Genotoxicity of 50 Hz magnetic field in human SH-SY5Y neuroblastoma cells

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ABSTRACT
There have been discussions on the genotoxicity of extremely low frequency (ELF) magnetic field (MF) over the past two decades concerning appliances emitting electromagnetic field. The current study was designed to establish whether ELF MFs might cause genotoxic effect on human SH-SY5Y neuroblastoma cells. Numerous studies have suggested that there is a risk of cancer associated with exposure to ELF MFs. The results of the studies have been, however, inconsistent on mutagenicity and carcinogenicity of ELF MFs. In this study human SH-SY5Y neuroblastoma cells were exposed to 50 Hz MF (100 µT) for 24 h, after which they were co-exposed with menadione at different concentrations for the last 1 h. After the exposure, the samples were analyzed by alkaline comet assay to evaluate immediate DNA damage and repair, and micronucleus frequency was used to assess the persistent DNA damage. Mere menadione exposure and co-exposure to MF + menadione caused increased immediate DNA damage. Pre-exposure to MF decreased DNA repair rate as compared with menadione exposure alone. Pre-exposure to MF seems to decrease menadione-induced micronucleus frequency. In conclusion, pre-exposure to MF seems to affect menadione-induced DNA damage responses.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>4NQQ</td>
<td>4-nitroquinoline 1-oxide</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BLM</td>
<td>Bleomycin</td>
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<tr>
<td>CA</td>
<td>Chromosome aberration</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DMBA</td>
<td>7, 12-Dimethylbenz (a)anthracene</td>
</tr>
<tr>
<td>ELF</td>
<td>Extremely low frequency (0-300 Hz)</td>
</tr>
<tr>
<td>EMF</td>
<td>Electromagnetic field</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMS</td>
<td>Ethylmethanesulfonate</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz, cycles per second, the unit of frequency</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>ICNIRP</td>
<td>International Commission on Non-Ionizing Radiation Protection</td>
</tr>
<tr>
<td>IF</td>
<td>Intermediate frequency (300 Hz-100 kHz)</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared (300 GHz-300THz)</td>
</tr>
<tr>
<td>MF</td>
<td>Magnetic field</td>
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<tr>
<td>MN</td>
<td>Micronuclei</td>
</tr>
<tr>
<td>MMC</td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>MMS</td>
<td>Methyl methanesulphonate</td>
</tr>
<tr>
<td>MQ</td>
<td>Menadione</td>
</tr>
<tr>
<td>NIEHS</td>
<td>National Institute of Environmental Health Sciences</td>
</tr>
<tr>
<td>OTM</td>
<td>Olive Tail Moment</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>RF</td>
<td>Radiofrequency (100 kHz-300 GHz)</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>SCE</td>
<td>Sister chromatid exchange</td>
</tr>
<tr>
<td>SCENIHR</td>
<td>Scientific Committee on Emerging and Newly Identified Health Risks</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet (radiation)</td>
</tr>
<tr>
<td>VIS</td>
<td>Visible light (400-750 nm)</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</tbody>
</table>
CONTENT

ACKNOWLEDGEMENTS .................................................................................................................. 3

Abbreviations ................................................................................................................................. 4

1. INTRODUCTION .......................................................................................................................... 7

2. LITERATURE REVIEW .................................................................................................................. 8

2.1 50Hz ELF Magnetic field ......................................................................................................... 8

2.2 Genotoxicity ............................................................................................................................... 9

2.3 Genotoxicity of 50 Hz ELF Magnetic Field ............................................................................... 10

3. AIM OF THE STUDY ..................................................................................................................... 15

4.1 Cell line and culture conditions ............................................................................................... 16

4.2 Exposure to 50 Hz magnetic fields ............................................................................................ 16

4.3 Exposure protocol ..................................................................................................................... 17

4.4 Comet assay ............................................................................................................................. 18

4.5 Micronucleus assay using flow cytometry ................................................................................. 19

4.6 Statistical analysis ..................................................................................................................... 21

5. RESULTS ....................................................................................................................................... 22

5.1 Immediate DNA damage and DNA repair ................................................................................. 22

6. Discussion ....................................................................................................................................... 26

7. Conclusion ...................................................................................................................................... 28
1. INTRODUCTION

Electromagnetic fields (EMFs) from alternating current sources, such as power lines, distribution of electricity and use of electrical appliances, with frequencies in the range of 3 – 300 Hz and wavelengths from 100,000 to 1000 km are called extremely low frequency fields (ELFs). In the last decade, a lot of focus and concern has been directed to the exposure of all living things (including human) to various forms of environmental electromagnetic fields (EMFs), due to their possible carcinogenicity. Since these EMFs are generated during the generation and distribution of electricity as well as during the use of electrical appliances, everyone is continuously exposed to the field. Also, the era of industrialization has lead to a serious increase in the exposure, (WHO, 2007).

The possible health effect of this exposure was studied in the late 1970s by Wertheimer and Leeper, when they showed a positive association between exposure to residential EMF and childhood leukemia in their study on cancer in children living near power lines. Subsequently, there has been a great deal of studies focus on the possible relationship between EMFs and most biological processes (Santora et al., 1997). Based on the review of series of studies (in vivo, in vitro and epidemiological) on the carcinogenicity of ELF MFs, the International Agency for Research on Cancer (IARC) classified ELF MFs as “possibly carcinogenic to humans (IARC 2002). Although there is currently no generally accepted explanation for the mechanism of carcinogenic effects of weak ELF MFs, they are classified as possible carcinogens. Several different types of studies are therefore needed to clarify the interaction between biological systems and the ELF MFs as well as their health impacts.

This study therefore tried to look at the possible genotoxic effects of 50 Hz magnetic field on human cells by exposing the human SH-SY5Y neuroblastoma cells to 50 Hz magnetic field and menadione at different concentration levels.
2. LITERATURE REVIEW

2.1 50Hz ELF Magnetic field

Electromagnetic spectrum consists of electromagnetic waves grouped according to their frequencies or wavelengths. Depending on their ability to ionize molecules (movement of an electron to a higher energy state; Kwan-Hoong Ng 2003), the electromagnetic spectrum is usually divided into ionizing and non-ionizing radiations. Ionizing radiations, such as gamma rays, cosmic rays and X-rays, have enough energy per quantum to break bonds between molecules while the non-ionizing radiations do not carry enough energy to do so. Electromagnetic field spectrum is presented in Fig. 1.

![Electromagnetic Spectrum Diagram](image)

**Figure 1.** The electromagnetic spectrum (Adapted from Markkanen 2009)

Some of the electromagnetic fields (EMFs) from alternating current sources, such as power lines, distribution of electricity and use of electrical appliances, with frequencies in the range of (3 – 300) Hz and wavelengths from 100,000 to 1000 km are called extremely low frequency (ELF) fields. Their strength depends on a lot of factors, such as distance from the source, number of sources and type of source. For instance, EMFs like 50 Hz EMF, with long
wavelength, are strongest close to their sources and weaker with distance. While naturally occurring 50 Hz EMF levels are extremely low, higher level fields are associated with sources like power lines. Numerous studies have also looked at occupational exposure to ELF magnetic fields and some suspected biological effect like brain cancer and leukaemia (Kheifets 2001). The elevated risk of leukaemia and brain tumours among finnish workers exposed to ELF magnetic field (Juutilainen et al., 1990)

Specifically, EMFs with very short wavelengths have the energy capacity to be deposited into the body while those with very long wavelength (at low frequencies such as 50 Hz) do not. The possible cocarcinogenic effects of ELF may require repeated long-term interaction with known carcinogens (Juutilainen et al., 2000). The basis of guidelines for limiting human exposure to ELF MFs is about 10 mT at 50 Hz, higher than these could cause adverse biological effects (ICNIRP 1998). According to Kumlin (2004), although direct effects of ELF magnetic fields on biological tissues have been reported, which includes the stimulation of excited tissues due to the induction of electric currents, the intensity of the 50 Hz magnetic field must be about 10 mT for these effects to occur. The biological effect(s) can be positive or negative, permanent or temporary and in some instances, may not have any health effect at all. It is also note worthy that the occurrence of biological effects does not automatically mean health effects.

### 2.2 Genotoxicity

In all living organisms, maintenance of genomic integrity is so essential in order to have accurate transfer of genetic materials from one generation to another. Their genetic material is however continuously facing a number of both intracellular and extracellular stresses which could trigger several cellular responses that may lead to the instability of the cell. Therefore, harmful effects on genetic material that results in the lost of its integrity and possibly, changes in the genetic materials (mutagenicity) and/or the development of tumor (carcinogenicity) are called genotoxicity. According to Norbury and Hickson (2001), the cells induces reactions to damage on genomic materials such as, cell cycle arrest, activation of transcriptional programs, enhancement of DNA repair and apoptosis may even be initiated when the extent of damage is high.
DNA damage may be repaired (which is needed for survival) and may or may not result into damages which are inheritable. Different assays could be used to measure the initial damages and they include but not limited to, assay of phosphorylation of histone H2AX (γ-H2AX) and Comet assay (Watters et al., 2009), which was used in this study. It is also worthy of note, that the measurement of the damages caused, was done before the repair processes is finished and so may not be interpreted as harmful consequences.

2.3 Genotoxicity of 50 Hz ELF Magnetic Field

The accuracy of genomic information or features (usually encoded as DNA) transfer from one organism’s generation to another, depends mainly on the free replication of the organism’s DNA. Several studies have been carried out to ascertain the consequences of DNA exposure to these stress factors as well as their possible remedies. Consequences to stress factors (including physical and chemical agents) suggested and studied includes DNA damage formation and repair (Svedenstal et al., 1999, Ivancsits et al., 2002; 2003, Cooke et al, 2003; Focke et al., 2008; & Luukkonen et al., 2011;), cell proliferation (Robinson et al., 2002), and chromosomal aberrations (Nordenson et al., 1994), just to mention a few. The result of most of these studies showed conflicting findings and thereby makes it difficult to currently arrive at a conclusive result. Some experimental findings shows that (Sommer and Lerchl 2004 but it may induce the risk of tumor growth in sensitive subpopulations (Sommer and Lerchl 2004). In 1994, Nordenson et al showed that there was no increase in the frequency of chromosomal aberrations, in human amniotic cells exposed to 50 Hz EMF at 30 mT for 72 h. Human lymphocytes exposed to 50 Hz and there was no significant effect on chromosome aberration, sister chromatid exchanges and single-strand breaks (Maes et al., 2000, Hone et al 2003, Verheyen et al., 2003 Luceri et al., 2005). Svedenstal and Johanson (1998) showed that there was no increase of micronucleated erythrocytes in adult mice exposed for 90 days to a 14 µT magnetic field. Kumlin et al., (2005) reported the effect of 50 Hz (100 µT) MF exposure on melatonin production in female CD2F1 (BALB/c x DBA/2) mice and the results of the findings showed increase in the sensitivity of the pineal gland. The 50 Hz magnetic field exposure (1 µT and 100 µT) has no effect on the body weight gain or survival rate of mouse and lymphoma incidence did not differ between exposed and sham-
exposed animals (Sommer and Lerch 2004). Also, study by Svedenstal et al (1999) using the
brain cells of CBA mice exposed to 50 Hz 0.5 mT for 2 h, 5 days or 14 days showed no
genotoxic effect and after 14 days increase in the DNA strand break. Yokus et al., (2008)
conducted studies on rat leucocytes exposed to 50 Hz 100 and 500 µT for 2 h/day during 10
month, the result reveled oxidative DNA damage was increased in both 100 µT and 500 µT
ELF-MF exposed groups. There could also be also effect at as was reported by (Mariucci et
al., 2010) on CDI mice exposed to 50 Hz MF, 1 mT for 1 or 7 days (15 h / day), the result
shows an increase in primary DNA damage in all cerebral areas of the exposed mice
compared to controls. Kim et al., (2010) conducted studies on DNA double-strand breaks in
IMR90 (human lung fibroblast) primary cells and HeLa (human cervical carcinoma) cell,
using time-varying magnetic field 6 mT for 30 min (every 24 h for 3 days), the results
revealed that repetitive exposure to MF with extremely low frequency can induce DNA
DSBs. Mouse macrophages exposed to 50 Hz MF, 1 mT, for 12, 24 or 48 h. shows no
increase of micronucleus frequency (Frahm et al., 2006). Udroiu et al., (2006) conducted
studies on clastogenicity and aneuploidy in newborn and adult mice exposed to 50 Hz
magnetic field, the result shows a significant increase in MN in MF exposure, also no
significant effect was recorded on exposed adult mice. The exposure of Wistar rats to 50 Hz 1
mT for (1 day for 4 h), and long term (4 h/day for 45 days) do not show any statistically
significant difference in chromosome aberration between the negative control group and the
exposed group (Erdal et al., 2007). Focke et al., (2008), who studied human fibroblast cell
lines exposed to 50 Hz, 1 mT ELF-EMF for 15 h, reported that there was also no significant
difference between exposed and non-exposed cells, with respect to DNA damage formation
and repair as well as cell cycle progression. There was no significant effect on DNA strand
breaks in cerebella cells of immature mice exposed continuously to a 60 Hz magnetic field at
1 µT 2 h (McNamee et al., 2002). E. coli transfected (pTN89) exposed to 60 Hz 5 mT
continuously for 4 h. showed no genotoxicity effect on the bacteria strain (Koyama et al.,
2004). Verschaeve et al., (2011) reported a genotoxicity investigation of extremely low-
frequency ELF magnetic field (MFs 50 Hz 100 and 500 µT, 1 and 2 h exposure using
salmonella typhimurium bacteria, the result shows ELF-MF do not induce SOS-Based
mutagenicity in S.typhimurium bacteria. These results were supported by Williams et al
(2006), when they reported that exposure of salmonella strains of bacteria to 60 Hz 14.6 mT
for 4 h did not induce single or double-strand breaks in DNA. Several studies have reported
increased in genotoxicity properties of ELF magnetic field alone both in vitro and in vivo
exposure. Winker et al., (2005) conducted studies on human fibroblast cells exposed to 50
12 Hz, 1 mT ELF-MF for 2 – 24 h and the results revealed a time-dependent increase in micronuclei and chromosomal aberrations. This was supported by Fatigoni et al., (2005), who reported that 50 Hz MF of 1 mT is genotoxic in the Trad-MN bioassay of tradescantia exposed for 1.6 and 24 h. There was increase in the genotoxic potential due to intermittent EMF exposure and dose and time-dependent single and double-strand DNA breaks reported in human diploid fibroblasts cells exposed to 50 Hz sinusoidal 1000 µT MF for 24 h and 50 Hz sinusoidal 20 h respectively (Ivancsits et al., 2002 and 2003). Sister chromatid exchange (SCE) in dividing human peripheral blood lymphocytes were exposed to 50 Hz (sinewave or squarewave), 1 µT or mT in either a pulsed (4 s on/4 s off) or continuous form for 72 h, the result shows a significant increase in the number of SCEs/ cell in the grouped experimental condition compared to the controls (Wahab et al., 2007). Lai and singh (1997) reported an increase in both single and double-strand DNA breaks in the brain cells of male Sprague-Dawley rats exposed to 60 Hz MF at 0.1, 0.25 and 0.5 mT for 2 h. Rat-I fibroblasts, WI-38-diploid fibroblasts and HL-60 leukaemia cell were exposed to 50 Hz MF (0.5, 0.75, and 1 mT) for 24, 48, and 72 h, after 24 h exposure there were a dose-dependent increase in SSB was found in all cell lines (Wolf et al., 2005). Others such as Ravindra et al., (2010) and Sarimov et al., (2011) studied human peripheral blood leucocytes exposed to 0.2, 0.4, 0.6, 0.8 and 1.0 mT at 50 Hz for 15, 1h and whole human peripheral blood samples exposed to 50 Hz 5 – 20 µT for 15 – 180 minutes respectively. According to their findings, the DNA integrity in human peripheral blood leucocytes was altered by way of increase in DNA damage (Ravindra et al., 2010) while increases in chromatin conformation in human lymphocytes were dependent on donor (Sarimov et al., 2011). Robison et al., (2002) discovered decreased DNA repair rates in HL-60 and HL-60R cell lines and not in Raji cell line of human promelocytic leukeamia exposed to 0.15 mT 60 Hz for 4 and 24 h.

Since exposure to EMF usually occur along side other stress factors (physical and chemical), co-exposure with other factors or agents have also been studied several of the co-exposure studies showed that the genotoxicity of MF may be altered by co-exposure with other stress factors. In other words, their effects could be synergic, antagonistic or neutral depending on the agent or factor involved as well as dose and time of exposure. For example, although male Sprague-Dawley rats, using an in-vivo newborn rat astrocyte micronucleus assay, exposed for 24, 48 and 72 h did not show any genotoxic effect when exposed to 50 Hz 7.5 – 10 mT MF alone, it however increased the genotoxic activity of Cisplatin upon co-exposure for the same period of time and exposure parameter (Miyakoshi et al., 2005). There was
reported increase in the oxidative cellular damage in Rabbit RBCs upon co-exposure to 50 Hz 0.2-0.5 mT for 90 min together with Ascorbate and FeSO$_4$ (Fiorani et al., 1997). Magnetic fields were also reported to act as a promoter or as a co-carcinogenic factor in X-ray induced micronuclei of CHO-K1 cells when the cells were co-exposed to X-rays (1 Gy) and 50 Hz 5 mT for 24 h, while exposure to the ELF field alone could not induce micronuclei in CHO cells (Ding et al., 2003). The co-exposure effects however may vary from one treatment to another depending on factors such as exposure conditions and agents. Jurkat cells (human lymphoblastoid T-cells) tested for possible genotoxic and/or co-genotoxic activity of 50 Hz 5 mT with Xenobiotic Benzene (1, 4-BD, and 1, 2,4-BT), for 1 and 24 h showed increased frequency of MN upon exposure for 24 h to 5 mT (50 Hz) MF and no effect of benzene to the genotoxic activity after 1 h exposure (Pasquini et al., 2003). Villarini et al., (2006) conducted studies on DNA damage in human peripheral blood leukocytes together with a 50 Hz, 3 mT for 30, 60, or 120 min. Cells were simultaneously exposed to N –methyl-N’-N-nitro-N-nitrosoguanidine (MNNG) or 4-nitroquinolin 1-oxide (4NQO), with co-exposure to MF increased DNA damage measured by alkaline comet assay in co-exposure compared to the cells exposed to MNNG alone. When they combined MF exposure and 4 NQO, DNA damage was decreased compared to the cells exposed to the mutagen alone. There could also be no effect at all, as was reported by Heredia-Rojas et al., (2004) who tested mouse germ cells (in vivo) for meiotic chromosome aberrations and sperm morphology after co-exposure to 60 Hz 2.0 mT MF for 72 h (continuous 8 h daily for 10 consecutive days) and colchicine (2 h after MF exposure). Human lymphocytes co-exposed to 50 Hz and vinblastine shows no significant effect on chromosome aberration, sister chromatid exchanges and single-strand breaks (Verheyen et al., 2003). There was no synergic or antagonistic effect from combined exposure of human blood samples to 50 Hz 1 mT for 48 h and 5 min exposure to X-rays (Testa et al, 2004). Human peripheral blood cells co-exposed to 0.23, 0.47 and 0.7 mT (at 50 Hz) for 12 h and Gy X-rays, showed no enhancement of aberration yields in cells held in the magnetic fields (Lloyd et al., 2004). Falone et al., (2007) conducted studies on cell viability in human neuroblastoma cells (SH-SY5Y), exposed to 50 Hz, 1 mT, for up to 96 h, and co-exposed with hydrogen peroxide (H$_2$O$_2$) the results shows that MF exposure alone increased cell viability. There was also no detectable effect of MF 2.0 mT co-exposed with Mitomycin-C (72 h) on meiotic chromosome aberrations of male mice germ cells (Heredia-Rojas ., 2004). Juutilainen et al., (2006) reported a meta-analysis of data from in vitro studies and short-term animal studies that have combined extremely low frequency magnetic fields with known carcinogens or other toxic physical or chemical agents, and the
results of reviewed were positive, suggesting that magnetic fields do interact with other chemical and physical exposures.

A study on human diploid fibroblasts (ES-1) with hydrogen peroxide or mitomycin C (MMC) after exposure to 50 Hz for 24 h, to induce genotoxic effects, did not show any increase in micronucleus frequency (Scarfi et al., 2005). The result of so many more studies on co-exposure such as the induction of genotoxic and cytotoxic effects in cultured human lymphocytes exposed to 0.1 mT sinusoidal 50 Hz for 72 h showed no increases in induction of genotoxic and cytotoxic effects in cultured lymphocytes with (MMC) and hydroquinone (HQ) co-exposure (Zeni et al., 2001), while exposure of human glioma cells (glioma cell line UVW) to ELF-EMF 50Hz 1 mT for 12 h, increased the mutagenic capacity of 0.3 and 3 Gy gamma 2.6 and 2.75 upon co-exposure to ionizing radiation (Mairs et al., 2007). Also, co-exposure of human fibroblast cells CCD-986sk to 60 Hz 0.8 mT for 28, 88, 180, or 240 h and Bleomycin, to access the effect on delayed chromosomal instability, showed increased cytotoxicity of Bleomycin by co-exposure (Cho et al., 2007). Murine fibroblast cells tested for adaptative responses and cell cycle arrest by co-exposure to 50 Hz, MF of 100 or 300 µT for 24 or 48 h and UV or MQ for 20 or 60 min, showed altered cellular responses to subsequent exposure to MQ, decreased G1 cells and increased G2/M (G1 and G2 are phases in cell cycles Markkanen et al., 2008). After studying that co-exposure of EMF can lead to increased genotoxic effect and to test the rate of DNA damage, repair, micronucleus frequency and relative survival of the cells, Fedrowitz and Löscher (2008) conducted studies on mammary tumours (female Fischer 344 rats) exposed to 50 Hz 100 µT for 24 h/day 7/day/week 26 weeks and co-exposed to 7,12-dimethylbenz(a) anthracene (DMBA), the results shows that MF exposure increased the mammary tumourigenesis compared to control exposed rats. Luukkonen et al., (2011) exposed Human SH-SY5Y neuroblastoma cells, pre-treated with menadione and Methyl methanesulfonate (MMS) for 3 h, to 50 Hz MF of 100 µT for 24 h. The result showed that exposure of cells to MF at 100 µT alters cellular responses to menadione. E. coli transfected (pTN89) exposed to 60 Hz 5 mT and hydrogen peroxide (H₂O₂) continuously for 4 h. showed 2 fold increase in mutations on the bacteria strain (Koyama et al., 2004).
3. AIM OF THE STUDY

To evaluate the genotoxicity of extremely low frequency magnetic field on human SH-SY5Y neuroblastoma cells

The specific objective of the present studies was;

- to examine the exposure of the human SH-SY5Y neuroblastoma cells to 100 µT 50 Hz magnetic field and menadione at different dosage levels (0.1 µM, 1 µM, 10 µM, 15 µM, and 25 µM)
4. MATERIALS AND METHODS

4.1 Cell line and culture conditions

Human neuroblastoma (SH-SY5Y) cells (obtained from Dr Sven Påhlman, University of Uppsala, Sweden) were grown in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum (FBS) and 50 unite/ml penicillin and 50 µg/ml streptomycin. SH-SY5Y cells were incubated (+37 °C, 5% CO₂) in 75 cm² cell culture flasks (Nunc A/S, Roskilde, Denmark). Cells were harvested during exponential growth with 0.02 Ethylenediaminetetraacetic acid (EDTA) (Merck KGaA, Darmstadt, Germany) in Ca²⁺- and Mg²⁺- free phosphate buffer saline (PBS). The density was adjusted to 3x10⁶ per dish, and the 100 000 cells were sub cultured in 48-well plastic plates approximately 20 h before each experiment.

4.2 Exposure to 50 Hz magnetic fields

Cells cultures were incubated inside a temperature –controlled incubator (Heraeus HERACell, Kendro Laboratory Products, Hanau, Germany), with 5% CO₂. The cultured cells were placed at the center of a pair 340 mm x 460 mm coil system in order to ensure even distribution of the magnetic field. (The MF exposures were applied at a magnetic flux density of 100 µT for 24 h.) The current (sinusoidal 50 Hz) were generated from Wavetek Waveform Generator model 75 (Wavetek, San Diego, CA, USA) and amplified by a Peavey M-3000 Power Amplifier (Peavey Electronics corp., Meridian, MS, USA). Holaday H1-3624 ELF Magnetic Field Meter and Holaday ELF Magnetic Field Sensor P/N 491017 (Holaday Industries, INC., Eden Prairie, MN USA) was used to monitored Magnetic flux density.
4.3 Exposure protocol

**Comet assay**

<table>
<thead>
<tr>
<th>Pre-culture</th>
<th>20 h</th>
<th>MF</th>
<th>24 h</th>
<th>MQ</th>
<th>1 h</th>
<th>Comet assay</th>
</tr>
</thead>
</table>

Change of medium | Change of medium + (menadione)

During the comet assay analysis, the cells (3x10^6) were pre-cultured for 20 h, after that the medium was changed, then the cells were exposed to MF for 24 h and the medium was changed again, then cells was further exposed to menadione for 1 h.

**Micronucleus assay**

<table>
<thead>
<tr>
<th>Pre-culture</th>
<th>20 h</th>
<th>MF</th>
<th>24 h</th>
<th>MQ</th>
<th>3 h</th>
<th>72 h</th>
<th>incubation</th>
</tr>
</thead>
</table>

Change of medium | Change of medium +MQ | Change of medium

In micronucleus assay, the cells were pre-cultured for 20 h; the medium was changed after pre-cultured. The cells were exposed to MF (50 Hz) for 24 h and after exposure, the medium was changed, following 3 h exposure to MQ, the medium was then changed and the cells were further incubated for 72 h, to enable the cells to recover and multiplied before the analysis.
The cells were categorized into four: group I, control not exposed to MF or menadione; group II, exposed to MF alone; group III, not exposed to MF but treated with a menadione; and group IV, exposed to MF and treated with menadione. Menadione has been used experimentally as a chemotherapeutic agent for cancer, also is a quinone that undergoes one-electron reduction in the mitochondrial respiratory chain, followed by transferring one-electron to molecular oxygen, producing $O_2^-$ (Luukkonen et al., 2009.) Menadione was used to induce cellular oxidative stress, and subsequent genotoxicity.

### 4.4 Comet assay

The single cell gel electrophoresis assay (also known as the Comet assay) is a relatively simple way to analyze and measure DNA damage in individual mammalian cells. Over the electrophoresis step of the assay, DNA fragments migrate away from the nucleus forming a tail. The shape, size, and the fragment content of the tail reveal the total of DNA moved out of the nucleus which is proportional to DNA damage (Luukkonen et al., 2009). The electrophoresis of samples and the unwinding of DNA were conducted under alkaline conditions (pH>13) so that the comet assay can detect DNA double-strand and single-strand breaks related with partial excision repair sites, DNA-DNA/DNA-protein cross-links, and alkali labile sites (Tice et al., 2002).

MQ was dissolved in distilled H$_2$O to attain a 100 mM stock solution, which was stored at -20°C and diluted with fresh medium prior to use. For MQ and MF + MQ exposure, after 24 h exposure to MF, cells were washed with 6 ml fresh medium, added 6 ml fresh medium, exposed to 25 µM of MQ. The positive control was treated with a higher concentration of MQ alone (50 µM ) further exposed the cells for 1 h in a 5% C0$_2$ + 37 °C incubated environment.

Samples for the immediate DNA damage (0 min) were placed on ice and cells were washed with a 2 ml of fresh medium and detached with 3 ml of ice cold 0.02 % Ethylenediaminetetraacetic acid (EDTA) in PBS by scraping and suspended for 10 times. The samples for the DNA repair (allowed to repair for 7.5 and 15 min) were washed with a 2 ml of fresh medium, then 6 ml of fresh medium was added, and the cells were further incubated at 37 °C in an atmosphere of 95% air and 5% CO$_2$ for 7.5, and 15 min respectively. After
incubation cells were detached with 3 ml of ice cold EDTA in PBS by scraping and suspended 10 times. After exposure 15 µl (~ 2 x 10^4 cells) of this cell suspension was mixed with 75 µl of 0.5% low melting point agarose (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Mixture of molten agarose and cell suspension (80 µl) was carefully suspended and applied on a microscopic slide, which was precoversed with 1% normal-melting point agarose (SeaKem GTG agarose, FMC Bioproducts, Rockland, ME, USA). Cells were spread on a slide, covered with a glass cover slip and the slide was placed on ice for 5 min, then cover slips were carefully detached and slides were placed in a dark box. Slides with embedded cells were incubated in a + 4°C refrigerator for 1 h in lysis buffer [2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 1% sodium lauroyl sarcosinate, 1% Triton X-100, pH 10]. After lysis treatment, the slides were placed in an electrophoresis unit (Gibco-BRL, Horizon 20.25, and Gaithersburg, MD, USA) for 10 min, allowing DNA to unwind in the electrophoresis buffer (1mM EDTA and 300mM NaOH, pH> 13, +4°C). This was instantly followed by electrophoresis for 10 min at 24V and 300 mA. Following electrophoresis, the slides were washed gently to remove alkali, immersed in a neutralization buffer (0.4 M Tris, pH 7.5) 3 times for 5 min and fixed with 96% ethanol for 1 min. The slides were coded and stained with 20 µg/ml ethidium bromide in dark to prevent fading of the EtBr fluorescence. The analysis of one hundred nuclei per sample were scored for each experiment point from different slides, under fluorescent microscope (Axio Imager.A1, Carl Zeiss, Göttingen, Germany) with the Comet assay IV (Perspective Instruments Ltd., UK) image analysis system. To quantify the total of DNA damage computer generated OTM values (tail length multiplied by fraction of total DNA in the tail) were used.

4.5 Micronucleus assay using flow cytometry

The technique applied in the measurement of micronuclei frequency, is such that a sequential staining method was applied to the treated samples, cells were first stained with ethidium monoazide bromide (EMA), photo-activated with visible light (light bulb), stained with SYTOX Green and Florence beads. DNA from death cell/necrotic cells with compromised cell membranes was labeled with both EMA and SYTOX Green, which can be distinguished from EMA-negative and SYTOX Green-positive. Micronucleus assay (MN) using flow cytometry was used to detect small membrane bound DNA fragments, extra nuclei in the cell
cytoplasm that signify chromosome fragments excluded from nuclei at the cell division. MN assay reveal high concordance with chromosome aberration analysis, but it is executed more rapidly and require less technical expertise (Shi et al., 2010). The double staining used allows differentiation of the nuclei of negative and positive cells.

For SH-SY5Y cells (100 000/well), were sub cultured in a flat-bottomed 48well cell culture plate, for 20 h at 37 °C in a humidified atmosphere and 95% air 5% CO₂. After the incubation medium was changed, cell cultures were exposed to 50 Hz magnetic field for 24 h and after that incubated further for 72 h. Final concentration of (MMS) served as concurrent positive controls to ensure the performance of the assay) 12.5 µg/ml, and menadione, (0.1µM, 1µM, 10 µM, 15 µM, and 25 µM). Exposure time for chemicals was 3hours. Medium was removed from the wells of the plate and it was placed on ice for 20 min. After incubation, 150 µl / well of cold (+4 °C) EMA-solution was added and samples were light activated under the table lamp. The distance between the plate and light bulb (60 W) was 15 cm. Plates were placed without lid on ice under the lamp for 30 min. EMA-solution was removed from the wells and cells were washed with 500 µl of cold (+4 °C) PBS-FBS-solution. 250 µl of Lysis I solution [deionized water, and consisted of 0.584 mg NaCl/ml 1 mg sodium citrate/ml, 0.3 µl IGEPAL/ ml, 0.5mg RNase A/ml and 0.4 µM SYTOX Green] was added and the plates were incubated for 1 h at 37 °C, protected from light. After that 250 µl of lysis II solution [deionied water, 85.6 mg sucrose/ml 15 mg citric acid/ml, and 0.4 µM SYTOX Green] (to which was added one drop of fluorescent beads Peak Flom™ Green Flow Cytometry reference beads 6 µm Invitrogen molecular probes USA.) was added to each plate and was incubated for 30 min at room temperature (+20 °C) (protected from light), the samples were transferred into flow cytometer tubes for analysis.

Flow cytometric examination was performed using Becton Dickinson FACSCalibur cytometer [Becton Dickinson, San Jose CA, USA] with an argon ion laser (488 nm) as the excitation source. Data were acquired using Cell Quest™ V 3.3 software (Becton Dickinson, USA).
4.6 Statistical analysis

Statistical analysis of the experiment was performed using compare means, the mean ± SE of DNA damage and repair was calculated for each set of samples exposed cells. The normality of the data was estimated by compare means analysis of variance (one-way ANOVA) with LSD were used for comparing the DNA damage and repair between different exposures. All analysis was done using the SPSS package version 19. Differences were considered to be significant when the P values were (P<.05).
5. RESULTS

5.1 Immediate DNA damage and DNA repair

Figure 1. The effect of 50 Hz magnetic field (MF) and Menadione (25 µM) on the immediate DNA damage and repair of human SH-SY5H neuroblastoma cells. The samples cells were continuously exposed to 100 µT, 50 Hz ELF-MF; control cells were grown in a similar cell culture incubator. After 24 h incubation, cells were harvested and counted. The counted cells were exposed MF and menadione before comet assay analysis. Data are mean ± SE (n= 3) p-value <0.05 is statistically significant.

The result (Figure 1) show that there was increased in immediate DNA damage at the time of 0 min and decrease DNA repairs at times 7.5 and 15 minutes, for all the treatments. The DNA damage in MQ treatment and MF+MQ is higher than the DNA damage in control treatment. No significant difference was recorded between immediate DNA damage for MQ treatment alone and MQ + MF treatment. An interaction between MF + MQ treatment reflected the drastic decreased in repair due to addition of MQ to the co-exposure cell,
indicating that the MF did not have significant effect on MQ-induced DNA damage, but did slow down the repair efficiency. Also, the highest immediate DNA damage was recorded for the positive control with a highest concentration of 50 µM of menadione (OTM = 9.67±7.09) which showed statistically significant p-value (<0.05)

There is little bit repair efficiency in MF treatments alone compared to control with only MQ than the treatment with MQ + MF, indicating that repair process of MQ, was possibly reduced or altered by MF. The result therefore showed that there is no significant effect of MF on MQ DNA damage, but only alters the repair process in a combined treatment of both of them.
5.2 Persistent DNA damage

Table 2. The effects of 50 Hz magnetic field (MF) and menadione (MQ), Methyl methanesulphonate (MMS) on micronucleus frequency in human SH-SY5Y neuroblastoma cells, Cells were exposed to 100 µT, 50 Hz ELF-EMF; control cells were grown in a similar cell culture incubator. After 20 h subculture, cells were harvested and counted before exposure to MF, MQ at different concentrations (0.1µM, 1µM, 10 µM, 15 µM, and 25) µM and MMS. MMS was used as positive control. Data are mean ± SE (n = 3)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Micronuclei (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.072 ± 0.006</td>
<td></td>
</tr>
<tr>
<td>MMS12.5µg/ml</td>
<td>0.086 ± 0.023</td>
<td>0.993</td>
</tr>
<tr>
<td>MQ 0.1 µM</td>
<td>0.058 ± 0.005</td>
<td>1.000</td>
</tr>
<tr>
<td>MQ 1.0 µM</td>
<td>0.050 ± 0.006</td>
<td>0.984</td>
</tr>
<tr>
<td>MQ 10 µM</td>
<td>0.089 ± 0.027</td>
<td>0.994</td>
</tr>
<tr>
<td>MQ 15 µM</td>
<td>0.129 ± 0.036</td>
<td>0.971</td>
</tr>
<tr>
<td>MQ 25 µM</td>
<td>5.312 ± 2.570</td>
<td>0.002</td>
</tr>
<tr>
<td>MF</td>
<td>0.079 ± 0.016</td>
<td></td>
</tr>
<tr>
<td>MF+MMS12.5µg/ml</td>
<td>0.067 ± 0.003</td>
<td>0.988</td>
</tr>
<tr>
<td>MF + MQ 0.1 µM</td>
<td>0.194 ± 0.145</td>
<td>0.895</td>
</tr>
<tr>
<td>MF + MQ 1.0 µM</td>
<td>0.068 ± 0.021</td>
<td>0.989</td>
</tr>
<tr>
<td>MF + MQ 10 µM</td>
<td>0.110 ± 0.040</td>
<td>0.967</td>
</tr>
<tr>
<td>MF + MQ 15 µM</td>
<td>0.107 ± 0.017</td>
<td>0.970</td>
</tr>
<tr>
<td>MF + MQ 25 µM</td>
<td>3.548 ± 1.676</td>
<td>0.002</td>
</tr>
</tbody>
</table>

P-value <0.05 is statistically significant

We observed an increased micronucleus frequency in (SH-SY5Y) cells at highest concentration 25 µM MQ being 5.312 ± 2.570 which showed statistical significant different P-value (<0.05) compared to control sample. MQ 0.1 µM to MQ 15 µM did not show statistical significant different compare to control sample. In the absence of MQ, MF alone did not have any effect on micronucleus frequency. When we combined MF + MQ at the same concentrations, there was significantly higher increase in the treatment of 25 µM of MF
+ MQ groups compared to control sample. In result of a co-exposure treatment MF + MQ the same concentration 25 µM MQ p-value (<0.05) there is a little bit decreases in micronucleus frequency. Our result suggests that highest concentration MQ have effect on micronucleus frequency however, MF alone did not have effect on micronucleus frequency. MMS was used as a positive control samples.
6. Discussion

The present study was to investigate the effect of 50 Hz MF and menadione on the immediate DNA damage and repair, and the micronucleus frequency in human SH-SY5Y neuroblastoma cells. Currently among the different types of non-ionising radiation, the low frequency MF is reported to cause possible genotoxic effect in most Studies. The result of the current study showed that there was immediate DNA damage and repair for all the treatments. Sarimov et al., (2011) reported that there was increase and decrease in chromatin conformation in human lymphocytes exposed to 50 Hz for 180 minutes. In exposed lymphocytes, the chromatin conformations are individual and strongly depend on the initial state of chromatin Sarimov et al., (2011). The results from previous studies conducted on human jurkat cell exposed to 50 Hz revealed that there were no direct DNA damaged (Moretti et al., 2005) and this result also conformed to the findings of Nordenson et al., (1994), Svedenstal et al., (1999), Falone et al., (2007), Luukkonen et al., (2011), Ruiz-gomez (2010) and Sarimov et al., (2011) and contrary to previous findings by Moretti et al., (2005), Focke et al. (2008), Neighs (et al., 2008)

The results also showed that there was no significant differences in the cells exposed to MF alone when compared to control (Figure 1), and this is in agreement with the findings of Focke et al., (2008) who reported there was no significant differences between the cells exposed to MF and non-exposed cells However, Markkanen et al., (2008) reported that exposure to MF, alters the subsequent exposure to menadione, as growth phase (G1) cells decreased while gap between DNA synthesis and mitosis (G2/M) increased. Also, Luukkonen et al., (2011) reported that MF pre-treatment of cell before menadione exposure alters cellular response to menadione. The reason this results did not agree with this results of Markkanen et al., (2008) and Luukkonen et al., (2011) may be due to differences in the time and duration of exposure, as well as exposure method.

The DNA damage was followed by a repair process which decreased with time in all the treatments. According to the comet assay results, the DNA repair was least in the MF + menadione treatment, meaning that there could be some synergistic effect between the MF and MQ treatments. This agrees with the report of Moretti et al., (2005) and Falone et al., (2007) on the synergistic effects of MF with other stress agents while it contradicts the finding of Testa et al., (2004) which did not record any synergistic effects on Human blood
samples from combined exposure. The reason may simply be that the MF combined better with the menadione and reduced the repair process.

Exposure to MFs alone did not have a genotoxic effect on DNA damage but co-exposure to ELF MFs did increase the genotoxic activity induced by menadione. Furthermore, highest concentration of menadione (25 µM) caused persistent DNA damage to the exposed cells while concentrations less than 25 µM shows no significant differences on persistent DNA damage when compared to the control (table 1). Co-exposure of MF with highest concentration of menadione (25 µM) also causes persistent DNA. As reported by Miyakoshi et al., (2005), genotoxicity activity of cisplatin was increased by the co exposure to 50 Hz. Also Moretti et al., (2005) reported that co-exposure of 50 Hz and xenobiotics increased the genotoxicity while Cho et al., 2007, also reported that the co-exposure of 50Hz and bleomycin increase the cytotoxicity of human fibroblast cells. This result is therefore in agreement with the findings while it is contrary to the findings of the study conducted by Nieghs et al., (2008) who reported that there was no effect of co exposure of 50 Hz and dimethylbenzene anthracene to CD-1 mice.

The results of the experimental study on the micronucleus assay revealed that there was increase in the value of the micronuclei frequency when the cells were exposed to highest concentration of MQ. Also there were no significant differences between MF exposed cells alone and control samples, thus these results supports the finding of previous study conducted on male germ cells in mice exposed to a 60 Hz and 2.0 mT magnetic field (Heredia-Rojas et al., 2004).

The result of the current study indicates that the co-exposure of MF and 25 µM of MQ caused persistent DNA damage to the exposed cell. These results were in agreement with the findings of previous studies conducted on the co-exposure of 50 Hz MF and cisplatin on rat brain cell (Miyakoshi et al., 2005) and co-exposure of 60 Hz MF and Mitomycin-C treatment on mouse sperm cells (Heredia-Rojas et al., 2004). Moreover, addition of MQ to MF reduces little bit of repair efficiency when compared to MQ- only exposed samples.
7. Conclusion

The results of the present study indicate that 50 Hz extremely low frequency magnetic field alone did not have a genotoxic effect but pre-exposure to MF decreased DNA repair rate as compared with menadione exposure alone. Moreover, co-exposure to MF might affect the micronuclei frequency induced by menadione.
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