Biological effects of exposure to electromagnetic fields of 900 MHz are reduced by use of RayGuard equipment. Experimental study on CCRF-CEM cells in culture.

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Introduction

It is becoming increasingly evident that electromagnetic radiation (NIR) is able to produce effects on biological systems. There is a wide range of data documenting the ability of non-ionising radiation (NIR) to affect living organisms (Adey 1990) and there is also data regarding the biochemical and molecular mechanisms of cells both *in vitro* and *in vivo* with effects independent of thermal phenomena (Barnes 1996, Astumian *et al.*1995, Litovitz *et al.* 1993). Recent studies have shown that NIR affects cell metabolism and proliferation, inducing potentially damaging effects in all cell components, from the cytoplasmic membrane where the distribution of proteins is modified (Bersani *et al*. 1997), to the cytoplasm and to the nucleus where the NIR activates a series of sequential events involving intracellular enzymes (*signalling*) (Hill 1998) and molecules regulating cell growth. In order to examine the biological effects produced by NIR on cellular systems, we analysed the changes that may occur in lymphocytes exposed to radio frequencies and microwaves (RF/MW), using human leucemic T cell lines (CCRF-CEM) which are the system closest and most similar to human primary lymphocytes, and which could display variability due to, for example, the intrinsic characteristics of the donors. We studied the variation in cell proliferation and the course of the cell cycle via FACS analysis of these cells while exposed to 900 MHz electromagnetic fields with and without the protective **RayGuard** equipment, to assess whether the cell-protecting RayGuard system can cancel or reduce the effects of NIR on cells in culture documented in other publications

Materials and methods

CELL CULTURES

Human leucemic T cell lines CCRF-CEM, were cultivated in culture medium RPMI 1640 with addition of FCS 10%. The cells were cultivated in culture flasks of 8 cm diameter at a density of 1x 10^6 cells per capsule and exposed to electromagnetic fields of 900 MHz for 2, 4, 24, 48 hours with and without the RayGuard present. The same quantity of cells was used as a control in the same incubator outside the TEM cell. As a further system of control, the cells not exposed to fields were incubated in a different incubator from that containing the TEM cell.

SYSTEM OF CELL EXPOSURE TO 900 MHz

The cells were irradiated by an electromagnetic field produced by a sinusoidal signal generator between 800 and 1000 MHz with 5 dBm of power output generating an electric field calculated to be 4.89 V/m inside the TEM cell.

The electromagnetic field was produced using a broadband signal generator and sent to a TEM (Transverse Electro-Magnetic) cell in a frequency range between 800 and 1000 MHz terminating with a load suitably adapted for frequencies from 850 to 950 MHz, having characteristic impedance of 75 ? . The TEM cell, (Fig. 1), is a three plane transmission line with closed side walls, so as not to permit energy to radiate outside, thus ensuring electromagnetic isolation within the surrounding environment. When designed for frequencies greater than 800 MHz and characteristic impedance of 75 ? it produces a stable electromagnetic field inside the cell (0-2dBm). In order to expose cells to the field with the RayGuard present, the RayGuard was placed inside the TEM cell. The field inside the cell was mapped in order to check whether there were field perturbations which could affect the exposure of cells in the culture flasks.

SHAM EXPOSURE

In order to carry out a check on the effect of the equipment on the cells in culture but without exposure to the field, leucemic cells (CCRF-CEM) were cultivated for 2, 4, 24 and 48 hours within the TEM cell and outside it without exposure to electromagnetic radiation (generator switched off), with and without the RayGuard present. At the end of the sham exposure, the proliferation tests and FACS analysis were carried out as for the exposed samples.

CELL PROLIFERATION TEST

100 ?l/well of exposed and non-exposed cell suspension (5000 cells), with and without the RayGuard being present, were aliquoted on a 96-well ELISA plate. 12 wells for reading were prepared from each exposure sample to obtain statistically valid values. 50 ?g of MTT (3-?4,5-dimethylthiazol-2-yl?-2,5-diphenyl tetrazolium bromide) were added to each well, followed by incubation for 4 hours at 37°C. The MTT was separated in a NADP/NADPH dependent process, from metabolically active cells, in formazan salts which are insoluble in aqueous solution and form violet precipitates. 100 ?l of solubilising buffer (0.01M HCl, 10% SDS) were then added and the solution reincubated at 37°C overnight. The formazan crystals were thus solubilised and measured spectrophotometrically (550nm-690nm) using an ELISA plate reader. The colour intensity is directly correlated to the quantity of metabolically active cells present in the well at the moment of adding MTT. The index of proliferation was calculated by making a ratio between the directly measured value of the examined sample and a reference value. Standard deviation was calculated according to the usual rules of error analysis.

FLOW CYTOFLUORIMETRIC ANALYSIS (FACS)

In order to determine the amount of DNA, the cells were cultivated for 2, 4, 24 and 48 hours, with or without electromagnetic field and RayGuard being present, and were fixed in 70% ethyl alcohol at 4°C for 30 min. The nuclei were stained with 25 µg/ml of propidium iodide and incubated with 1mg/ml of RNases for 1 hour at 37 °C. The nuclear DNA content was determined using flow cytofluorimetry.

Results

PROLIFERATION TEST

An equal number of cells (1,000,000/ml culture medium) was used for cultivation using the experimental conditions described under „Materials and methods“. Examining the results of the proliferation test, which indicates the number of cells present at the end of the experiment, it is evident that in the exposed cells there is a statistically significant decrease ($p<0.01$) in the total number of cells for exposures of 48 hours. There was no significant difference observed between exposed and non-exposed cells for shorter exposure times (2, 4, 24 hours) (Fig. 2). The cells cultivated inside the TEM cell under the same culture conditions, but without administering the field (sham exposure) do not display any difference to the control group of cells (Fig. 3), suggesting that the TEM cell alone does not cause any alteration to cell proliferation in the absence of an electromagnetic field. The same results are obtained using the same sham conditions but with the RayGuard present inside the TEM cell.

The proliferation test on cells cultivated inside the TEM cell and exposed to an electromagnetic field of 900 MHz **with the RayGuard present**, does not display any significant difference in proliferation compared to

the control, even after 48 hours of exposure (Fig.4). The RayGuard therefore cancels the effect of electromagnetic field exposure on cell proliferation.

FLOW CYTOMETRY (FACS analysis)

In order to find out whether modifications to the cell cycle phases had taken place which were not attributable just to the quantity of cells present, the cell DNA content and thus the percentage of cells involved in the various cell cycle phases was determined using flow cytometry.

Analysis of the exposed samples showed a significant increase ($p<0.01$) (18.07% in the cells exposed to electromagnetic fields and 3.89% in the controls) in the number of cells undergoing apoptosis starting from two hours of exposure, this increase was also evident at 24 (7.98% in the cells exposed to electromagnetic fields and 4.03% in the controls) and 48 hours (3.38% in the cells exposed to electromagnetic fields and 1.37% in the controls) of exposure ($p<0.05$).

Furthermore the electromagnetic fields induce a significant increase in the number of cells duplicating (39.63% in the exposed cells and 22.6% in the controls) and a significant reduction in blocked cells (resting) (26.68% in the exposed cells and 40.06% in the controls) ($p<0.01$) after 48 hours of exposure.

Regarding the effects of electromagnetic fields **with RayGuard present**, the FACS analysis showed similar values to those obtained in the controls, both for cells in the various phases of the cell cycle and for the percentage of cells undergoing apoptosis. The presence of the RayGuard in the exposure cell therefore prevents the effects caused by electromagnetic radiation on cycling cells. (Tab.1 and Fig. 5)

Discussion

The molecular control mechanisms of the cell cycle have been the object of numerous research studies in recent years, particularly due to the growth of information regarding their role in cancer, a disease which involves dynamic changes to the genome.

Potentially carcinogenic effects of both low and high frequency NIR have been hypothesized based on the effects shown in various types of tumours (Byus et al, 1987; O' Brien et al 1994, 1998; Liburdy et al, 1993;Cain et al, 1993) including chronic lymphatic leucemia in children (Uckun et al, 1995; Feychting e Ahlbom 1993). This studies show that NIR can have genotoxic effects. It has been demonstrated that microwaves are able to induce breaks in the DNA double helix and breaks in a single strand of DNA are frequently associated with the emergence of tumours (Tice 1978; Ames 1989), with apoptosis (Walker et al. 1991; Prigent et al. 1993) and aging (Hart e Setlow 1974). Mutagenic effects of low level microwave radiation (2.45 GHz calculated as SAR 1.8 W/Kg) on DNA sequences in the brain and testicles of rats have been shown by Sarkar et al. 1993. Lai and Singh 1995 showed that microwaves and non-modulated fields (CW) cause breaks in brain tissue DNA and that these breaks occurred after 4 hours following a second exposure in a dose-dependent way, as also reported by Adey 1997.

Clinical evidence suggests that a defect in DNA damage repair mechanisms is a fundamental necessary process for carcinogenesis. Additionally Garaj-Vrhovac et al. 1990 showed that workers exposed to microwaves with a power density of 10-50 ?W/cm2 displayed 27.9% micronucleus formation following activation of the apoptotic process and 31.5 % of chromosomal aberrations compared to controls who displayed an incidence of micronucleus formation of 1.8% and incidence of chromosomal aberrations of 1.5%.

In order to find out whether the RayGuard could reduce or eliminate the damaging effects produced by NIR, tumour cells were exposed to electromagnetic fields of 900 MHz with the equipment present.

Tumour cells exposed to NIR were progressively reduced in number compared to the control culture. This reduction in the number of cells in culture is correlated to altered cell proliferation deriving from abnormal duplication of the genetic information (DNA) which cell division does not respond to, particularly in the longer exposure times (48 hours). This leads to the appearance of tumorigenic cells which, if not eliminated through the process of programmed cell death (apoptosis), could give rise to clones of highly aggressive tumour cells. In fact, the cells exposed to NIR for short periods show a considerable increase in apoptotic cells mainly at 2 and 24 hours of exposure. This finding suggests that the cells immediately respond to the damage signal induced by the electromagnetic fields, activating the apoptotic process. Since exposure extends for long periods, surviving cells that have avoided the apoptotic process continue with genomic duplication, giving rise to the selection of highly aggressive clones which have accumulated damage to the DNA.

The cell cultures exposed to electromagnetic fields in the presence of the RayGuard display values for cell proliferation and percentage of cells in the various phases of the cell cycle that are comparable to those of control cells not exposed to electromagnetic fields. The presence of the RayGuard during exposure to NIR appears to neutralize the damaging effects at genomic level induced by electromagnetic fields of 900 MHz. Further studies on the biological and molecular mechanisms underlying this phenomenon will need to be carried out in order to clarify in more detail these preliminary results.

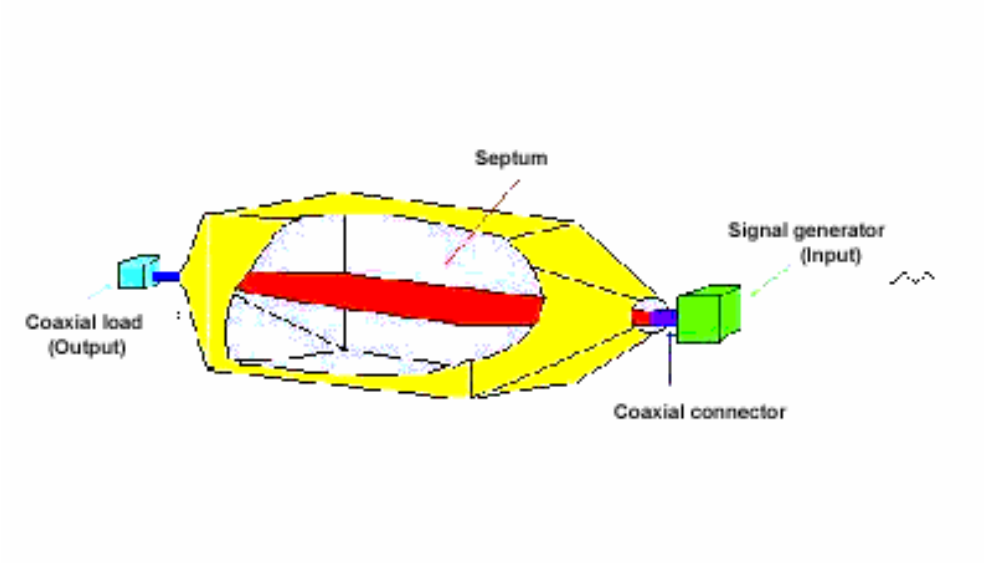

Fig. 1: Diagram of the TEM cell

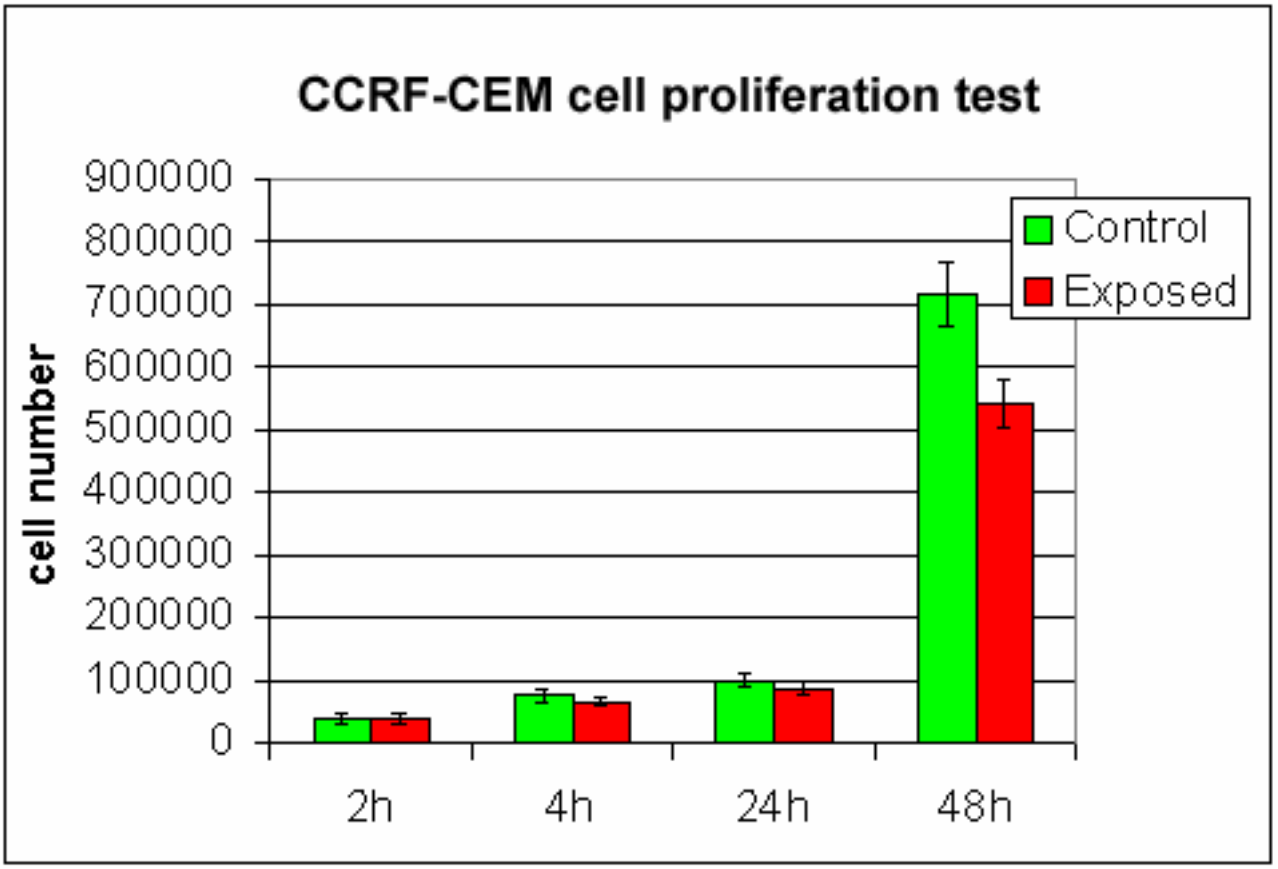

Fig. 2: Graph showing cell proliferation of CCRF-CEM leucemic cells in culture. The statistically significant reduction in the number of cells after 48 hours of exposure to a 900 MHz electromagnetic field is evident ($p < 0,05$).

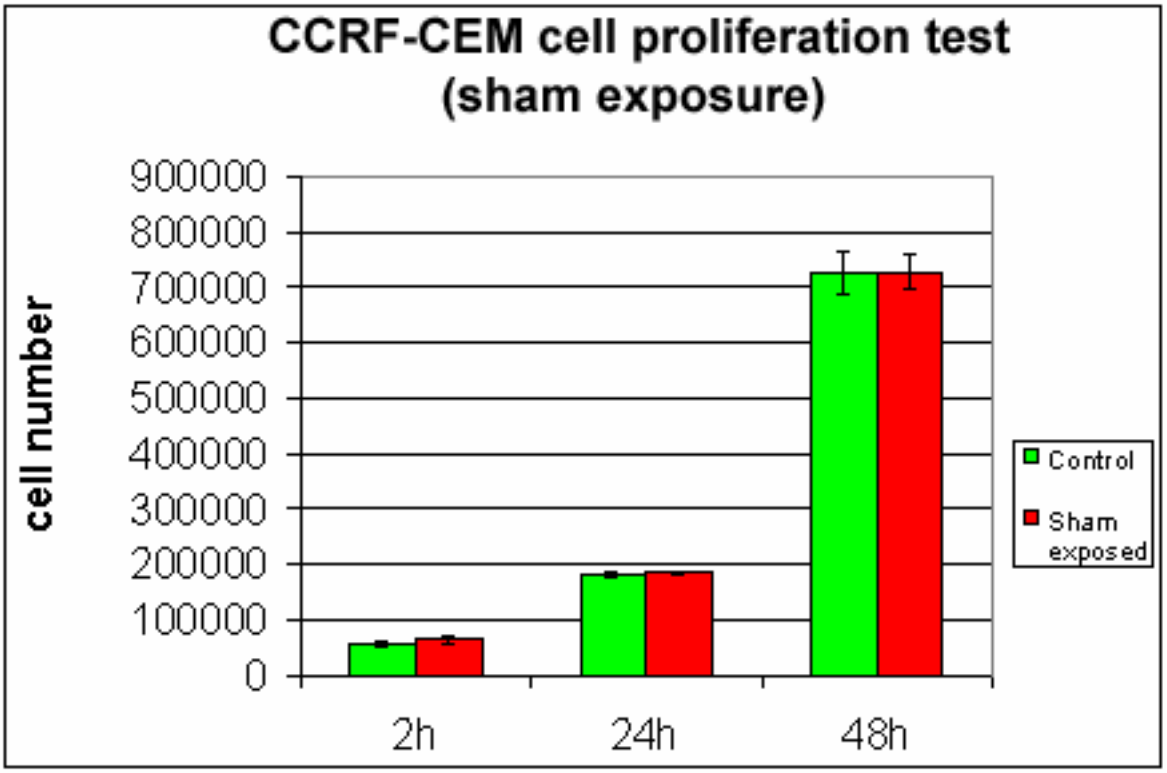

Fig. 3: Graph showing cell proliferation in CCRF-CEM T leucemic cell cultures. Sham exposure: the cells cultivated under experimental conditions but without electromagnetic field do not display any significant difference to the proliferation test.

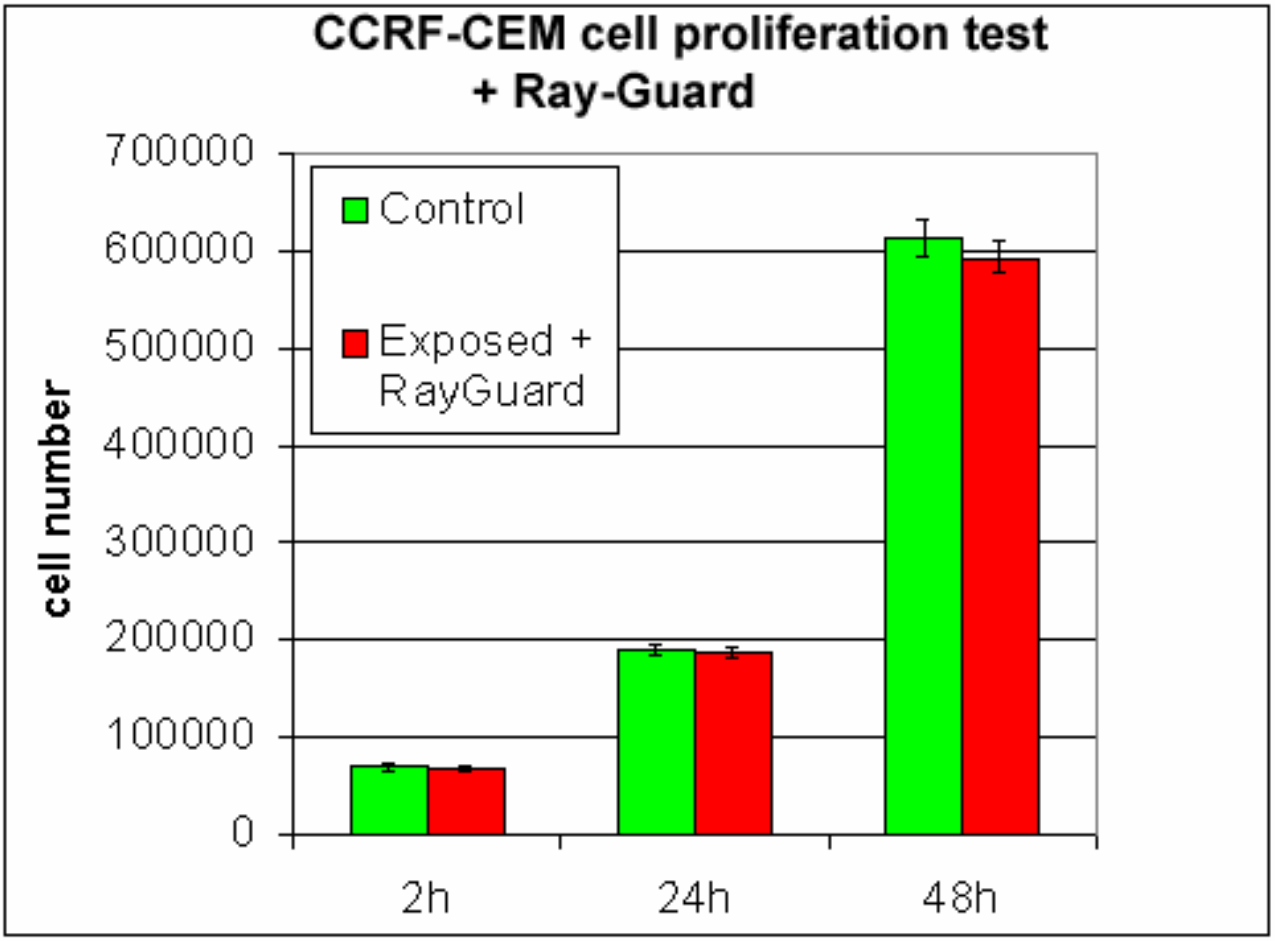

Fig. 4: Graph showing cell proliferation of CCRF-CEM T leucemic cells in culture. In the presence of the RayGuard, the cells exposed to an electromagnetic field of 900 MHz do not display any significant difference in cell proliferation at 48 hours.

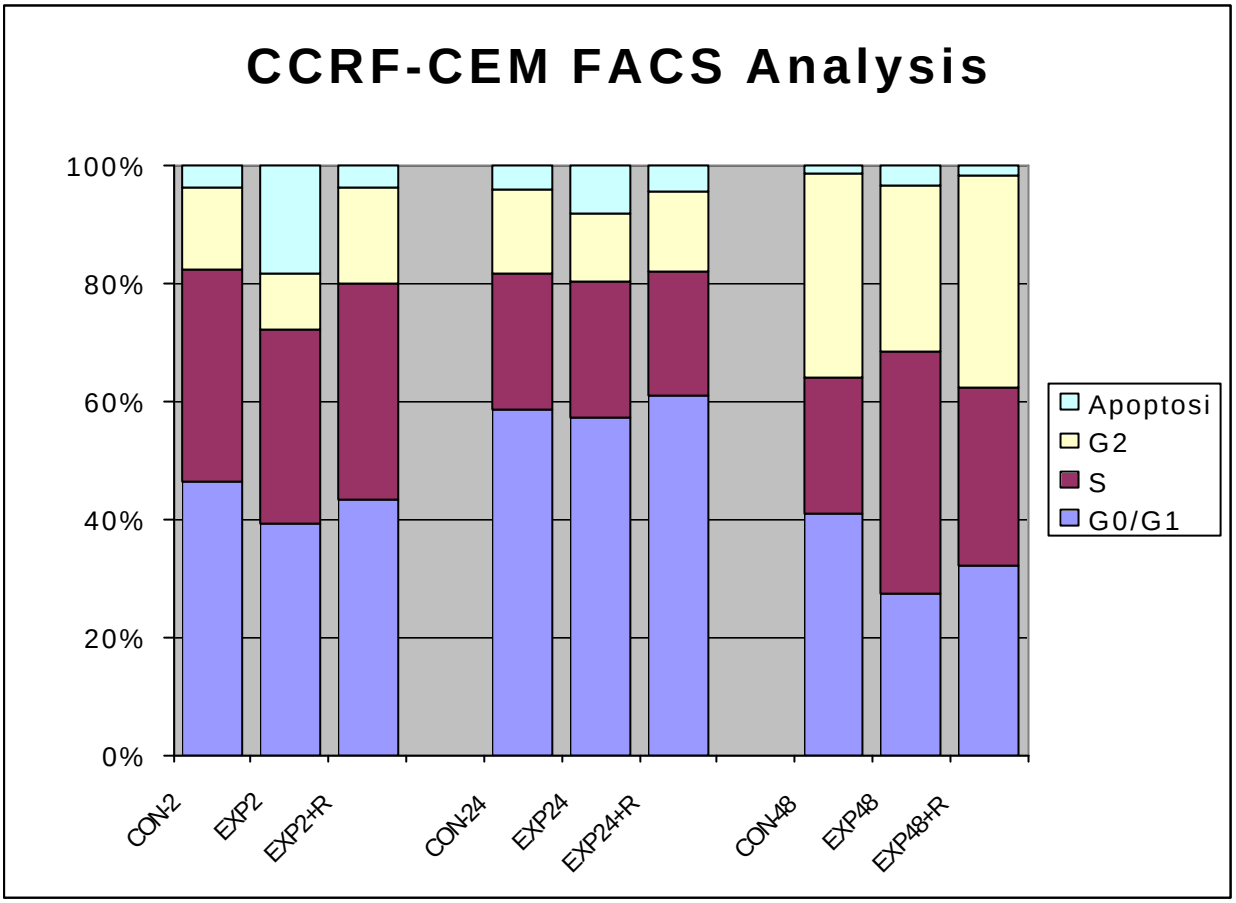

Fig. 5: GRAPH OF RESULTS OF FACS ANALYSIS
CEM leucemic cells exposed to electromagnetic field. The sections of the columns represent the percentages in the G0/G1, S, G2/M and apoptosis phases of the cell cycle.

2 HOURS EXPOSURE	G0/G1	S	G2/M	APOPTOSIS
CONTROL	45.75	35.55	13.42	3.89
EXPOSED NIR	38.5	32.38	9.13	18.07
EXPOSED+RAYGUARD	42.43	36.2	15.83	3.75

24 HOURS EXPOSURE	G0/G1	S	G2/M	APOPTOSIS
CONTROL	56.31	21.95	13.79	4.03
EXPOSED NIR	54.82	22.15	11.09	7.98
EXPOSED+RAYGUARD	58.31	20.15	12.98	4.22

48 HOURS EXPOSURE	G0/G1	S	G2/M	APOPTOSIS
CONTROL	40.05	22.60	33.97	1.37
EXPOSED NIR	26.68	39.83	27.62	3.38
EXPOSED+RAYGUARD	31.74	29.35	35.39	1.7

Tab. 1: FACS analysis of CCFR-CEM cells exposed to 900 MHz electromagnetic field with and without the **RAYGUARD** present. The values represent the percentages of cells in the various cell cycle phases. The percentages in red indicate where a statistically significant number of cells have altered cell cycle following exposure to electromagnetic fields. The values obtained from the control cells and the cells exposed to an electromagnetic field in the presence of the RayGuard are not statistically different (values in blue).

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