Assessment of the Effects of a Low Electromagnetic Field Stimulation on Proliferation of 5 Different Cell Lines In Vitro

Project CO11000

Sponsor:

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This report consists of 15 pages

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Contents

List of Abbreviations

EMEM	*Eagle's minimal essential medium*
EMS	*electromagnetic stimulation*
FCS	*fetal calf serum*
GLP	*good laboratory practice*
H_2O_2	*hydrogen peroxide*
JSW	*JSW-Research, Forschungslabor GmbH*
LDH	*lactate dehydrogenase*
MTT	*3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazoliumbromide*
OD(s)	*optical density(ies)*
PBS	*phosphate buffered saline*
QAU	*quality assurance unit*
R.I.	*reference item(s)*
SDS	*sodium dodecyl sulfate*
SOP	*standard operating procedure*
T.I.	*test item(s)*

1. GENERAL INFORMATION

This study is carried out to assess the effects of a weak-electromagnetic-field stimulation (EMS) on proliferation of isolated cells from various cell lines. Therefore cells from 5 different lines are incubated for different time periods in a CO_2 incubator and at distinct times a weak-electromagnetic-field stimulation is applied.

1.1. Aim of the Experiment

assessment of the effects of a weak-electromagnetic-field stimulation (EMS) on cell proliferation on isolated cells from fife different cell lines

1.2. Test and Reference Items

1.2.1. Test Items:

Name:	electromagnetic stimulation (EMS; electromagnetic-field-pillow)
Information about the T.I.:	available at the sponsor
Position of the electromagnetic field pillow	on the bottom of the CO_2 incubator in a clear distance of at least 10 cm from the culture plates

1.2.2. Reference Item:

No R.I. will be used

Name of the substance:	----------
Information about the T.I.:	----------
Position of the electromagnetic field pillow	----------

1.2.3. Controls:

For control purpose cells are raised under exactly the same conditions, however, in an incubator without a electromagnetic-field-pillow and therefore no EMS stimulation is applied.

1.3. Assay Conditions

Cell lines:

1) **HeLa** (cell line originated from human adenocarcinoma with epithelial morphology)

2) **Jar** (cell line originated from human choriocarcinoma with epithelial morphology)

3) **Jeg-3** (cell line originated from human choriocarcinoma with epithelial morphology)

4) **Neuro-2a** (cell line from mouse neuroblastoma with neuronal and amoeboid stem cell morphology)

5) **SH-SY5Y** (cell line from human neuroblastoma with epithelial morphology)

Nutrition media:	For **HeLa**, **Jeg-3** and **Neuro-2a** Eagle's minimum essential medium with 2 mM L-glutamine, 10% FCS and 1% gentamycin is used. **Jar** is cultured in RPMI 1640 with 2 mM L-glutamine, 10% FCS 1% gentamycin. **SH-SY5Y** cells need Eagle's minimum essential medium and Ham's F12 medium in a 1:1 mixture and supplemented with 10% FCS and 1% Penstrep.
Cell culture conditions:	of each cell line 10^4 cells per ml nutrition medium will be raised in 96-well plates in a CO_2 incubator.
Cell culture periods:	20, 44 and 68 hours
Intensity of the EMS:	40 µT
Pulse of the EMS:	square pulse, waveband
Time points of the EMS:	**day 1:** 3.00 p.m. **day 2:** 7.00 a.m. and 3.00 p.m. **day 3:** 7.00 a.m. and 3.00 p.m. **day 4:** 7.00 a.m.
Duration of each EMS:	24 minutes
Position of culture plates in the incubator:	Not directly on the pillow in a distance of at least 10 cm
Time of EMS exposures:	• 20 hours; 2 x EMS • 44 hours; 4 x EMS • 68 hours; 6 x EMS
Evaluation of the effects:	MTT proliferation assay LDH cell death assay
Duration of one single experiment:	5 days
Number of independent experiments:	2 for each cell line
Main SOPs used:	*MET006, MET011, MET012*

1.4. Sponsor/Testing Facility/Responsible Personnel

Sponsor:	SANTERRA Handels GmbH; Ahornstr. 30, D-83451 Piding Austria Internationale Ärztegesellschaft für Energiemedizin Breitenfeldergasse 10, A-1080 Wien
Testing facility:	JSW-Research, Forschungslabor GmbH. Rankengasse 28, A-8020 Graz Institute of Histology and Embryology, K-F-Univ. Graz Harrachgasse 21/7, A-8010 Graz
Head of test facility management:	Dr. M. Windisch
Study directors:	Dr. B. Hutter-Paier, Univ. Prof. Dr. G. Dohr

Cell culture experiments:	E. Grygar, I. Hauser, N. Golob
Quality assurance unit (QAU):	Dr. I. Reinprecht

1.5. Archive

Archives of data:	During the study: All specimens, raw data and other documents generated during the course of this study will be stored at the JSW archives. After reporting: A detailed report will be sent to the sponsor.
Standard operating procedures (SOPs):	All works will be carried out according to the appropriate SOPs and followed for all stages of the study.
Staff safety:	Safety precautions operating within the department will apply to the study.

1.6. Study Dates

Code number of the study:	CO11000
Study initiation date:	19 March, 2002
Date of the Report:	2 May, 2002

2. METHODS

The effects of a weak-electromagnetic-field stimulation (EMS) on proliferation of cells from 5 different cell lines, namely of the human adenocarcinoma cell line HeLa, the human choriocarcinoma cell lines Jar and Jeg-3, the mouse neuroblastoma cell line Neuro-2a and the human neuroblastoma cell line SH-SY5Y, are investigated. Therefore 10^4 cells per ml nutrition medium are seeded in 96-well plates and incubated in a CO_2 incubator together with, or for control purpose without an electromagnetic field pillow. EMS, which lasts for 24 minutes, starts after preparation of the plates on day 1 at 3.00 p.m. and is continued on the next day (day 2) at 7.00 a.m. In general stimulation times are at 7.00 a.m. and 3.00 p.m. Incubation time of the culture plates last for three different time periods (20h, 44h and 68h) and finally cell proliferation and cell death are measured with the MTT-assay and the LDH-assay, respectively.

2.1. Cell Culture Conditions

2.1.1. General Preparations:

All items necessary are sterilised prior to the experiments. Stock solutions have been purchased already sterile and final solutions are mixed in the laminar airflow cabinet.

2.1.2. Cell Source:

For these experiments 5 different cell lines are used, namely of the human adenocarcinoma cell line **HeLa**, the human choriocarcinoma cell lines **Jar** and **Jeg-3**, the mouse neuroblastoma cell line **Neuro-2a** and the human neuroblastoma cell line **SH-SY5Y.**

2.1.3. Culture Medium:

In the experiments presented here different media are used. For **HeLa**, **Jeg-3** and **Neuro-2a** cells Eagle's minimum essential medium with 2 mM L-glutamine, 10% FCS and 1% gentamycin is used. **Jar** is cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% FCS and 1% gentamycin. **SH-SY5Y** cells need Eagle's minimum essential medium and Ham's F12 medium (1:1 mixture) and 10% FCS and 1% Penstrep are added to ensure optimal conditions for the cells.

2.1.4. Preparation of the Cells:

After thawing cells are grown under standard culture conditions for several days in T75 culture flasks in the appropriate media (mentioned above). Flasks are stored in an incubator at 37°C, 95% humidity and 5% CO_2 until cells are confluent.

2.1.5. Cell Counting and Determination of Viability:

Using a standard trypan blue dye exclusion test (PAA Laboratories) the number of cells and the cell viability can be determined, which is necessary for cell seeding. For cell counting one part of the cell suspension has to be diluted with 9 parts of trypan blue solution (270µl PBS and 180µl 0.5% trypan blue solution). Living cells and blue stained death cells are counted in a Bürker-Türk-hemocytometer. The total number of cells minus the stained dead ones gives the amount of vital cells and from these cells 10^4 /ml medium are added to a plate. In each well 1600 cells are seeded.

2.1.6. Plating out and Maintaining of Nerve Cells:

In the experiments described poly-d-lysine pre-coated 96-well microtiter plates (Biocoat Cat. No. 344461) have been used. When confluent cells are harvested (trypsin-solution) and seeded in a density of 10^4 cells per ml to 96-well plates and maintained in the CO_2 incubator for another 20, 44 and 68 hours. EMS, which lasts for 24 minutes, starts after preparation of the plates at 3.00 p.m. and is continued on the next day at 7.00 a.m. In general stimulation times are at 7.00 a.m. and 3.00 p.m. Controls are grown in an incubator without EMS. When preparing plates routinely, outside wells are filled with nutrition medium only to prevent evaporation. Plates are kept at 37°C, 95% humidity and 5% CO_2 without change of media for 20, 44 and 68 hours.

3. EVALUATION

3.1. Cell Proliferation Assay

At the end of each experiment the cell proliferation and viability of cultures is determined with the MTT assay as described in *SOP MET011* using a plate-reader (570nm). This assay is based on the reduction

of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyl tetrazolium bromide), to dark blue formazan crystals by mitochondrial dehydrogenases (succinate dehydrogenase). Since this reaction is catalysed only in living cells the assay can be used for the quantification of cell viability. For the determination of cell viability MTT solution is added to each well in a final concentration of 0.5mg/ml. After 2h the MTT containing medium is aspired. Cells are lysed with 3% SDS and formazan crystals are dissolved in Isopropanol/HCl. To estimate optical density a plate-reader (Anthos HT II) is used at wavelength 570nm. For control purpose as well as for calculation on each plate four wells of a high control (changing of the medium immediately before starting the MTT test) as well as four wells of a low control (addition of the toxic substance H_2O_2 prior to the MTT test) and four wells containing medium only, as the background controls, are generated. Cell proliferation rate is expressed in optical density (OD), however, values show in the figures are given in % (proliferation rate in four wells of the high control have been taken as 100%).

3.2. Cell Death Assay

Lactat dehydrogenase (LDH) is a stable cytoplasmatic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane. LDH activity in the cell culture supernatant is determined by a coupled enzymatic reaction whereby the tetrazolium salt INT is reduced to formazan. An increase in the amount of dead or plasma-membrane-damaged cells results in an increase of the LDH-enzyme activity in the culture supernatant. This increase in the amount of enzyme activity in the supernatant directly correlates with the amount of formazan formed during a limited time period. The water-soluble formazan dye shows a broad absorption maximum. At the end of each experiment described in this report the amount of death cells is determined with this cell death assay. Therefore 100µl of the cell free culture supernatants are collected and 100µl reaction mixture is added to each well (0.1M phosphate buffer (pH 7.0), sodium pyruvate and NADH). After incubation for 20 minutes absorbance (A_{492}/A_{690}) is measured using a plate-reader (Anthos HT II). For control purpose as well as for calculation on each plate four wells of a high control (changing of the medium immediately before starting the LDH test) as well as four wells of a low control (addition of the toxic substance H_2O_2 prior to the LDH test) and four wells containing medium only, as the background controls, are generated. Cell death is expressed in optical density (OD), however, values show in the figures are given in % (amount of proliferation rate in the four wells of the high control have been taken as 100%).

4. STATISTICS

Descriptive statistical analysis is performed.

5. RESULTS

5.1. Cell Proliferation Assay (MTT-Test)

The effect of an EMS on the proliferation rate of isolated cells from different cell lines cultured for 20, 44

or 68 hours are shown in the upper graph of the figures 1 to 5, respectively. Values in the figures are given in %, whereby the mean proliferation rate in the four wells of the high control (changing of the medium immediately prior the MTT-test) has been taken as 100%. 10^4 cells of each cell line have been grown in 96-well-plates for 20, 44 and 68 hours and have been stimulated twice a day. Control cells have been raised under the same conditions but these cells have not been stimulated. As shown in the figures 1 to 5 there is no major effect of a weak EMS on cell proliferation measured with the MTT test.

5.2. Cell Death Assay (LDH-Test)

The effect of an EMS on the amount of death cells from different cell lines cultured for 20, 44 or 68 hours are shown in the lower graph of the figures 1 to 5, respectively. Values in the figures are given in %, whereby the mean cell death rate in the four wells of the high control (changing of the medium immediately prior the LDH-test) has been taken as 100%. Like in the proliferation assay no major effects of an EMS on cell death can be shown (figures 1 to 5, respectively).

6. CONCLUSION

A weak twice a day electromagnetic stimulation (EMS) has no major effect neither on cell proliferation nor on cell death of 5 different cell lines namely of the human adenocarcinoma cell line **HeLa**, the human choriocarcinoma cell lines **Jar** and **Jeg-3**, the mouse neuroblastoma cell line **Neuro-2a** and the human neuroblastoma cell line **SH-SY5Y**

7. SIGNATURES

Study directors: …….....

Dr. B. Hutter-Paier, Univ. Prof. Dr. G. Dohr

.....

Date

8. APPENDIX

Figures 1 to 5

Figure 1: Effects of a weak-electromagnetic-field stimulation (EMS) on proliferation (MTT) and cell death (LDH) of HeLa cells maintained for 20, 44 and 68 hours in an incubator.

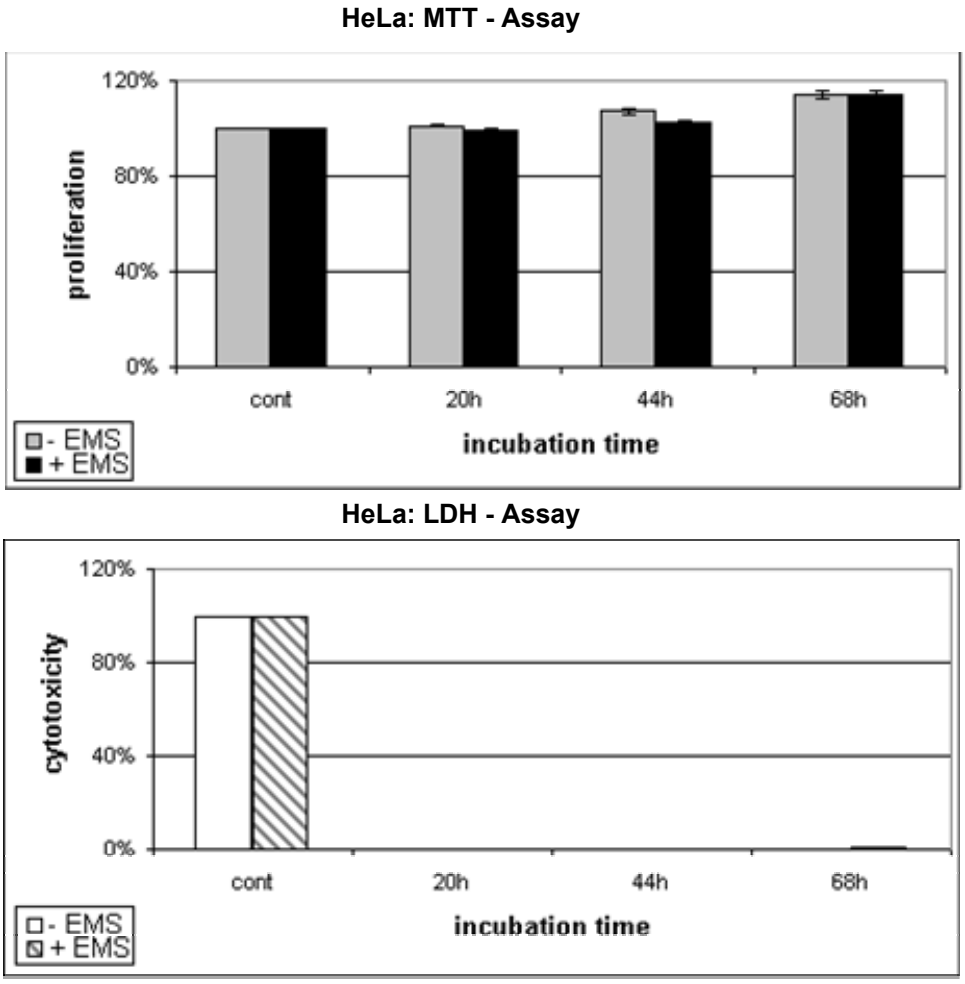

Figure 2: Effects of a weak-electromagnetic-field stimulation (EMS) on proliferation (MTT) and cell death (LDH) of JAR cells maintained for 20, 44 and 68 hours in an incubator.

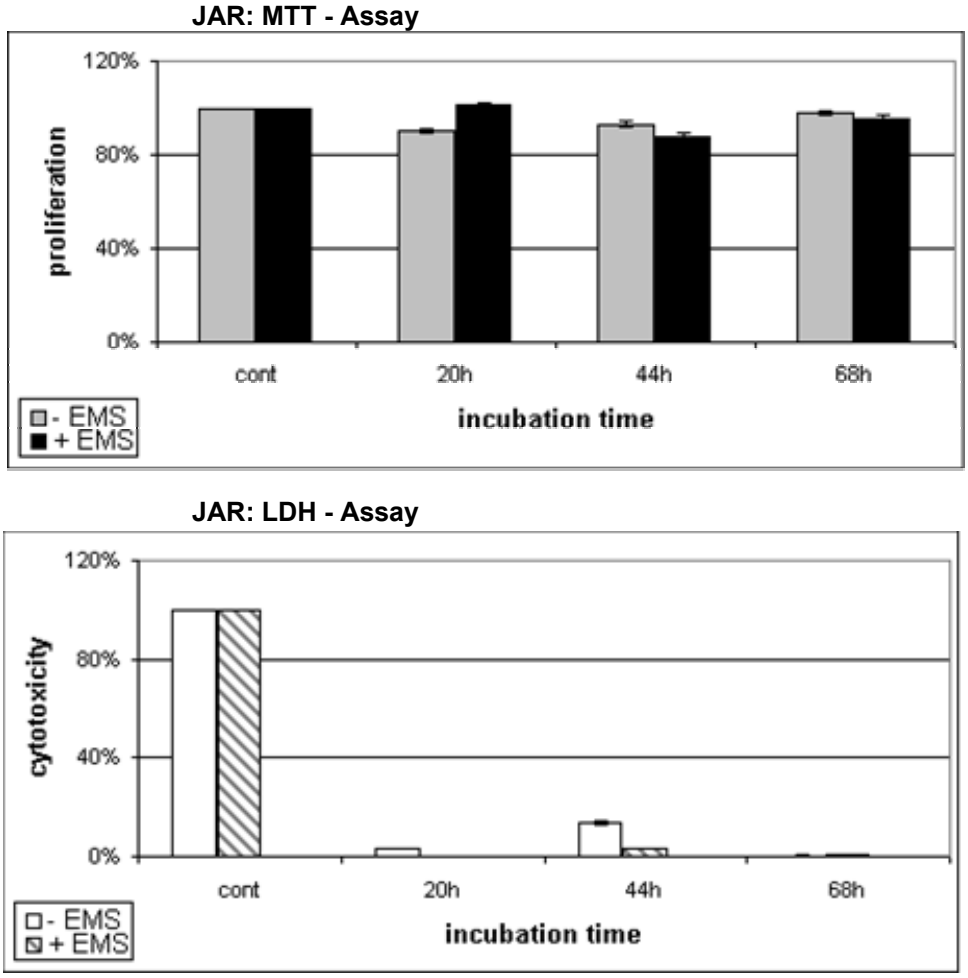

Figure 3: Effects of a weak-electromagnetic-field stimulation (EMS) on proliferation (MTT) and cell death (LDH) of JEG-3 cells maintained for 20, 44 and 68 hours in an incubator.

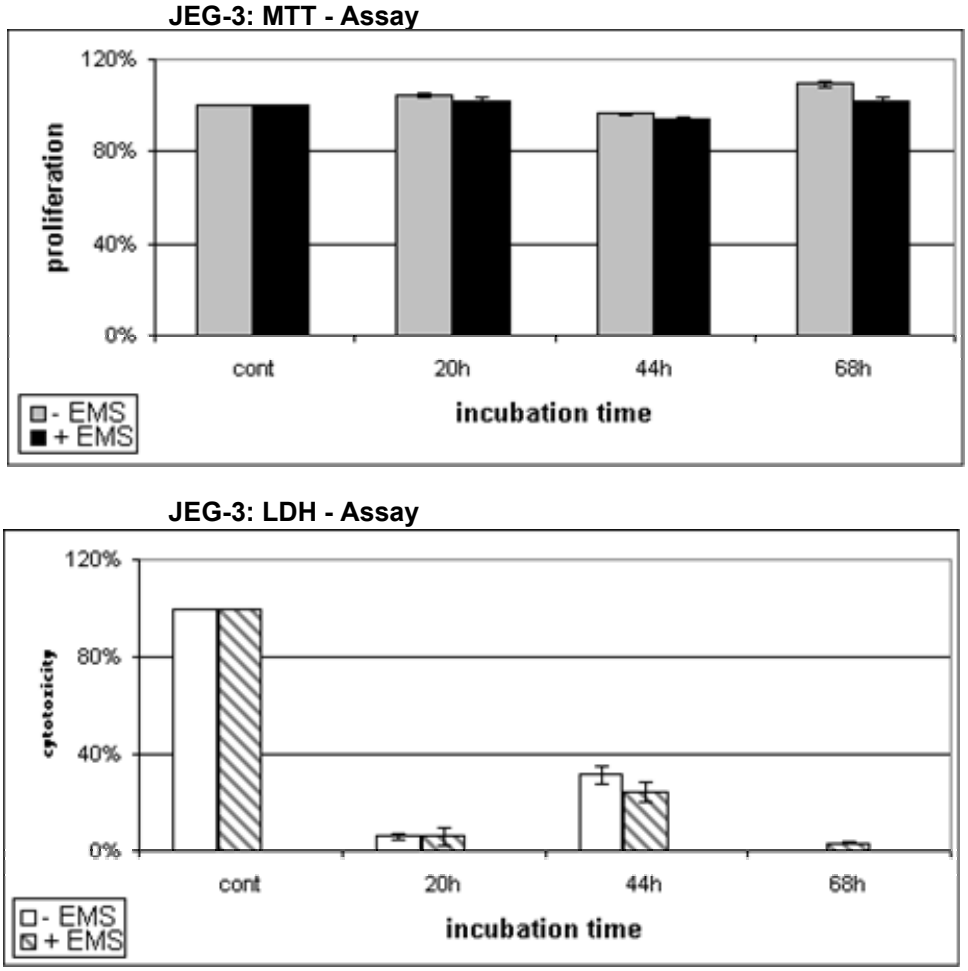

Figure 4: Effects of a weak-electromagnetic-field stimulation (EMS) on proliferation (MTT) and cell death (LDH) of Neuro-2a cells maintained for 20, 44 and 68 hours in an incubator.

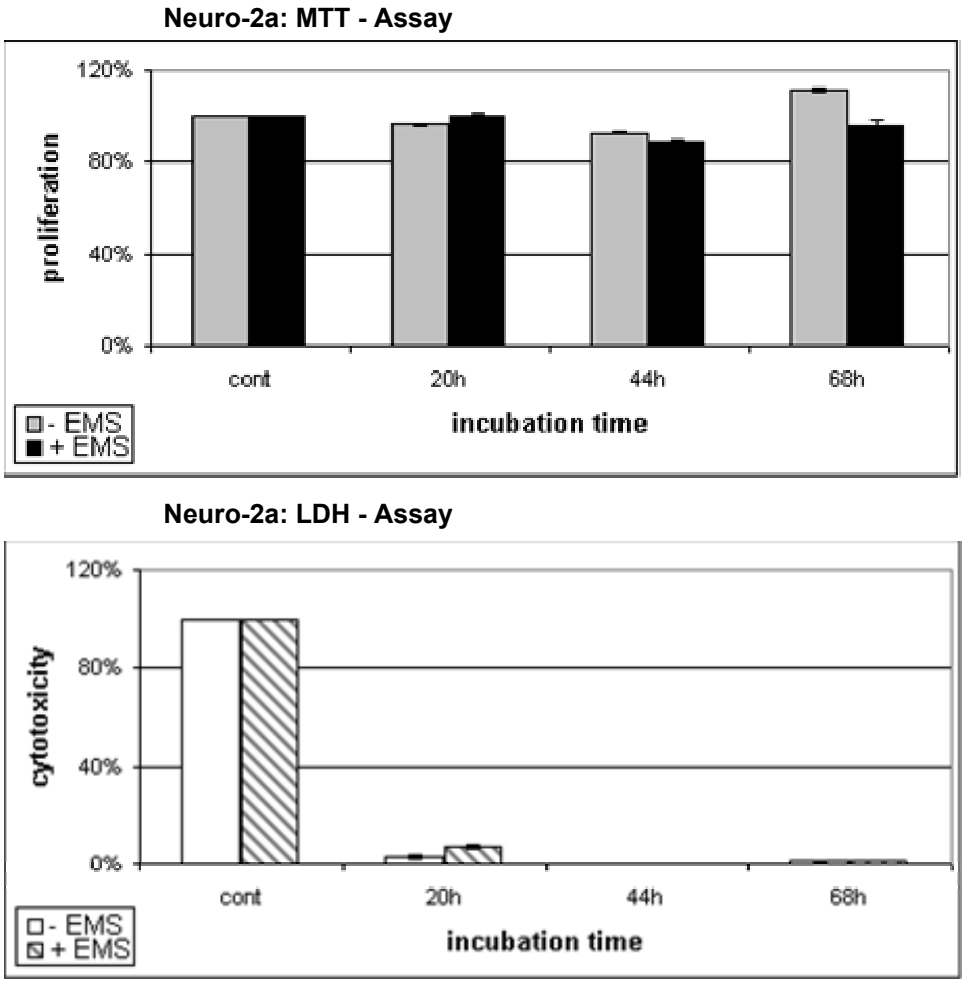

Figure 5: Effects of a weak-electromagnetic-field stimulation (EMS) on proliferation (MTT) and cell death (LDH) of SH-SY5Y cells maintained for 20, 44 and 68 hours in an incubator.