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# Thapsigargin blocks electromagnetic field-elicited intracellular $Ca^{2+}$ increase in HEK 293 cells

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## Abstract

Biological effects of electromagnetic fields (EMFs) have previously been identified for cellular proliferation and changes in expression and conduction of diverse types of ion channels. The major effect elicited by EMFs seems to be directed toward $Ca^{2+}$ homeostasis. This is particularly remarkable since $Ca^{2+}$ acts as a central modulator in various signaling pathways, including, but not limited to, cell differentiation and survival. Despite this, the mechanisms underlying this modulation have yet to be unraveled. Here, we assessed the effect of EMFs on intracellular $[Ca^{2+}]$, by exposing HEK 293 cells to both radio-frequency electromagnetic fields (RF-EMFs) and static magnetic fields (SMFs). We detected a constant and significant increase in $[Ca^{2+}]$ subsequent to exposure to both types of fields. Strikingly, the increase was nulled by administration of 10 μM Thapsigargin, a blocker of sarco/endoplasmic reticulum $Ca^{2+}$-ATPases (SERCAs), indicating the involvement of the endoplasmic reticulum (ER) in EMF-related modulation of $Ca^{2+}$ homeostasis.

**KEYWORDS**
calcium, electromagnetic fields, endoplasmic reticulum, intracellular dynamics

## 1 | INTRODUCTION

The last decade experienced an exponential increase in the daily usage of electronic instruments, such as smartphones, laptops and wireless devices, producing a broad range of electromagnetic fields (EMFs) (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, World Health Organization, and International Agency for Research on Cancer, 2002; Organization, W.H., 2007). This has led to higher intensities and prolonged exposure to users with respect to the electromagnetic radiation produced by these devices. Along with this, there is increasing interest in the biological impact of this exposure within the scientific community.

Numerous investigations have reported an association between chronic exposure to extremely low frequency

electromagnetic fields (ELF-EMFs), up to 300 Hz, and an increased risk of developing childhood leukemia (London et al., 1991; Savitz et al., 1988). Recently the WHO's International Agency for Research on Cancer listed radio-frequency electromagnetic fields (RF-EMFs), ranging from 10 MHz to 300 GHz, as potentially carcinogenic (Baan et al., 2011). On the other hand, an increasing number of studies reported EMFs beneficial role in the treatment of numerous chronic diseases, such as cancer, mood disorders and many forms of neurodegeneration (Jimenez et al., 2018; Martiny et al., 2010; Wyszkowska et al., 2019), paving the way for the therapeutic use of magnetic-field based techniques, such as Transcranial Magnetic Stimulation and Pulsed Electromagnetic Field (PEMF) stimulation (Hallett, 2000; Markov, 2007).

The biological effects of EMFs are varied and appear to affect cell physiology on many levels. Both ELF-EMFs and RF-EMFs have been shown to modulate cell proliferation (Choi et al., 2020; Grassi et al., 2004). They have been linked to direct changes in genetic and protein expression, such as modulation of ion transport rate in different types of ion channels (Czyz et al., 2004; Grassi et al., 2004; Haghani et al., 2013; He et al., 2013; Lacy-hulbert et al., 1998). Specifically, excitable tissues like those found in the central nervous system are particularly sensitive to EMFs. Here, RF-EMFs have been linked to abnormal brain development, activation of the autophagic pathway, decreased number and size of synaptic vesicles and reduced ion channels expression (Kim et al., 2018, 2019; Odaci et al., 2008). ELF-EMFs on the other hand, have been shown to affect synaptic plasticity, increase glutamate and GABA release, increase ROS levels, and facilitate all forms of endocytosis (Balassa et al., 2013; Duan et al., 2014; Morabito et al., 2010; Sun et al., 2016).

Amongst all these effects however, particular emphasis has been placed on the modulation of $Ca^{2+}$ dynamics. Several studies have identified $Ca^{2+}$ channels as the principal modulators of EMF impacts on cellular dynamics (Buckner et al., 2015; Cui et al., 2014). The involvement of $Ca^{2+}$ is remarkable, since this cation plays a primary role as a second messenger in the modulation of a variety of physiological functions, including, but not limited to, gene expression, cell motility and survival, muscle contraction, membrane excitability, neurotransmitter release and stress response (Ebashi & Endo, 1968; Hardingham et al., 1997; Neher & Sakaba, 2008; Nicotera & Orrenius, 1998; Reddy et al., 2011; Tian et al., 2010; Tsien, 1983). It is well established that the relative concentrations of $Ca^{2+}$ in the various subcellular compartments are finely regulated by a set of proteins including membrane channels and intracellular transporters (Bronner, 2001). The pivotal role of these proteins is reflected by the great number of channelopathies caused by their dysfunction, such as many forms of neoplasia, epilepsy, neuropathic pain and

neurodegeneration (Dolphin, 2016). Radiofrequency radiation has been reported to decrease pan-$Ca^{2+}$ channel expression in mouse hippocampus and hypothalamus, increase cytosolic $[Ca^{2+}]$ in stem-cell derived neuronal cells and decrease $Ca^{2+}$ binding proteins expression (Kim et al., 2018, 2019; Maskey et al., 2013; Titushkin et al., 2009). On the other hand, ELF-EMFs have been linked to an increase of $[Ca^{2+}]$ in various cells and tissues, amongst which mouse hippocampus and rat PC12 cells (Morabito et al., 2010; Sun et al., 2016; Yin et al., 2016), enhance the expression of voltage-gated calcium channels (VGCCs), and modulate their electrical properties (Duan et al., 2014; Lisi et al., 2006; Marchionni et al., 2006; Sun et al., 2016). However, results reported in literature are often conflicting and other studies report no effect on $Ca^{2+}$ current or perturbation of $Ca^{2+}$ homeostasis (Groot et al., 2014; Marchionni et al., 2006; Platano et al., 2007).

Additionally, the mechanism through which EMFs impact $Ca^{2+}$ dynamics has yet to be unraveled. The numerous evidence reporting a change in the expression and transport dynamics of VGCCs suggests the cellular membrane as the preferential target for EMF interaction (Adey, 1993). Furthermore, many studies suggest the direct involvement of specific VGCCs, such as L, P/Q, N and T subtypes (Buckner et al., 2015; Lisi et al., 2006; Marchionni et al., 2006; Sun et al., 2016; Titushkin et al., 2009). However, the large number of discordant results cannot be overlooked (Groot et al., 2014, 2016; Platano et al., 2007), with several studies suggesting the alteration in $Ca^{2+}$ homeostasis is independent of VGCC dynamics (Luo et al., 2014; Morabito et al., 2010).

HEK 293 cells are broadly used as a platform for the heterologous expression of diverse subtypes of $Ca^{2+}$ channels, such as T-type and L-type, in the study of EMF exposure (Cui et al., 2014; Hristov et al., 2018) and they are particularly interesting due to their numerous neuronal characteristics such as sensitivity to the neurotransmitter acetylcholine, bradykinin and neurotensin (Vetter & Lewis, 2010). However, little is known about the endogenous response of HEK 293 cells to EMFs, as their native $Ca^{2+}$ dynamics are still a matter of debate (Berjukow et al., 1996; Bugaj et al., 2005; Varghese et al., 2006).

Here, we aim to determine the specific mechanistic pathway involved in the alteration of internal $Ca^{2+}$ homeostasis in HEK 293 cells, under exposure to RF-EMFs and SMFs.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell Culture

The human embryonic kidney cell line HEK 293 (Sigma-Aldrich, UK) was cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific,

UK), supplemented with 10% heat inactivated fetal bovine serum (Thermo Fisher Scientific, UK), 1% Penicillin/ Streptomycin (Thermo Fisher Scientific, UK) and 1% L-Glutamine (Sigma Aldrich, UK) stock solutions. On the day prior to each experiment cells (confluence 60–70%) were divided and seeded in 96-well plates (Thermo Fisher Scientific, UK) on a volume of 100 µl/well. For some experiments, cells were directly seeded in Standard Extracellular Physiological Saline (120 mM NaCl, 4 mM KCl, 2mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose). Each well was checked for full confluence before the experiments.

## 2.2 | Determination of intracellular $[Ca^{2+}]$

Intracellular $[Ca^{2+}]$ changes were assessed using Fluo-4 AM (Abcam, UK). Cells were seeded in two 96-wells microplates (Thermo Fisher Scientific, UK) on the day prior to the experiment to obtain full confluence on experiment day. On experiment day, culture media was removed, and each plate washed with Standard Extracellular Physiological Saline (120 mM NaCl, 4 mM KCl, 2mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose) to remove any trace of dead cells or culture media. Both plates were then incubated for 1 h (38°C, 5% $CO_2$) in Standard Extracellular Physiological Saline containing 1 µM Fluo-4AM (Thermo Fisher Scientific, UK), 50 µl/well. After incubation time, plates were washed again and resuspended in 50 µl of Standard Extracellular Physiological Saline. A first measurement of intracellular $[Ca^{2+}]$ was acquired using a Clariostar Plate reader (BGM Labtech, Germany), pre-adjusted for gain and focus level, and used as baseline. Plates then underwent exposure protocols (details in the below section). Fluorescence intensity reflecting intracellular $[Ca^{2+}]$ was measured at $T_1$ (15 min) and $T_2$ (30 min) (Figure 1). The baseline was subtracted to the measurement taken at each timepoint and the relative increases of the sham and the exposed groups compared.

To assess the origin of the detected increase, some experiments were performed in $Ca^{2+}$-free solution (120 mM

NaCl, 4 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose). To evaluate minimum responses, 10 µM of EGTA (Sigma-Aldrich, UK) was also added to dye solution in some experiments. To interfere with intracellular and extracellular $Ca^{2+}$ dynamics various compounds were also used and added to the dye incubation solution (Figure 1): as $Ca^{2+}$ channel blocker,10 µM Nifedipine (Sigma-Aldrich, UK); to block SERCAs on the ER, 10 µM Thapsigargin (Santa Cruz Biotechnology, USA); to block RyRs, 10 µM Dantrolene (Sigma-Aldrich, UK). Data were recorded as individual well scans.

## 2.3 | EMF exposure conditions

The sham group was submitted to the same conditions as the EMF group, but without EMF irradiation. In the RF-EMFs experiments, a mobile telephone (Nord, OnePlus) was used as a source of radiation (Figure 2a). The mobile telephone was operated in test mode and was powered through a stabilized power supply, so that the antenna power supply as well as the field intensity were constant. In this diagnostic configuration the cell phone produced a constant field having a measured intensity of 0.4 mT. The maximum radiation was achieved in a 50 × 50 mm square corresponding to the location of the mobile telephone antenna. Plates were exposed for a total of 30 min and intracellular $[Ca^{2+}]$ evaluated every 15 min. For maximum radiation, the EMF source was placed directly on the top of plate's lid (Figure 2a). The Specific Absorption Rate (SAR) as stated by producing company (OnePlus) was 1.27 W/Kg. Both sham and exposed plates were placed throughout the whole experiment on a heating plate producing a measured average temperature of 38°C. A thermal probe was placed in a test microplate well, loaded with experimental solution and used to monitor the temperature over exposure to phone cell activity and SMF generator. Temperature was monitored over 30 min before each experiment and no major fluctuations were recorded as a consequence of exposure
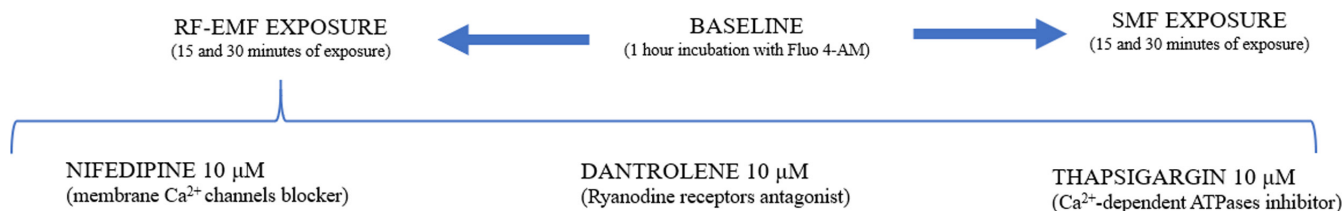
**RF-EMF EXPOSURE**
(15 and 30 minutes of exposure)
⟵
**BASELINE**
(1 hour incubation with Fluo 4-AM)
⟶
**SMF EXPOSURE**
(15 and 30 minutes of exposure)

**NIFEDIPINE 10 µM**
(membrane $Ca^{2+}$ channels blocker)
**DANTROLENE 10 µM**
(Ryanodine receptors antagonist)
**THAPSIGARGIN 10 µM**
($Ca^{2+}$-dependent ATPases inhibitor)

**FIGURE 1** Flow chart of the experimental protocol. The impact on intracellular $[Ca^{2+}]$ of oscillating (RF-EMFs) and static (SMFs) fields was initially assessed. To elucidate the origin of the detected increase, the RF-EMFs experiments were then repeated by using different compounds. These were: 10 µM Nifedipine as $Ca^{2+}$ channels blocker; 10 µM Thapsigargin as blocker of $Ca^{2+}$-dependent ATPases (SERCAs) inhibitor; 10 µM Dantrolene as antagonist of Ryanodine receptors of (RyRs)
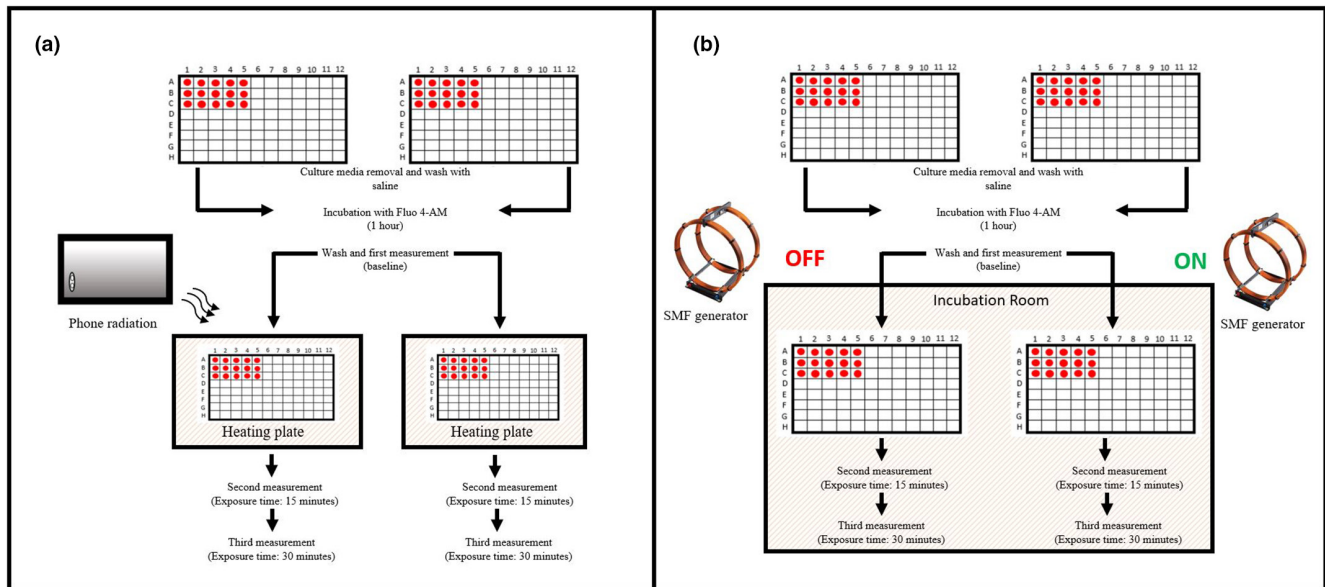
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**FIGURE 2** Experimental arrangement of RF-EMF and SMF experiments: plates were incubated for 1 h with Fluo-4AM $Ca^{2+}$ dye. A first measurement was used as baseline. For RF-EMF exposure (a), a cell phone was used as source of radiation. Both plates were then placed on a heating plate at 38°C. The cell phone's antenna was directly placed on the charged wells (red dots in the figure) for maximum radiation. Sham group was subjected to the same condition of the exposed group, but for the presence of the phone. For SMFs (b), we used a custom generator composed of two parallel Helmholtz coils powered by a DC supply. Both plates were incubated at a monitored temperature of 38°C. Sham group was subjected to the same condition of the exposed group, but the generator was turned off. In both cases, plates were exposed for 30 min and fluorescence intensity, reflecting intracellular $[Ca^{2+}]$, measured after 15 and 30 min

conditions. A thermal probe was placed in a test microplate well, loaded with experimental solution and used to monitor the temperature over exposure to phone cell activity and SMF generator. Temperature was monitored over 30 min before each experiment and no major fluctuations were recorded as a consequence of exposure conditions. In SMF experiments the plates were placed in a SMF generator consisting of two Helmholtz Coils (Figure 2b) powered by a direct current (DC) generator and used to produce a constant SMF of 1 mT (measured before and after each experiment). Differences smaller than ±1%, in respective to the center, in a cylindrical volume of 60 mm in diameter × 60 mm in length, minimum. The SMF generator was placed in an incubation room (38°C) for the duration of the experiments. The sham plate was subjected to the same conditions of the exposure groups, but the SMF generator was off.

## 2.4 | Statistical Analysis

Statistical analysis was performed using Graph Pad Prism® software version 6 for Windows ($p < 0.05$). Error bars represent standard error of the mean (SEM). T-tests were used to determine whether a difference is seen between the means of two unrelated groups of data. The "$n$" value

reported refer to microplate wells. For each condition 3 independent experiments were carried out.

All experimental procedures were approved by and adhered to the guidelines of ethical committee of University of Surrey, Guildford, UK.

## 3 | RESULTS

### 3.1 | EMFs triggers intracellular $Ca^{2+}$ release

To compare the impact of the electric and the magnetic component of the exposure, the effect of different frequency fields was assessed. Specifically, the exposures consisted of oscillating radiofrequency fields (RF-EMFs) and static magnetic field (SMFs), constant in time and direction, and thus, with a 0 Hz frequency. For the first we choose to use a mobile phone, closely resembling the exposure deriving from smartphones and wireless devices usage. For the second, a static magnetic field generator consisting of two Helmholtz coils connected to a DC power supply. As we were interested in the rapid effects of EMFs, we focused our investigations on early timepoints (15 and 30 min).

A rise in dye fluorescence, reflecting an increase in intracellular $[Ca^{2+}]$, was observed as result of both types of exposures. In the RF-EMF exposed cohort, this was

significantly different at $T_1$ (15 min) and $T_2$ (30 min) ($p$-value $< 0.0001$) when compared to sham. Additionally, in this group when compared between $T_1$ and $T_2$, the increase in intracellular $Ca^{2+}$ was significantly increased ($p$-value 0.0002) in $T_2$ when compared to $T_1$ suggesting a time dependent effect on intracellular increases albeit acute.

The SMFs, on the other hand, showed no difference between sham and exposed groups at $T_1$, but at $T_2$, the exposure showed a significant increase ($p$-value $< 0.0001$) when compared to the sham (Figure 3b). Similar to the RF-EMF group, when compared between $T_1$ and $T_2$, the intracellular $Ca^{2+}$ was significantly increased in $T_2$ ($p$-value 0.0009) when compared to $T_1$, also suggesting a time dependent effect on intracellular $Ca^{2+}$ increase. The temperature generated by phone cell activity and SMF generator were monitored with a thermal probe over 30 min before experiments. Since in both cases they were always lower than the incubating temperature of the plates ($37.5°C$), the thermal effects were considered to be not significant. No significant difference in temperature was detected when the SMF generator was switched on or turned off.

As, contrary to SMFs, acute exposure to RF-EMFs is not commonly reported to increase cytosolic $[Ca^{2+}]$, we decided to focus our investigation on this type of fields. In order to characterize the origin of the detected increase, the RF-EMF experiments were repeated in $Ca^{2+}$-free solutions, aiming to evaluate the involvement of intracellular $Ca^{2+}$ stores. We observed no significant difference between $Ca^{2+}$-free and standard physiological solution at both $T_1$ and $T_2$. This suggested an intracellular origin for the RF-EMF-elicited increase in $Ca^{2+}$.

## 3.2 | Confirmation of the involvement of membrane $Ca^{2+}$ channels

Further experiments were undertaken to clarify the exact mechanism for the observed change in intracellular $Ca^{2+}$. In order to investigate the involvement of membrane $Ca^{2+}$ channels, cells were administered with 10 μM of the $Ca^{2+}$ channel blocker Nifedipine. All the drugs were directly added to the dye solution so that their impact was evaluated both when compared to baseline and to the field exposures.

Nifedipine treatment significantly reduced intracellular $[Ca^{2+}]$ ($p$-value 0.0002), confirming the presence of nifedipine-sensitive channels on the membrane of HEK 293 cells (Figure 4a). Moreover, it strongly reduced the increase of the exposed group when compared to control ($p$-value $< 0.0001$), suggesting the involvement of these channels in the modulation of the increase triggered by EMFs exposure (Figure 4b). The bigger reduction of fluorescence intensity achieved in the EGTA-treated group in respect to Nifedipine-treated one proves that a significant fraction of intracellular $Ca^{2+}$ is likely to originate from intracellular reservoirs, or it is translocated through nifedipine-insensitive channels.
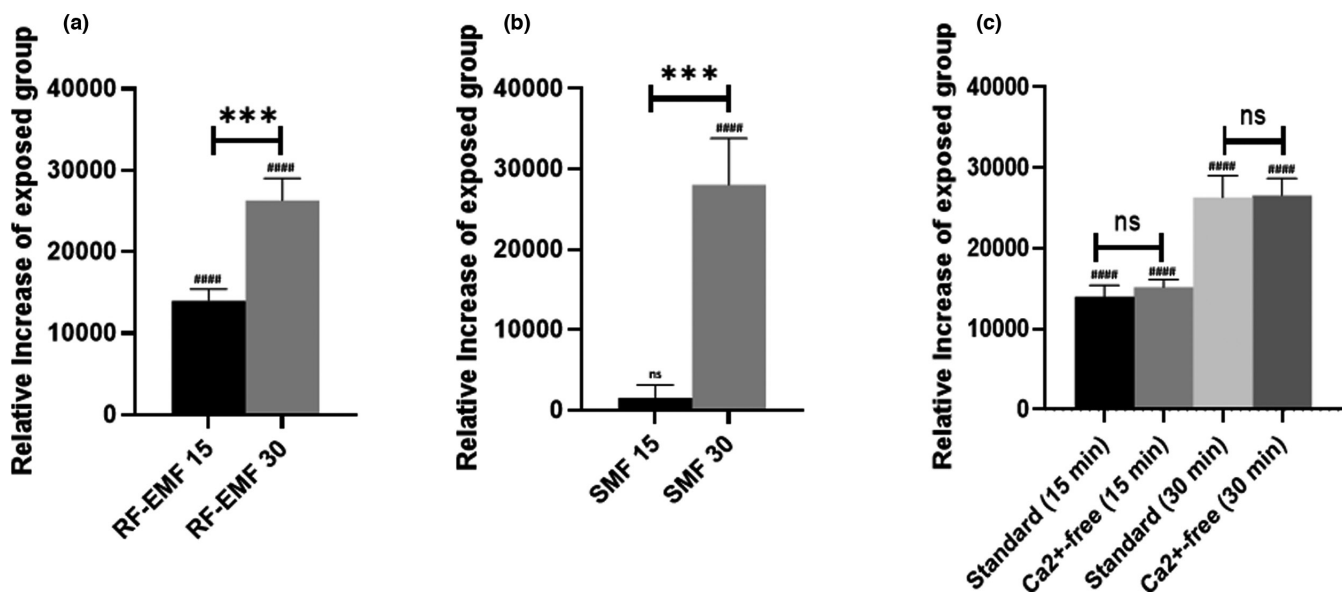


**FIGURE 3** SMF and RF-EMFs increase intracellular $[Ca^{2+}]$ in HEK 293 cells. Data are displayed as relative increase of exposed group when compared to sham. (*) represent inter-conditions significance while (#) display significance when compared to sham. (a) RF-EMFs increase basal $[Ca^{2+}]$ The difference with sham group is significant at both $T_1$ and $T_2$. (b) SMFs lead to a similar increase as already found with RF-EMFs. However, the increase is delayed when compared to what observed with RF-EMFs, and only significant at $T_2$. (c) Comparison between RF-EMF elicited increase in standard and $Ca^{2+}$-free conditions. The extracellular presence of $Ca^{2+}$ makes no difference on the detected $[Ca^{2+}]_i$, suggesting an intracellular origin for the elicited increase. $N = 45$ microplate wells from 3 independent experiments. $p$-value $< 0.001$
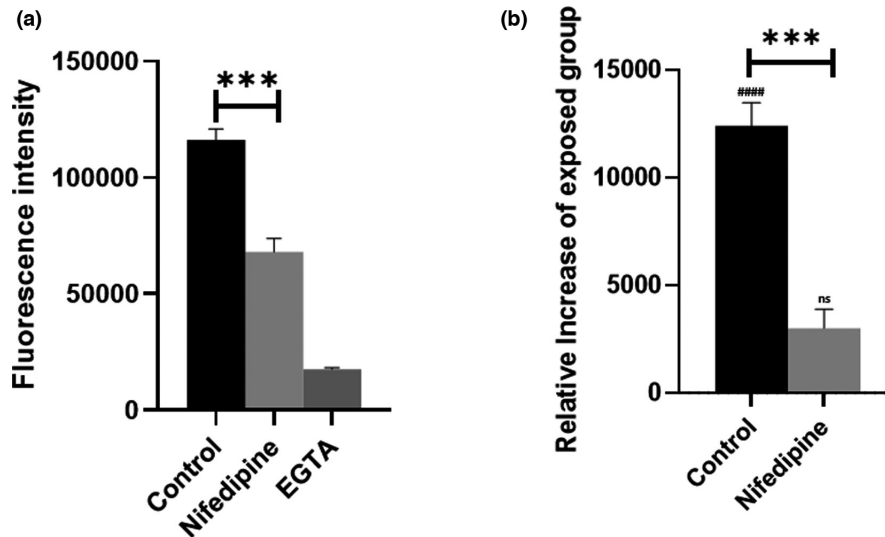
**FIGURE 4** Nifedipine administration confirms the presence of Nifedipine-sensitive channels on the membrane of HEK 293 cells, as their involvement in the increase in intracellular $Ca^{2+}$ elicited by RF-EMFs. Data in (b) are displayed as relative increase of exposed group when compared to sham. (*) represent inter-condition significance while (#) display significance when compared to sham. (a) The intracellular $[Ca^{2+}]$ is lowered by the addition of 10 μM Nifedipine. Response to $Ca^{2+}$ chelator EGTA was observed to be minimal. (b) Administration of 10 μM Nifedipine is sufficient to null any difference between sham and exposed group at $T_2$ exposure. $N = 45$ microplate wells from 3 independent experiments. $p$-value < 0.001

## 3.3 | Blockage of ER replenishment impairs RF-EMF effects

The impact of Nifedipine, mainly thought to target cell membrane $Ca^{2+}$ channels, was apparently in contrast with the intracellular origin suggested by the $Ca^{2+}$-free experiments described above. However, Nifedipine was also shown to interfere with the dynamics of intracellular $Ca^{2+}$ stores (Curtis & Scholfield, 2001; Rosales & Brown, 1992), mainly represented, in non-excitable cells, by the endoplasmic reticulum (ER). In order to elucidate the role of intracellular $Ca^{2+}$ stores, we decided to target the ER-cytoplasm $Ca^{2+}$ exchanges at different levels. In the ER, $Ca^{2+}$ release is mainly mediated by the activity of Ryanodine Receptors (RyRs). Hence, we assessed the impact of Dantrolene (10 μM), a commonly used antagonist of RyRs. Dantrolene treated group displayed no difference when compared to the control, both in the sham (Figure 5a) and exposed group (Figure 5b).

On the other hand, cytoplasmic $Ca^{2+}$ withdrawal is mainly mediated by sarco/endoplasmic reticulum $Ca^{2+}$-ATPases (SERCAs). These transporters are responsible for the replenishment of ER stores by consequence of the release of $Ca^{2+}$ in the cytoplasm. To inhibit the activity of SERCAs, cells were treated with 10 μM Thapsigargin, a commonly used SERCA blocker. An initial intracellular $Ca^{2+}$ increase was detected ($p$-value < 0.0001), in line with the blockage of ER replenishment as with the activation of non-selective $Ca^{2+}$-permeable cation channels triggered by depletion of intracellular stores. Strikingly,

in this group the increase at both $T_1$ and $T_2$ exposure was minimal, and no significant difference was observed between the two timepoints. Moreover, the relative increase was significantly different ($p$-value < 0.0001) to that of control and dantrolene-treated group, both at $T_1$ and $T_2$ (Figure 5b). This highlights the direct involvement of an internal $Ca^{2+}$ release, deriving from the ER, in the intracellular $Ca^{2+}$ increase elicited by EMFs.

All together these results suggest a cell membrane-independent mechanism, involving the ER intracellular $Ca^{2+}$ release in the modulations of $Ca^{2+}$ homeostasis perturbations elicited by EMFs.

## 4 | DISCUSSION

Our investigation on EMFs effect on intracellular $[Ca^{2+}]$ in HEK 293 cells revealed an important link between exposure to both static and oscillating magnetic fields and augmented presence of cytoplasmic $Ca^{2+}$. This outcome agrees with the abundant evidence found in literature about this correlation (Duan et al., 2014; Luo et al., 2014; Morabito et al., 2010; Prina-Mello et al., 2006). It is, however, in conflict with the results of other studies, showing no effect on basal $[Ca^{2+}]$ as consequence of exposure to RF-EMFs (O'Connor et al., 2010). This discrepancy could be due to the extreme variability of effects elicited by electromagnetic radiation, that is thought to be dependent on the frequency and the intensity of the field, the exposure time and the model studied (Bertagna et al., 2021). As a
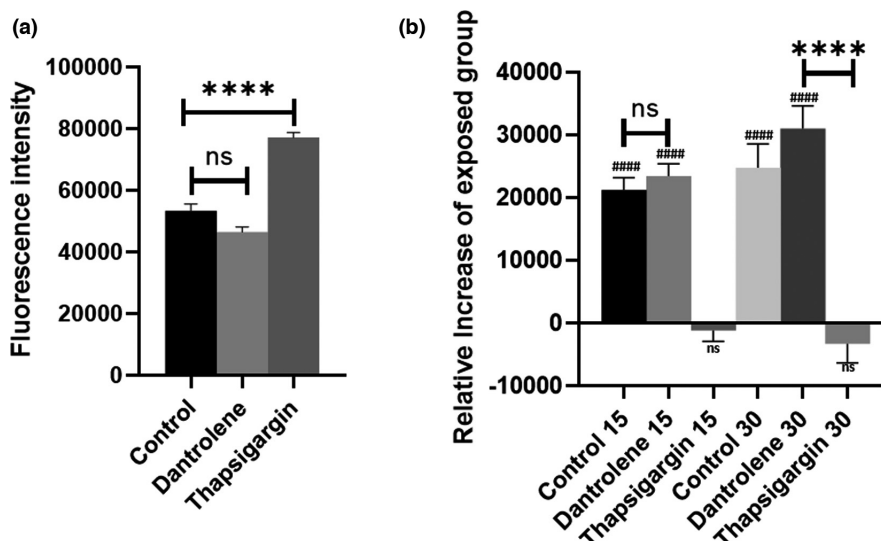
**FIGURE 5** Blockage of ER replenishment impairs RF-EMFs response. Data in (b) are displayed as relative increase of exposed group when compared to sham. (a) Baseline after incubation with 10 μM Dantrolene and 10 μM Thapsigargin. The block of SERCAs operated by Thapsigargin raise intracellular $Ca^{2+}$, impairing ER replenishment. The effect of dantrolene is not significant in line with the poor functional expression of RyRs in HEK 293 cells. (b) Administration of Thapsigargin is sufficient to completely null any difference between sham and exposed group at both $T_1$ and $T_2$ Dantrolene treated group displays no significant difference when compared to control. $N = 45$ microplate wells from 3 independent experiments. $p$-value $< 0.001$

consequence, the results of this study have to be related to the particular protocol used and cell line studied here. Nonetheless, the comparison between the two different exposure platforms used here (RF-EMFs and SMFs) supports the finding indicating oscillating EMFs, characterized by a continuous electromagnetic wave, to be more biologically active than SMFs, in virtue of their greater perturbation of electromagnetic homeostasis of the cell (Panagopoulos et al., 2015).

The relative concentrations of $Ca^{2+}$ in different subcellular compartments are finely regulated by different types of transporters, amongst which ion channels are thought to play a primary role (Carafoli & Crompton, 1978). In line with this, an involvement of these proteins in the modulation of EMF biological effects is well established. Indeed, the particular sensitivity of ion channels, as their role in controlling the different ionic concentration underlying the majority of cellular signaling pathways, makes them a perfect target for EMF effects (Funk et al., 2009). This is particularly evident in excitable tissues expressing voltage-gated ion channels (VGCs) as the central nervous system, where an electromagnetic wave, mainly in virtue of its electrical field component, can easily displace the charges on the protein voltage sensor, modifying the gating dynamics and the conductance of the channels themselves (Bertagna et al., 2021; Mathie et al., 2003). Both RF-EMFs and SMFs have been shown to affect VGC activity in many ways, including their expression, presence in the membrane, gating and inactivation dynamics (Kim et al.,

2018, 2019; Marchionni et al., 2006; Shen et al., 2007; Sun et al., 2016).

Despite their pivotal contribution, the dynamics of intracellular $Ca^{2+}$ exchanges are not exclusively determined by cell membrane channel activity and thus may be affected by EMFs at different levels, partially or totally independent by cell membrane dynamics. Indeed, as fluctuations in intracellular $Ca^{2+}$ results are the most commonly reported effect of EMF exposure (Bertagna et al., 2021), many studies state these perturbations to be independent by cell membrane dynamics (Luo et al., 2014; Morabito et al., 2010). Here, we observed an increase in intracellular $[Ca^{2+}]$ in $Ca^{2+}$-free conditions similar to the one reported in standard physiological conditions. This suggested an intracellular origin for the increase in $Ca^{2+}$. Importantly, a disruption in internal $Ca^{2+}$ homeostasis is linked to the activation of apoptotic pathways and numerous other pathways involved in cell survival (Orrenius et al., 2003). Notably, changes in apoptotic and autophagic pathways, are commonly reported as a result of EMF exposure (Kim et al., 2018), albeit this modulation could be a consequence of $Ca^{2+}$ homeostasis disturbance.

Here, we focused on the role of the endoplasmic reticulum (ER) that, along with mitochondria, is primarily involved in the control of cytoplasmic $[Ca^{2+}]$. Dantrolene treatment was proven to have no effect on the EMF-elicited $Ca^{2+}$ increase, displaying no difference when compared to the control group that received the same type of exposure. However, this could be due to the poor functional expression of endogenous RyRs in HEK 293 cells, as previously

shown by Western Blotting analysis (Tong et al., 1999). The functional presence of RyRs in HEK 293 cells is matter of debate, as different studies report conflicting results (Querfurth et al., 1998; Tong et al., 1999). Nonetheless, our results seem to support a limited involvement of these transporters in the intracellular $Ca^{2+}$ ER-cytoplasm efflux of HEK 293 cells, that could be conversely controlled by other proteins, as inositol 1,4,5-trisphosphate receptor ($IP_3R$) (Aoyama et al., 2004). It will be therefore important, in future investigations, to assess the role of these proteins in EMF-elicited $Ca^{2+}$ modulation.

We were able to null EMF response by impairing the replenishment of the ER through the administration of 10 μM Thapsigargin, a specific blocker SERCAs. Despite the presence of $Ca^{2+}$ in the external solution, the intracellular $Ca^{2+}$ was mostly abolished, suggesting a direct involvement of the ER release in the detected $Ca^{2+}$ raise. Moreover, no difference was observed between sham and exposed group, indicating a direct involvement of the ER internal stores in the EMFs modulation. These results are in line with both the involvement of ER in the intracellular $Ca^{2+}$ increase detected and with the involvement of a membrane independent mechanism, modulating EMF effects, as already observed as consequence of exposure to extremely low-frequency fields in entorhinal cortex neurons (Luo et al., 2014).

Noteworthily, the ER stress response is one of the numerous signaling cascades modulated by $Ca^{2+}$. This pathway is initiated within ER and was found to be critical for cell survival. ER stress is accompanied by alterations in $Ca^{2+}$ homeostasis and altered level of reactive oxygen species (ROS). Interestingly, altered oxidative stress has been linked to both ER abnormal function and different fields exposure (Morabito et al., 2010; Pooam et al., 2020), as to the pathogenesis of multiple human disorders, as cancer and neurodegenerative diseases, that seem to be also modulated by EMFs (Chio & Tuveson, 2017; Van Raamsdonk et al., 2017).

It is important to stress the early timepoints on which we focused this study. The rapid effects observed are in line with a broad literature reporting basal $Ca^{2+}$ increase as result of acute (<24 h) exposure to different frequencies and intensities of EMFs, but different, and sometimes opposite, effects are reported for chronic exposure. This controversy could be maybe resolved by considering the time-dependent cellular adaptation to perturbances of $Ca^{2+}$ homeostasis, that could involve both fast and slow regulations, as different (positive and negative) feedback loops.

A consensus on the specific contribution of $Ca^{2+}$ internal stores in EMF-elicited alteration of $Ca^{2+}$ homeostasis is still missing, as diverse mechanisms involving different cellular compartments could have a different impact depending on the tissue. Nonetheless, this research contributes to the understanding of the cellular effect elicited by EMF on internal $Ca^{2+}$ dynamics of HEK 293 cells and sheds a light on the key involvement of the ER in this modulation.

## CONFLICT OF INTEREST

The authors declare no competing interests.

## AUTHOR CONTRIBUTION

F.B., K.J., R.L., J.M., and S.R.P.S. designed the research. F.B., K.J., and R.L. analyzed the data. F.B. performed experiments. F.B., K.J., and R.L. wrote the paper. All authors approved the final manuscript.

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