Assessing the combined effect of extremely low-frequency magnetic field exposure and oxidative stress on LINE-1 promoter methylation in human neural cells



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Abstract

Extremely low frequency magnetic fields (ELF-MF) have been classified as "possibly carcinogenic", but their genotoxic effects are still unclear. Recent findings indicate that epigenetic mechanisms contribute to the genome dysfunction and it is well known that they are affected by environmental factors. To our knowledge, to date the question of whether exposure to ELF-MF can influence epigenetic modifications has been poorly addressed. In this paper, we investigated whether exposure to ELF-MF alone and in combination with oxidative stress (OS) can affect DNA methylation, which is one of the most often studied epigenetic modification. To this end, we analyzed the DNA methylation levels of the 5′untranslated region (5′UTR) of long interspersed nuclear element-1s (LINE-1 or L1), which are commonly used to evaluate the global genome methylation level. Human neural cells (BE(2)C) were exposed for 24 and 48 h to extremely low frequency pulsed magnetic field (PMF; 50 Hz, 1 mT) in combination with OS. The methylation levels of CpGs located in L1 5′UTR region were measured by MassARRAY EpiTYPER. The results indicate that exposures to the single agents PMF and OS induced weak decreases and increases of DNA methylation levels at different CpGs. However, the combined exposure to PMF and OS lead to significant decrease of DNA methylation levels at different CpG sites. Most of the changes were transient, suggesting that cells can restore homeostatic DNA methylation patterns. The results are discussed and future research directions outlined.

Keywords (separated by '-'): DNA methylation - Epigenetics - LINE-1 - Retrotransposition - Extremely low frequency magnetic field - Oxidative stress

Footnote Information: Gianfranco Giorgi and Chiara Pirazzini are co-first authors.

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ORIGINAL ARTICLE

# Assessing the combined effect of extremely low-frequency magnetic field exposure and oxidative stress on LINE-1 promoter methylation in human neural cells

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**Abstract** Extremely low frequency magnetic fields (ELF-MF) have been classified as "possibly carcinogenic", but their genotoxic effects are still unclear. Recent findings indicate that epigenetic mechanisms contribute to the genome dysfunction and it is well known that they are affected by environmental factors. To our knowledge, to date the question of whether exposure to ELF-MF can influence epigenetic modifications has been poorly addressed. In this paper, we investigated whether exposure to ELF-MF alone and in combination with oxidative stress (OS) can affect DNA methylation, which is one of the most often studied epigenetic modification. To this end, we analyzed the DNA methylation levels of the 5′untranslated region (5′UTR) of long interspersed nuclear element-1s (LINE-1 or L1), which are commonly used to evaluate the global genome methylation level. Human neural cells (BE(2)C) were exposed for 24 and 48 h to extremely low frequency pulsed magnetic field (PMF; 50 Hz, 1 mT) in combination with OS. The methylation levels of CpGs located in L1 5′UTR region were measured by MassARRAY EpiTYPER. The results indicate that exposures to the single agents PMF and OS induced weak decreases and increases of DNA methylation levels at different CpGs. However, the combined exposure to PMF and OS lead to significant decrease of DNA methylation levels at different CpG sites. Most of the changes were transient, suggesting that cells can restore homeostatic DNA methylation patterns. The results are discussed and future research directions outlined.

**Keywords** DNA methylation · Epigenetics · LINE-1 · Retrotransposition · Extremely low frequency magnetic field · Oxidative stress

Gianfranco Giorgi and Chiara Pirazzini are co-first authors.

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## Introduction

Extremely low frequency magnetic fields (ELF-MF) are pervasive in today's society. Indeed, people are exposed to increasing electromagnetic fields generated by power lines and ordinary electric and electronic devices on a daily basis. In 2002, the International Agency for Research on Cancer surmised that ELF-MFs increase the risk of neoplastic malignancies and classified them as "possibly carcinogenic to humans" (IARC 2002).

Various in vivo and in vitro studies have been carried out to understand the molecular mechanisms behind the biological effects induced by ELF-MF, but a clear picture has not yet emerged. Moreover, the assessment of genotoxicity by standard genotoxicity assays has given conflicting results, so the question whether ELF-MF can be involved in carcinogenesis or in cancer progression is still unanswered

(Vijayalaxmi and Prihoda 2009). Some reports suggested that ELF-MF exposure alone is not genotoxic but it can increase DNA damage in the presence of a genotoxic agent. Therefore, further research dealing with co-exposure evaluation should be considered.

Recent evidence suggests that non-genotoxic epigenetic mechanisms, such as DNA methylation, microRNA, long noncoding RNAs, histone code etc, are involved in aging and disease development and, in particular it is known that DNA methylation may play a key role in tumorigenesis and tumor progression (Klutstein et al. 2016).

DNA methylation, the most studied epigenetic mechanism, is a biochemical process where a methyl group is added to DNA nucleotides and, in mammals it typically occurs at cytosines in a CpG dinucleotide. DNA methylation may have a role in the control of gene expression by acting on regulatory elements. Cancer cells often show hypermethylation of the promoter region of specific genes and hypomethylation of the promoter region of repetitive elements, including long interspersed nuclear elements (LINE-1s or L1s) (Klutstein et al. 2016; Cruickshanks et al. 2013; Schulz 2006).

L1 elements constitute approximately 17% of the human genome. A full length L1 element is about 6 kb and consists of a 5′ untranslated region (5′UTR) with sense and antisense promoter activity, two open reading frames (ORF1 and ORF2), encoding proteins involved in retrotransposition and 3′ untranslated region (3′UTR) with polyadenylation site. Recently, an additional ORF (ORF0) has been reported in the primate lineage and it has been suggested that it could play some positive regulatory role in the retrotransposition process (Denli et al. 2015). After transcription, the L1 retroelement can be inserted into another genomic site by target-primed reverse transcription (TPRT) mechanism. L1 insertion can cause insertional mutagenesis, DNA double-strand breaks, exonisation or shuffling of genetic material, resulting in genetic instability (Iskow et al. 2010).

Several studies have shown an inverse correlation between L1 expression and the methylation status of the CpG island in L1 5′-UTRs (Bourc'his and Bestor 2004). Indeed tumor cells often show both low DNA methylation levels of the L1 5′-UTR promoter region and high L1 retrotransposition activity (Schultz 2006), with consequent alterations of gene expression and genomic instability. Moreover, recent evidence suggests that alterations of the L1 promoter methylation level might be involved in several cell processes, including cell replication timing and chromatin organization (Belan 2013).

Emerging data indicate that changes in L1 5′-UTR methylation levels can be induced by environmental factors (Bollati et al. 2007; Pogribny and Beland 2013). It has been suggested that L1 5′-UTR methylation evaluation should be included in health risk assessment of environmental (Vrijheid et al. 2014; Chappell et al. 2016).

To the best of our knowledge, only one paper addressed the issue of evaluating the effects of ELF-MF exposure on DNA methylation, reporting that methylation changes occurred in mouse spermatocyte-derived GC-2 cell line under exposure to ELF-MF (Liu et al. 2015).

The aim of this study is to assess whether the exposure to ELF-MF, alone and in combination with oxidative stress (OS), induces changes in methylation of L1 5′UTR region in human cells. A combined exposure was tested to simulate condition of real life, where the simultaneous exposure to ELF fields and other stress agents normally occurs. OS was chosen as co-stressor having been shown to affect DNA methylation (O'Hagan et al. 2011) and to contribute to tumorigenesis and tumor progression (Kryston et al. 2011; Li et al. 2015).

A pulsed magnetic field (PMF) was used since it is produced by several devices and is widely used in clinical applications. Moreover, it was shown to be biologically effective in our previous investigations (Del Re and Giorgi 2013; Del Re et al. 2012).

We used the BE(2)C human cell line, which is representative of neuronal cell type (Biedler et al. 1978) because ELF-MF effects on neuronal cells appear interesting for the risk assessment. Indeed, epidemiological studies suggested a possible relationship between Alzheimer's disease, brain tumors and ELF-MF exposure (Qiu et al. 2004; Li et al. 2009).

## Materials and methods

### Cell culture and treatments

Neuroblastoma BE(2)C cells were kindly provided by Prof. Della Valle (University of Bologna, Italy), and were maintained in Dulbecco's modified Eagle's medium (DMEM, EuroClone, Milano, Italy), supplemented with 10% heat-inactivated fetal bovine serum (FBS, EuroClone), 100 UI/ml penicillin (Sigma, Ronkonkoma, NY, USA) and 100 µg/ml streptomycin (Sigma), in a humidified 5% carbon dioxide air atmosphere at 37 °C.

24 h before PMF/Sham exposure, BE(2)C cells were seeded into 3 cm petri dish at the density of 75,000 cells/dish.

BE(2)C cells were exposed to 300 µM $H_2O_2$ (Sigma) for 1 h. Control cultures were treated with equivalent volumes of distilled water. This dose has been largely used in studies dealing with oxidative stress and does not greatly affect the cell viability of our cellular model, as previously reported (Giorgi et al. 2011, 2014).





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## Exposure system and field characteristics

The exposure system has been previously described (Del Re et al. 2012) and is shown in Fig. 1. It consisted of two systems, each composed by two sets of horizontal Helmholtz coils of 25 cm diameter, with 40 (20 + 20) turns that were double-wrapped to obtain wound (active coil) or counter-wound configuration. In the counter-wound configuration, the current is the same as in the active coil but the MF is zero (sham). The coils are powered by a home-made DC current amplifier, connected with a signal generator Model 33120A (Agilent Technologies, Loveland, CO, USA). Both the active and the sham coils were maintained in the same 5% $CO_2$ incubator (B-5060, Heraeus, Hanau, Germany) at a constant temperature of 37 °C, and at a sufficient distance to minimize the stray field from the active coil in such a way as to have in the Sham coils a magnetic field ≤1/50 of the field in the active system. The background field within the incubator was also measured: the static component of the local magnetic field was 16.9 μT (horizontal component 10.8 μT, vertical component 13.0 μT), the AC component was on the order of 0.1 μT, as measured with a very sensitive probe (EMDEX II, Enertech Consultants, Campbell, CA).

The system was controlled by means of a PC which, through an appropriate software and a switching system, randomly selected the active and sham coil system. All experiments were conducted in blind and only at the end of the experiments was the code decrypted. To have a field uniformity within 5%, the samples were placed within a virtual cylinder (about 11 cm in height, and 4 cm in diameter), centered with respect to the coil system. A bipolar pulsed-square wave magnetic field was chosen (Fig. 1b), with an intensity 0-peak of 1 mT, a 50-Hz repetition frequency, and a duty cycle 50%. The rise time τ of the square, from peak to peak, was about 0.6 ms, resulting in an average rate of change of magnetic flux density of 3.3 T/s. The MF was measured by means of a Bell gaussmeter (F.W. Bell 7010, Division of Test and Measurement, Orlando, FL); the error in the magnetic flux-density values was on the order of 2%.

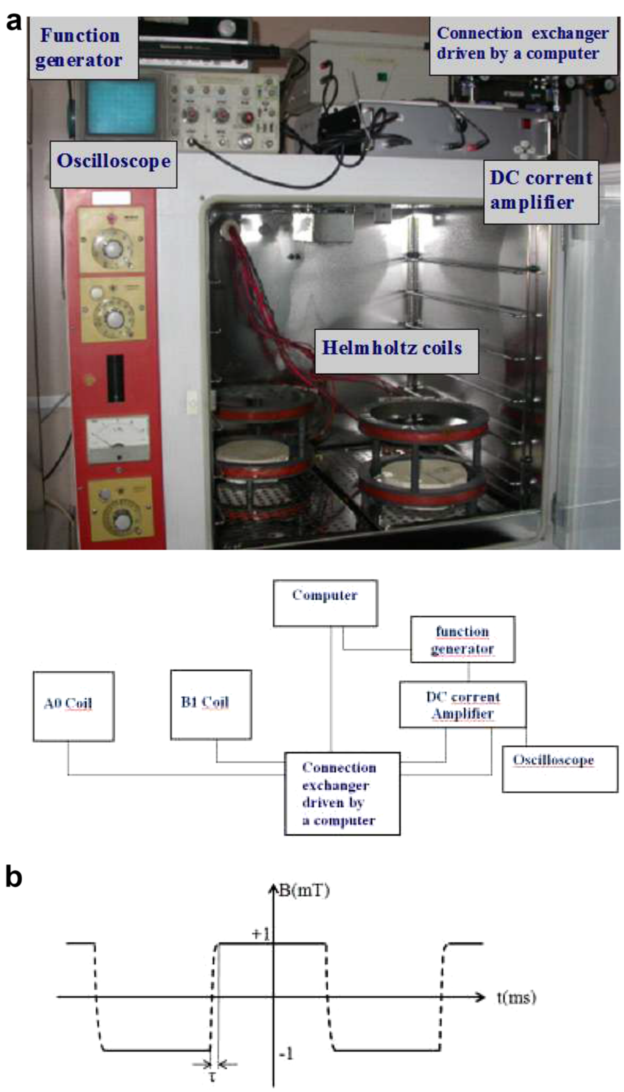


**Fig. 1** The exposure system (**a**) and PMF signal wave shape (**b**). The rise time τ of the square was about 0.6 ms

## DNA extraction and sodium bisulfite treatment

Genomic DNA was extracted by QIAmp DNA Mini Kit (QIAgen, Hilden, Germany) according to manufacturer's instructions. 1 μg was treated with sodium bisulfite using the EZ methylation kit (Zymo-Research, Irvine, CA). The treatment converts unmethylated cytosine into uracil, leaving methylated cytosine unchanged. In this way, variations in the sequence are produced according to DNA methylation status of the original DNA molecule.

## Methylation analysis

MassARRAY EpiTYPER technology (Sequenom) was used to quantitatively measure the methylation status of CpG sites within L1 5′UTR region (Accession No. X58075) (Fig. 2). 10 ng of bisulphite-treated DNA were PCR-amplified using the following primers: forward strand primer: AGGAAGAGAGTTTATTAGGGAGTGTTAGAT AGTGGG; reverse strand primer: CAGTAATACGACTCA CTATAGGGAGAAGGCTTCTATACCCTACCCCCA AAAATAAA.

By using these primers, we evaluated DNA methylation levels of 24 CpG units (i.e. regions containing one or multiple CpG sites), containing 28 CpG sites (Table 1).



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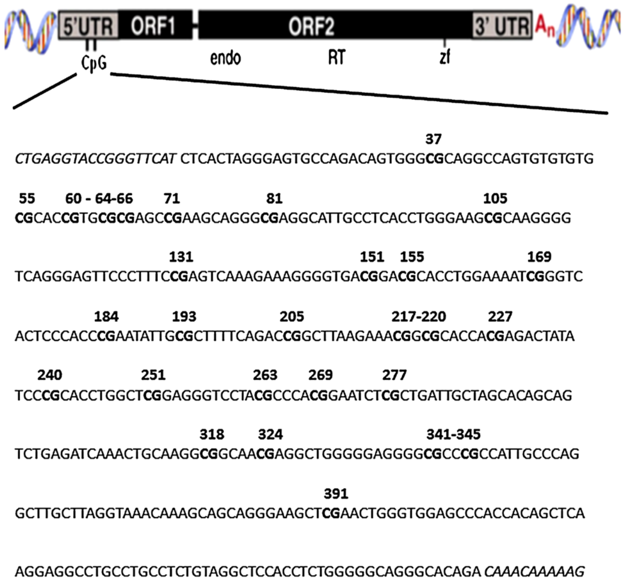


**Fig. 2** Schematic structure of an L1 element and CpG sites of the L1 5′UTR region. The sequence represents a 466 base pair fragment (Accession No. X58075). Numbers refer to locations of the CpG units interrogated for their methylation level by MassARRAY EpiTYPER

**Table 1** 24 CpG units containing 28 CpG sites of the L1 5′UTR region

| CpG units | Number of CpG sites |
|---|---|
| CpG_37 | 1 |
| CpG_55 | 1 |
| CpG_60-64-66 | 3 |
| CpG_71 | 1 |
| CpG_81 | 1 |
| CpG_105 | 1 |
| CpG_131 | 1 |
| CpG_151 | 1 |
| CpG_155 | 1 |
| CpG_169 | 1 |
| CpG_184 | 1 |
| CpG_193 | 1 |
| CpG_205 | 1 |
| CpG_217-220 | 2 |
| CpG_227 | 1 |
| CpG_240 | 1 |
| CpG_251 | 1 |
| CpG_263 | 1 |
| CpG_269 | 1 |
| CpG_277 | 1 |
| CpG_318 | 1 |
| CpG_324 | 1 |
| CpG_341-345 | 2 |

## Statistical analysis

Student's $t$ test was used to evaluate differences in methylation levels. A $p$ value <0.05 was considered to correspond with statistical significance.

## Results

To verify whether PMF exposure alone or in combination with OS would affect DNA methylation level of the L1 5′UTR region, BE(2)C cells were exposed or sham-exposed to PMF using the exposure system shown in Fig. 1. In the first hour of exposure, samples were subjected or not to OS (hydrogen peroxide 300 μM, 1 h). After 24 and 48 h of exposure, DNA methylation was evaluated by MassARRAY EpiTYPER technology, which is a highly accurate and sensitive method for the quantitative analysis of DNA methylation. We focused on a part of the L1 5′UTR region which is 466 bp in length and includes 24 CpG units (Fig. 2). The comparison of methylation levels of all CpGs among all the samples showed that the methylation level of 10 CpG units was modified depending on the type of treatment.

Effects of PMF exposure alone are shown in Fig. 3. After 24 h of PMF exposure, 3 CpGs (37, 217-220, 318) exhibited a significantly increased methylation level as compared to the CpGs from sham exposed samples (Fig. 3a). After 48 h of PMF exposure no significant differences were observed at any CpGs (Fig. 3b).

Results on the effects of OS alone are shown in Fig. 4. After 24 h three CpGs (184, 205, 277) exhibited significantly less methylation as compared to the CpGs from control samples (Fig. 4a). Also in this case, after 48 h no significant differences were observed at any CpG units (Fig. 4b).

Results about the effects of PMF exposure in combination with OS are shown in Fig. 5. After 24 h 5 CpG units (37, 131, 184, 217-220, 318) exhibited significantly less methylation as compared to the CpGs from samples exposed to PMF alone (Fig. 5a). After 48 h only 2 CpG units (60:64:66, 217-220) showed lower methylation levels than control (PMF) (Fig. 5b).

## Discussion

Epigenetic processes, including DNA methylation, are a molecular interface mediating the interaction between genome and environment. Changes in global genome methylation have been observed in association with exposure to such factors as air pollution (De Prins et al. 2013), gamma radiation (Kumar et al. 2011) low-levels of benzene





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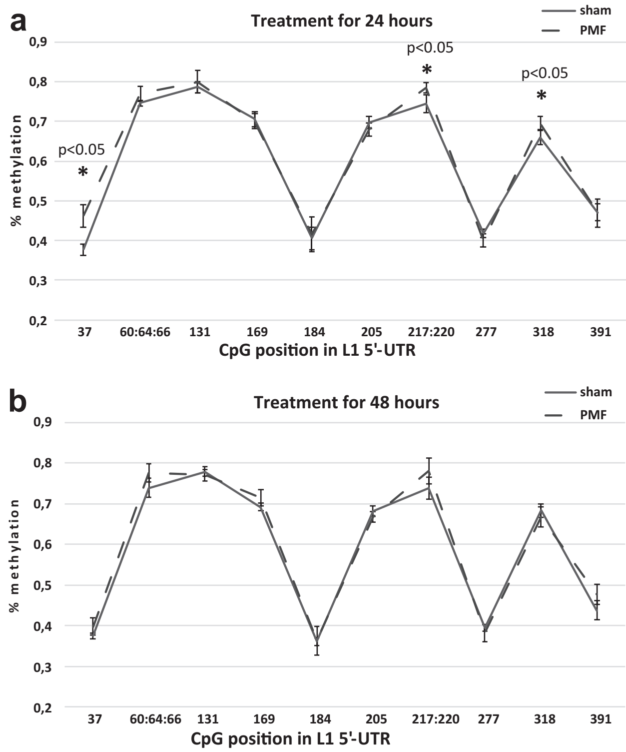


**Fig. 3** CpG dinucleotide methylation percentage of L1 5′UTR region in cells exposed to PMF (*dashed line*) or exposed to sham (control, *continuous line*) for **a** 24 and **b** 48 h. *Error bars* represent SEM of the values obtained from three independent experiments

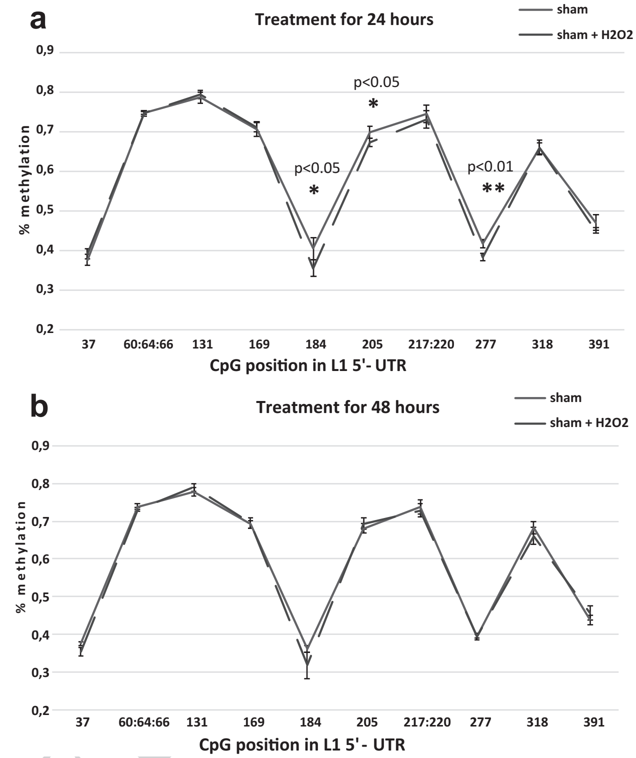


**Fig. 4** CpG dinucleotide methylation percentage of L1 5′UTR region in cells subjected to oxidative stress (300 μM $H_2O_2$ for 1 h) (*dashed line*) or distilled water (control, *continuous line*) after **a** 24 and **b** 48 h from the treatment. *Error bars* represent SEM of the values obtained from three independent experiments

(Bollati et al. 2007), cigarette smoke (Liu et al. 2010), synthetic compounds such as perfluoroalkylacids (Watkins et al. 2014), various genotoxic and non-genotoxic carcinogens (Pogribny and Beland 2013) and nutritional factors (Bacalini et al. 2014). However, the effects of ELF-MF on DNA methylation in human cells has never been studied. Therefore, we analyzed the DNA methylation levels of the L1 5′UTR region, which is commonly investigated as a surrogate for global genome methylation (Yang et al. 2004), in BE(2)C cells.

We showed that the exposure to PMF can interfere with DNA methylation inducing a slight increase in DNA methylation levels of some CpGs located in the L1 5′UTR region. Moreover, we found that OS alone induced a small and transient decrease of DNA methylation levels at some CpG units, whereas the combined exposures to PMF and OS induced a methylation decrease in 10 CpG units (Fig. 5). Therefore, in the presence of OS, the slight increase of methylation, induced by the exposure to PMF alone, disappeared.

The relationship between ELF-MF and oxidative stress has been largely debated and it has been proposed that ELF-MF can both induce ROS production and activate antioxidants, depending on the specific conditions tested (Manikonda et al. 2014; Di Loreto et al. 2009). Here, we observed that PMF synergistically contributes to OS effects. However, after 48 h of exposure methylation changes became undetectable. This result seems to be in line with the most recent evolutionary theories about the role of DNA methylation changes in humans (Klironomos et al. 2013; Flores et al. 2013; Giuliani et al. 2015). These theories suggested that methylation changes seem of crucial importance for rapid response to new stimuli, and in particular when new stimuli (in this case PMF + H202) arise. The data suggest that the environmental change from a normal condition—more than the constant exposure itself—increase DNA methylation variability, at least at the cellular level. The molecular mechanisms involved in these changes need to be validated in future studies but we can speculate as follows. DNA methylation patterns are dynamic states resulting from a continuous balance of methylation and demethylation. The ‘maintenance methyltransferase’ DNMT1 mainly maintains the methylation patterns across replication cycles, while de novo DNMT3A and DNMT3B enzymes mainly introduce methyl groups into unmethylated sites (Jurkowska et al. 2011). Currently, not much is known about the effects of electromagnetic



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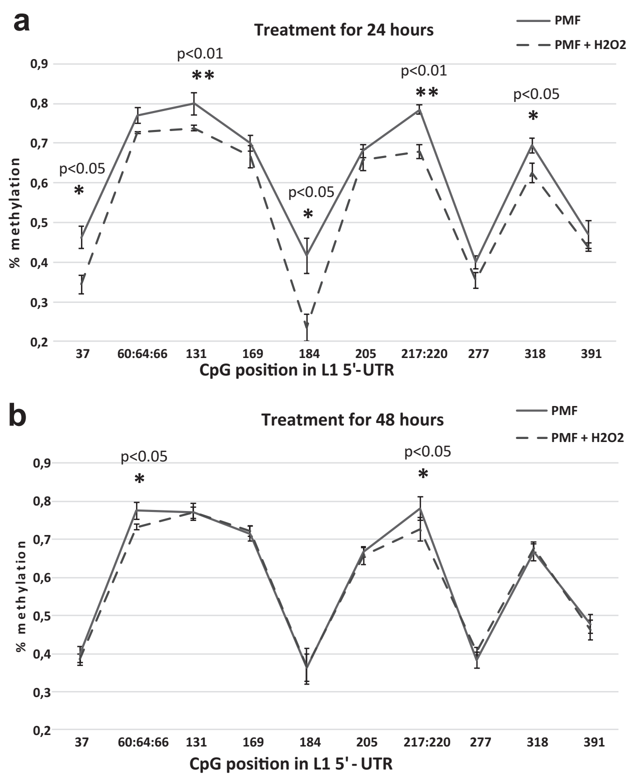


**Fig. 5** CpG dinucleotide methylation percentage of L1 5′UTR region in cells exposed to PMF and subjected to oxidative stress (300 μM $H_2O_2$ for 1 h) (*dashed line*) or distilled water (control, *continuous line*) after **a** 24 and **b** 48 h from the treatment. *Error bars* represent SEM of the values obtained from three independent experiments

fields on these enzymes, but a recent paper suggests that DNMT1 and DNMT3B activity can be modulated by intermittent ELF-MF exposure, depending on the magnetic field intensity (Liu et al. 2015). Kloypan et al. (2015) found that OS can induce LINE-1 hypomethylation and they observed that this effect was mediated through the depletion of S-adenosylmethionine (SAM) which is the classical methyl donor for methyltrasferases.

In our model, therefore, modulation of methyltransferase could be the mechanism responsible for the observed PMF effect, according with Liu et al. (2015). In addition, depletion of SAM could be the underlying reason for the OS effect, according to Kloypan et al. (2015). Finally, in the combined exposure, the presence of OS could determine an insufficient quantity of SAM, inhibiting the methyltransferase activity and, therefore, masking the increase of methylation induced by the PMF exposure alone.

Our data stimulate two methodological considerations. The first is about the time of exposure. Most studies on the relationship between DNA methylation alterations and environmental factors are epidemiological ones, which usually do not investigate the effects of different exposure times. We analyzed two different exposure times and we found different results, showing that the effects were transient. Therefore, whenever possible, it is strongly recommended to analyze various exposure times. The second consideration is about the CpG sites that are affected by ELF-MF/OS exposure. The MassARRAY EpiTYPER approach allows to quantitatively evaluate DNA methylation levels of multiple adiacent CpGs, providing more detailed information with respect to other commonly used approaches, such as the COBRA (combined bisulphite restriction analysis polymerase chain reaction) assay. We analyzed 24 CpG units and we observed that methylation changes occurred preferentially at specific CpG. This observation is in agreement with findings by Nüsgen et al. (2015), who observed that some specific CpG units within 5′-UTR L1 region are more prone to be subjected to methylation modifications. Our data suggest that it is important to analyse as many CpG sites as possible, since we do not known which sites are sensitive in each cell type and eventually affect gene transcription.

Emerging evidences reveal that microvesicles represent an important mechanism of cell to cell communication and that they can be involved in epigenetic processes including DNA methylation (Qian et al. 2015). Recently, it has been reported that microvesicles are released from cells upon activation by various stimuli including radiation (Jella et al. 2014) and ELF-MF exposure (Stratton et al. 2013). This aspect should be investigated, to verify whether it could be involved in the epigenetic alterations which we observed.

In conclusion, our results suggest that only some CpG units within L1 5′-UTR region could be subjected to methylation modification by PMF and OS exposure and that these alterations are, in any case, transient. The biological relevance of these transient variations of DNA methylation levels needs to be elucidated; they are at the forefront of important mechanisms of what is generally called "epigenetic stress". We hypothesize that these variations can explain some conflicting results obtained until now in in vitro cell systems after ELF exposure. Further studies are needed to clarify this point and to elucidate the epigenetic effect of ELF-MFs alone and in the presence of OS, also considering different cell types and exposure scenarios.

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**Compliance with ethical standards**

**Conflict of interest** The authors report no conflicts of interests. The authors alone are responsible for the content and writing of the paper.





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