engl. Krebszellen Project CO11000

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

Project CO11000

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This report consists of 15 pages

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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.

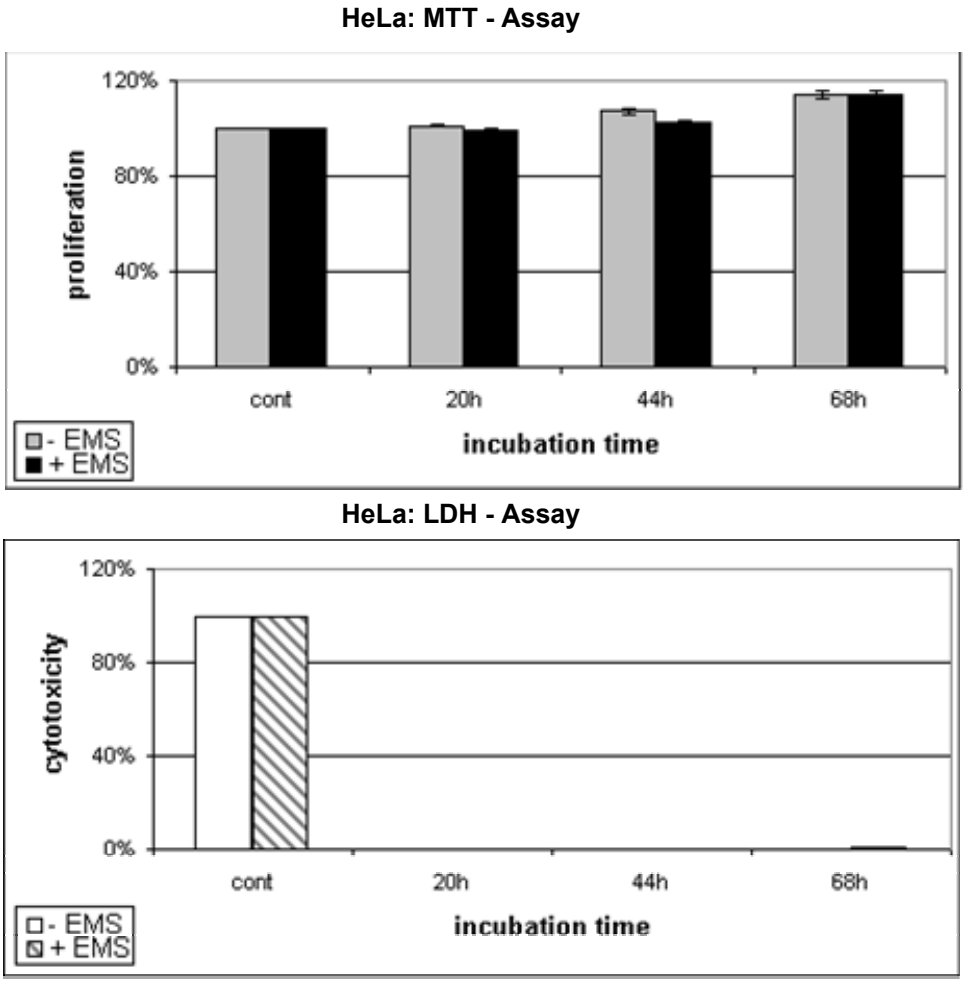


Figure 2: Effects of a weak-electromagnetic-field stimulation (EMS) on proliferation (MTT) and cell death (LDH) of JAR cells maintained for 20, 44 and 68 hours in an incubator.

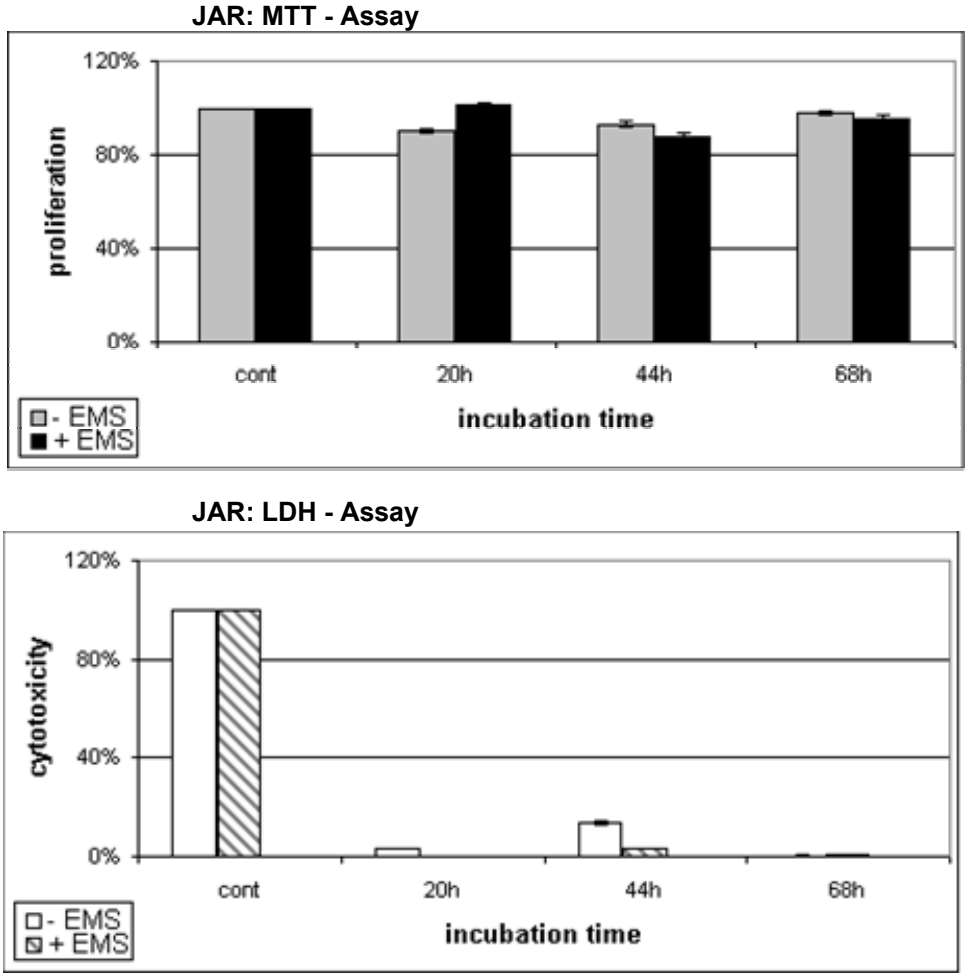


Figure 3: Effects of a weak-electromagnetic-field stimulation (EMS) on proliferation (MTT) and cell death (LDH) of JEG-3 cells maintained for 20, 44 and 68 hours in an incubator.

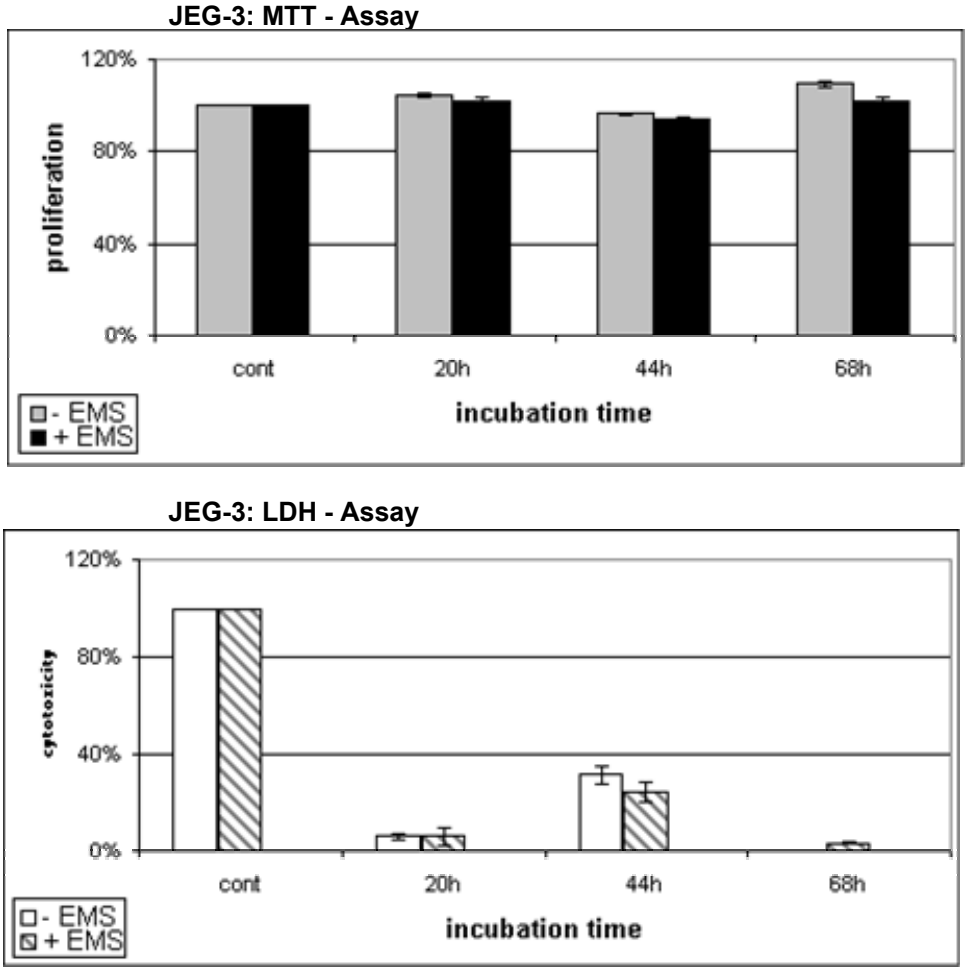


Figure 4: Effects of a weak-electromagnetic-field stimulation (EMS) on proliferation (MTT) and cell death (LDH) of Neuro-2a cells maintained for 20, 44 and 68 hours in an incubator.

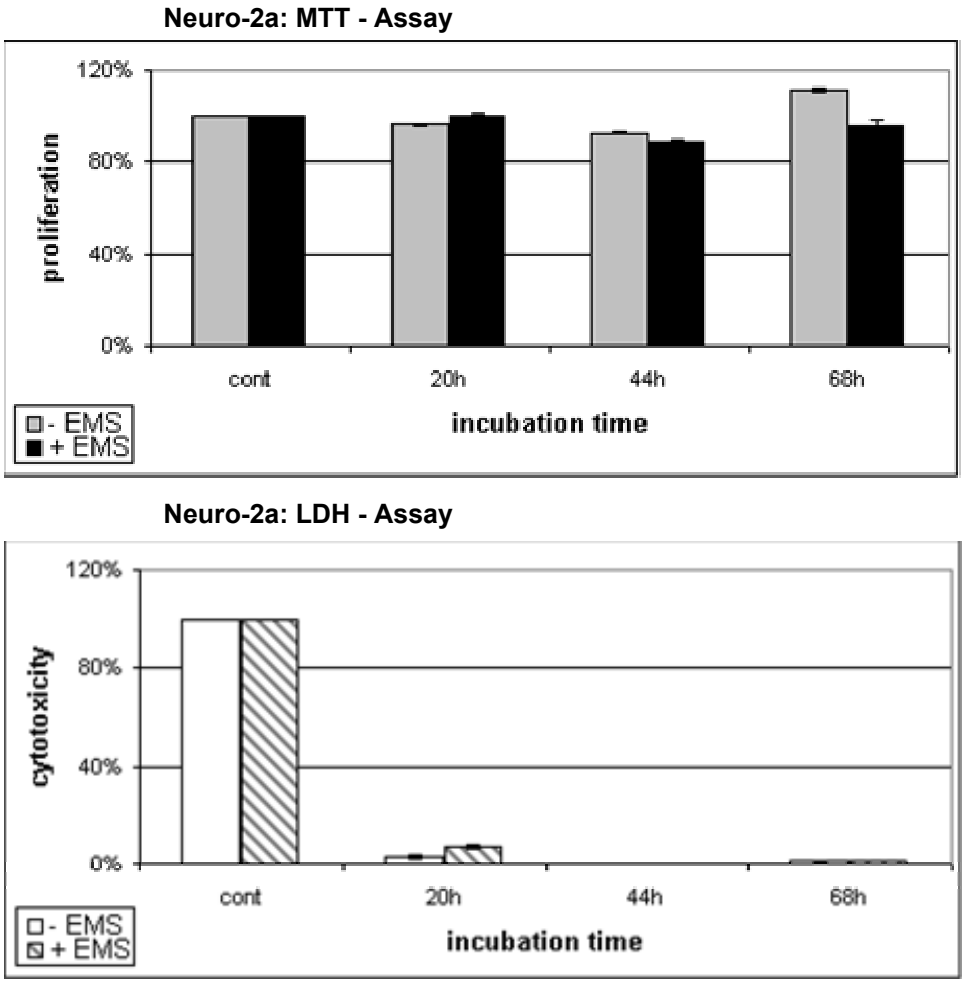
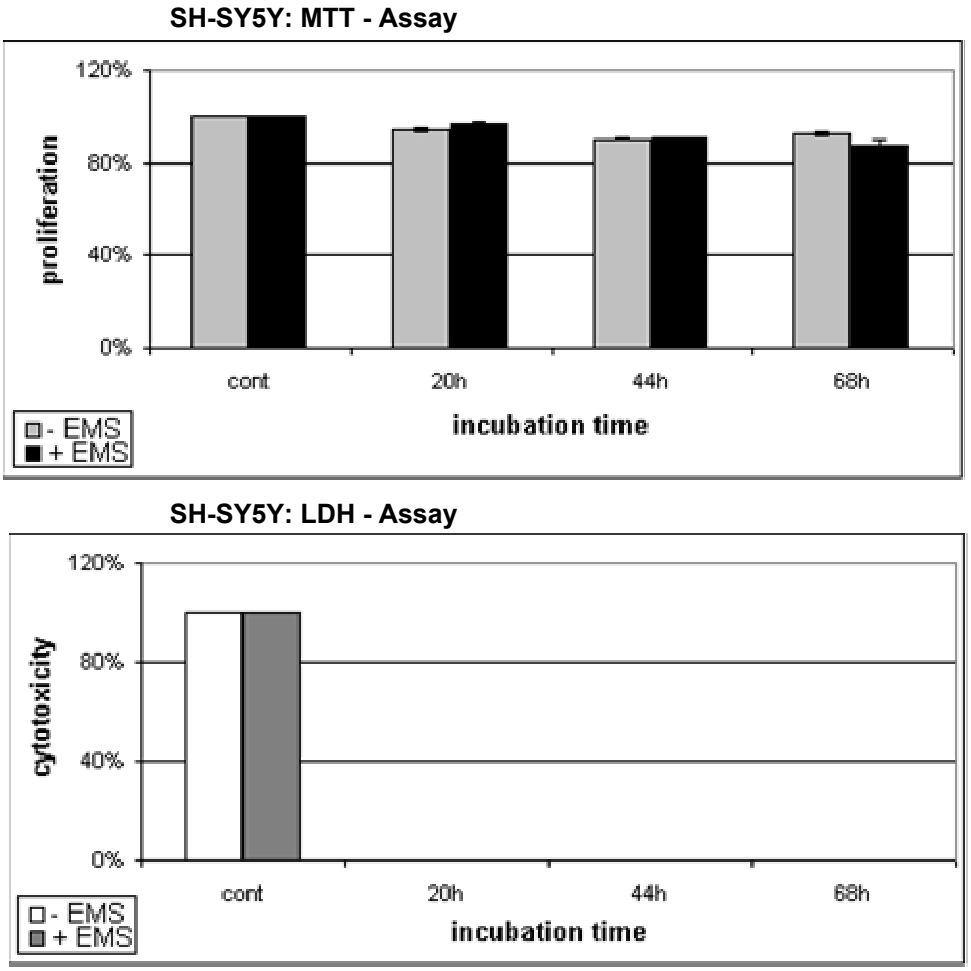


Figure 5: Effects of a weak-electromagnetic-field stimulation (EMS) on proliferation (MTT) and cell death (LDH) of SH-SY5Y cells maintained for 20, 44 and 68 hours in an incubator.



Nervenzellen gesamt komprimiert

1. GENERAL INFORMATION

This study has been carried out to assess possible neurotrophic or neuroprotective effects of a low electromagnetic field on isolated cortical neurons. Therefore, the influence of an electromagnetic stimulation (EMS) has been be investigated in a *5% Serum Assay* and in a *Low Serum Cell Stress Assay*. Both assay allow the estimation of the neurotrophic activity of an electromagnetic field on cell cultures. The study has been performed according to standard *SOPs* as well as to the study protocol.

1.1. Aim of the Experiment

The study has been carried out to the neurotrophic and neuroprotective capacity of an electromagnetic field equipment on isolated cortical neurons from chick embryos in a ***Normal 5% Serum Assay*** and in a cell death inducing ***Low Serum Cell Stress Assay*** with or without the addition of FGF-2.

1.2. Test Conditions:

Cell culture assays:	A) Normal 5% Serum Assay B) Low Serum 2% Cell Stress Assay
Test groups for each assay:	1. Cells maintained in the CO_2 incubator with electromagnetic field equipment. 2. Cells incubated with FGF-2 and maintained in the CO_2 incubator with electromagnetic field equipment. 3. Cells maintained in the CO_2 incubator without electromagnetic field equipment. 4. Cells incubated with FGF-2 and maintained in the CO_2 incubator without electromagnetic field equipment.
Duration of a single experiment:	8 days
Exposure period:	The electromagnetic field equipment has been in the incubator for the whole experimental period. The exposure cycle chosen was 24 minutes, the intensity 200%.
Exposure mode:	The electromagnetic field has been generated two times a day at 7.00 a.m. and 3.00 p.m. except at the first day with one exposure cycle at 3.00 p.m. and on the eighth day with only one exposure cycle at 7.00 a.m.
Cell source:	Telencephalon neurons from 9-day-old chicken embryos (Lohman Brown hybrid or White Leghorn hybrid).
Nutrition medium:	2% assay: EMEM with 1g glucose/l, 2% FCS, 0.01% Gentamycine and 2mM L-Glutamine. 5% assay: DMEM with 5g glucose/l, 5% FCS, 0.01% Gentamycine and 2mM L-Glutamine.

Number of independent experiments:	4
Evaluation of the effects:	MTT viability assay (displaying the amount of viable cells), Phase contrast images

1.3. Test Substance

Substance and batch numbers:	FGF-2 (bFGF, basic Fibroblast Growth Factor; Sigma)
Batch number:	38H8423
Storage conditions at JSW:	4-8°C in the refrigerator
Expiry date:	according to the suppliers specification
Application period:	for the whole experimental period of 8 days
Vehicle for dilution:	nutrition medium
Administration dose:	0.8ng, 1.6ng, 3.1ng, 6.25ng, 12.5ng, 25ng/ml medium

1.4. Controls

For control purpose neurons will be raised with nutrition medium only, no test substance has been added.

1.6. Evaluation/Statistics/Raw data:

Evaluation of the effects:	MTT viability assay, phase contrast images
Statistical analysis:	Primarily descriptive statistical analysis has been performed, however, significant differences between controls and FGF-2 treated cells have been evaluated with a two-tailed Students t-test for two samples with the same variance. Differences have been considered as significant on an error level of $p<0.05$. Data are represented as mean value ± standard deviation.
Handling of raw data:	Raw data are stored in the archive of JSW. Copies of raw data are part of the final report.

1.7. Study Dates

Study initiation date:	12 June, 2001
Start of the experiments:	23 July, 2001
Study completion date:	31 August, 2001

1.8. Findings of the study:

This report describes the effects of an electromagnetical stimulation (EMS) on neurons treated with or without FGF-2 and maintained under normal 5% FCS and low serum 2% FCS cell culture conditions. Therefore in one incubator an electromagnetic field pillow has been installed and turned on twice a day (24 minutes, 200%). For control purpose cells have been raised also without this electromagnetic stimulation. The effects of this treatment have been evaluated after eight days with a MTT viability assay and by estimating differences of neuritic growth or arborisation by the means of phase contrast images. The general conclusions that can be drawn are:

1. Due to the MTT data there is a clear neuroprotective influence of the electro magnetic field stimulation on cortical neurons maintained under low serum cell stress conditions. One can state here that under electro magnetic stimulation the effects of the naturally occurring growth factor FGF-2 can be additionally enhanced.
2. There is only a small effect in the normal 5% FCS cell assay can be described.
3. It seems to play a pivotal role where in the incubator microplates have been maintained (distance to the pillow).
4. No general toxic effects of the substances have been observed throughout the whole experiment, although in microplates stored in the incubator equipped with the electromagnetic field pillow at the third day a colour changing of the medium has been noticed.

2. METHODS

2.1. Cell Culture Conditions

2.1.1. General Preparations:

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

2.1.2. Culture Medium:

In the presented experiments Eagle's Minimal Essential Medium (EMEM) supplemented with a low concentration of FCS (2%) has been used. Since EMEM misses the amino acid L-Glutamine, required for growth and differentiation, it has been added to the nutrition medium in a concentration of 5mg/ml as well as 0.1mg Gentamycine/ml, to prevent cell cultures from an infection with mycoplasm or other unwanted micro-organism. For each experiment the nutrition medium is freshly prepared in the laminar airflow cabinet under sterile conditions.

2.1.3. Cell Source:

Lohman Brown chicken embryo hybrids have been used. One-day-old fertilised eggs are purchased from a local chicken breeder (Schlierbach Geflügel GmbH, Austria) and stored in the lab under appropriate conditions (12 ± 0.3°C and 80 ± 5% humidity). At embryonic day 0 eggs are transferred into a breeding incubator and stored under permanent turning until embryonic day 9 at 38 ± 0.5°C and 55 ± 5% humidity. For isolation of neurons per experiment 3 to 4 chicken embryos have been used. The age of the embryos is very critical, since only in a particular period of development the brain almost exclusively contains nerve cells and less than 5% glia (*Pettmann, B., Louis, J.C. and Sensenbrenner, L.M. (1979) Nature 281:378-380*).

2.1.4. Preparation and Dissociation:

Eggs have been wiped with 70% ethanol and cracked with large forceps at the blunt end. After decapitation of the embryo, the tissue covering the Telencephalon has been removed and hemispheres have been collected. After removing any loose tissue and remaining meningeal membranes, hemispheres have been transferred into a dish containing nutrition medium. Thereafter the tissue has been dissociated mechanically by using a 1ml pipette and by squeezing 3 times through a sterile nylon sieve with a pore size of 100 µm.

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. 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.

2.1.6. Plating out and Maintaining of Nerve Cells:

In the experiments described Poly-D-Lysin coated 24-well plates (Biocoat Cat. No. 344461) have been used. FGF-2 has been added to the wells in the appropriate concentrations once, from the first day onwards, while controls have been grown in nutrition medium only. The final amount of cells in each well is 3×10^5 /ml nutrition medium. Plates are kept at 37°C, 95% humidity and 5% CO_2 without change of media until 8 DIV. Neurons begin to extend processes after a few hours in culture.

2.2. Group Size/Dosages

Substances have been tested under identical conditions on two different days. FGF-2 has been administered in concentrations of 0.8ng, 1.6ng, 3.1ng, 6.25ng, 12.5ng and 25ng/ml nutrition medium.

3. EVALUATION

3.1. Viability Assay

At DIV 8 the viability of remaining nerve cells has been measured with a colorimetric MTT-reduction assay. 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 has been added to each well in a final concentration of 0.5mg/ml. After 2h the MTT containing medium has been aspired. Cells have been lysed with 3% SDS, Formazan crystals dissolved in Isopropanol/HCl. To estimate optical density a plate reader (Anthos HT II) has been used at wavelength 570nm. Neuronal viability is expressed in optical density (OD).

3.2. Phase Contrast Images

From each culture condition representative phase contrast images have been taken to evaluate morphological alterations according to the assay, to the treatment and the low electromagnetic field.

4. STATISTICS

MTT values (ODs) are expressed as mean ± standard deviation. Besides descriptive statistical analysis for statistical comparison a two-tailed Students t-test for two samples with the same variance and subsequent Bonferroni correction has been performed. Differences compared to the control have been considered as significant on an error level of $p<0.05$.

5. RESULTS

5.1. Observations

Dated and signed records of the observations outlined here were reported in the appropriate documentation.

5.2. Results of the Viability Assay

In the experiments presented here the effects of an electromagnetic stimulation (EMS) on neurons treated with or without FGF-2 and maintained under normal 5% FCS and low serum 2% FCS culture conditions are described. For these experiments in one incubator an electromagnetic field equipment has been installed and turned on twice a day. Electromagnetical stimulation lasted for 24 minutes and an intensity of 200 % has been chosen. For control purpose cells have been raised without this electromagnetic stimulation in an incubator located in a separate room. The effects of an EMS have been evaluated after eight days with a MTT viability test and by estimating differences of cellular growth and differentiation or neuritic arborisation with the help of phase contrast images.

It can be stated here that due to a week electromagnetical stimulation cortical neurons maintained under low serum cell stress conditions show an increased neuronal viability. The low serum cell stress assay, which has been described to be a good model for growth factor withdrawal, is described to induce apoptosis and neuronal cell death. This neurodegeneration can be partially inhibited by the addition of neurotrophic factors. In the experiments described here, the beneficial effects of the naturally occurring growth factor FGF-2 are significantly enhanced due to the EMS. As shown with the phase contrast images these effects are not those clearly visible when looking at the shape of the neurons and the arborisation. Nevertheless there are small effects on the arborisation on day 6 (6DIV) showing that EMS might stimulate arborisation or more likely protect cells from degeneration and MTT data are significantly speaking for a positive neuroprotective effect of such a week electromagnetical stimulation.

The effects of an EMS on neurons maintained in the normal 5% FCS cell assay are week compared to the not stimulated cells. Other than in the 2% assay, effects of FGF-2 cannot be additionally elevated due to an electromagnetical stimulation. In the normal 5% FCS assay the amount of serum alone seem to be high enough for cells to stay alive and no additional FGF-2 is needed for survival. Quite the contrary FGF-2 in higher concentrations decreases neuronal viability as reported in other studies but even under theses conditions EMS seem to be ineffective. In the phase contrast images it looks like if an EMS results in a quite good cellular response. Although we did not measure neuritic length and amount of arborisation it looks like that with ongoing culture duration the dendritic network is getting larger in stimulated compared to control cells.

What we noticed in this study is the fact that it seems to play a pivotal role, at least on neuronal viability, where in the incubator microplates and the electromagnetic pillow have been maintained. We tired to

figure out these effects a little bit more, and evaluated the viability of all neurones maintained under the same conditions as well as the viability of neurones grown in plates not directly placed on the magnetic pillow. And although the group of neurons maintained directly over the pillow is very small (n=2) it clearly turned out that in theses cells neuronal viability is clearly decreased. This is the reason why beneficial effects of an EMS are much only significant when these neurons have been excluded from the statistical calculation. Therefore it might be quite interesting to investigate different distances between the pillow and the microplates and neurons, respectively.

Independent from the culture condition, high or low FCS concentration or treatment with FGF-2, we noticed a colour changing of the nutrition medium in plates maintained together with the electromagnetic pillow. This change is due to a shift in pH since the red colour occurs from the pH indicator phenol red. Under conventional culture conditions this would indicate an increased metabolism of the cells or lacking of nutrition medium. Since we did not mention the same changes in the control incubator, changes must be due to the week electromagnetical stimulation. These changes in pH occurred in all plates independent from the FCS concentration and from the neuronal viability measured, so it could be very interesting to enlighten theses observations a little bit more, and try to figure out where these effects come from and whether they do have effects on neuronal physiology and metabolism.