
- Panagopoulos, D.J., 2019. Comparing DNA damage induced by mobile telephony and other types of man-made electromagnetic fields. *Mutat. Res.* 781, 53–62.
- Phillips, J.L., et al., 2009. Electromagnetic fields and DNA damage. *Pathophysiology* 16, 79–88.
- Pinto, R., et al., 2023. In vivo studies on radiofrequency (100 kHz–300 GHz) electromagnetic field exposure and cancer: a systematic review. *Int. J. Environ. Res. Public Health* 20.
- Qin, L., et al., 2015. CDK1 enhances mitochondrial bioenergetics for radiation-induced DNA repair. *Cell Rep.* 13, 2056–2063.
- Salehinyia, H., Farmanfarma, K.K., 2020. Brain cancer in the world: an epidemiological review. *Biomed. Res. Ther.* 7, 3898–3905.
- Santini, S.J., et al., 2018. Role of mitochondria in the oxidative stress induced by electromagnetic fields: focus on reproductive systems. *Oxid. Med Cell Longev.* 2018, 5076271.
- Schuderer, J., et al., 2004. High peak SAR exposure unit with tight exposure and environmental control for in vitro experiments at 1800 MHz. *IEEE Trans. Microw. Theory Tech.* 52, 2057–2066.
- Schuermann, D., Mevissen, M., 2021. Manmade electromagnetic fields and oxidative stress-biological effects and consequences for health. *Int. J. Mol. Sci.* 22.
- Sharma, A., et al., 2012. Histone H2AX phosphorylation: a marker for DNA damage. *Methods Mol. Biol.* 920, 613–626.
- Sharma, D., et al., 2022. Effect of mobile phone use on hearing in young adults: an observational study. *Indian J. Otolaryngol. Head Neck Surg.* 74, 3754–3757.
- Singh, M.M., et al., 2022. Does exposure to radiofrequency radiation (RFR) affect the circadian rhythm of rest-activity patterns and behavioral sleep variables in humans? *Biol. Rhythm. Res.* 53, 1414–1438.
- Sun, C., et al., 2016. Mobile phone signal exposure triggers a hormesis-like effect in Atm+/+ and Atm−/− mouse embryonic fibroblasts. *Sci. Rep.* 6, 37423.
- Sun, C., et al., 2018. Ataxia telangiectasia mutated deficiency does not result in genetic susceptibility to 50 Hz magnetic fields exposure in mouse embryonic fibroblasts. *Bioelectromagnetics* 39, 476–484.
- Viscomi, C., Zeviani, M., 2023. Experimental therapy for mitochondrial diseases. *Handb. Clin. Neurol.* 194, 259–277.
- Volkow, N.D., et al., 2011. Effects of cell phone radiofrequency signal exposure on brain glucose metabolism. *JAMA* 305, 808–813.
- Wang, J., Zhou, H., 2020. Mitochondrial quality control mechanisms as molecular targets in cardiac ischemia-reperfusion injury. *Acta Pharm. Sin. B* 10, 1866–1879.
- Wang, W., et al., 2016. Novel targets for mitochondrial medicine. *Sci. Transl. Med.* 8, 326rv3.
- Wescott, A.P., et al., 2019. Voltage-energized calcium-sensitive ATP production by mitochondria. *Nat. Metab.* 1, 975–984.
- Woods, J.J., Wilson, J.J., 2020. Inhibitors of the mitochondrial calcium uniporter for the treatment of disease. *Curr. Opin. Chem. Biol.* 55, 9–18.
- Wyszkowska, J., Pritchard, C., 2022. Open questions on the electromagnetic field contribution to the risk of neurodegenerative diseases. *Int. J. Environ. Res. Public Health* 19.
- Xie, W., et al., 2021. 900 MHz radiofrequency field induces mitochondrial unfolded protein response in mouse bone marrow stem cells. *Front. Public Health* 9, 724239.
- Xu, S., et al., 2013. Cell type-dependent induction of DNA damage by 1800 MHz radiofrequency electromagnetic fields does not result in significant cellular dysfunctions. *PLoS One* 8, e54906.
- Yang, H., et al., 2020. The effects of mobile phone radiofrequency radiation on cochlear stria marginal cells in sprague-dawley rats. *Bioelectromagnetics* 41, 219–229.
- Yu, G., et al., 2023. The ZIP9-centered androgen pathway compensates for the 2605 MHz radiofrequency electromagnetic radiation-mediated reduction in resistance to H(2)O (2) damage in Sertoli cells of adult rats. *Ecotoxicol. Environ. Saf.* 254, 114733.
- Zhang, D., et al., 2022a. Mitochondrial Ca(2+) homeostasis: emerging roles and clinical significance in cardiac remodeling. *Int. J. Mol. Sci.* 23.
- Zhang, S., et al., 2022b. Effects of mobile phone use on semen parameters: a cross-sectional study of 1634 men in China. *Reprod. Fertil. Dev.* 34, 669–678.