The effects of 60-Hz electromagnetic fields and teratogens on Drosophila melanogaster embryonic cultures: Analysis of heat shock proteins 23 and 70

Edmund James Koundakjian
THE EFFECTS OF 60-Hz ELECTROMAGNETIC FIELDS AND TERATOGENS ON DROSOPHILA MELANOGASTER EMBRYONIC CULTURES: ANALYSIS OF HEAT SHOCK PROTEINS 23 AND 70

A Thesis Presented to the Faculty of California State University, San Bernardino

In Partial Fulfillment of the Requirements for the Degree Master of Science in Biology

by
Edmund James Kountakjian
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ABSTRACT

Each day, human and other animal populations come into contact with substances that may be hazardous to themselves or to their developing embryos and fetuses. Since these agents are omnipresent, it is ideal to identify as many as possible and to limit exposure to them. In recent years, a number of developmental studies have suggested that electromagnetic fields (EMF) might act as a teratogenic physical agent. Here, *Drosophila* embryonic cells were used to assess whether exposure to magnetic fields result in a teratogenic response and furthermore if magnetic fields in conjunction with exposure to a selected teratogen serve as co-teratogens. Finally, the effect of magnetic fields acting as a general stressor to embryonic cells was assessed. In this study, a monoclonal anti-hsp70 antibody was utilized for hsp70 induction studies and a transgenic hsp23-β galactosidase *Drosophila* strain was used for the hsp23 induction studies; hsp23 was used to assess a teratogenic response and hsp70 to identify a generalized stressed state. Cultures were exposed to sinusoidal 60-Hz 100 mG or 1 G magnetic fields during different developmental times. Separate cultures were treated with well established chemical teratogens (hydroxyurea, retinoic acid, or cadmium
sulfate) with or without magnetic field exposure. Neither hsp23 nor hsp70 were induced by magnetic fields during any of the developmental stages nor were they induced from co-exposure with the three selected chemical teratogens. These results are consistent with those previously found in this lab (Nguyen, et al, '95). The results of this work do not concur with the belief that magnetic fields are teratogenic or act as a biological stressor as has been previously reported.
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INTRODUCTION

Each day, human and other animal populations come into contact with substances that may be hazardous to themselves or to their developing embryos and fetuses. These hazardous agents may be in the form of untested and illicit drugs, industrial by-products, environmental pollutants, ionizing radiation, or viruses (Wilson and Fraser, '77; Shepard, '86). Since these agents are omnipresent, it is ideal to identify as many as possible and to limit exposure to them. This is especially true for expectant mothers who need to be concerned not only with their own health but with that of their developing child who is subject to all the hazards that the mother faces but is dramatically more susceptible to them.

Development is a sequential process, composed of deliberate series of events and any slight aberration from the process may have serious consequences upon the organism. This is the focus of the field of Teratology, which is the study of the substances and mechanisms involved in developmental defects. A teratogen, literally a "monster maker", is any substance that affects the normal developmental program of an organism. The mechanisms operating include induced abnormal cell death, failure of
cell interactions, altered pattern of transcription &/or biosynthesis, or impeded morphological movements (Wilson and Fraser, '77).

Since the thalidomide tragedy in the 1960’s, there has been a concerted effort to identify potential human teratogens by exposing pregnant mammals (mice, rabbits, and rats) to these agents and assessing the developmental defects as seen in their pups. An effective alternative to these traditional in vivo studies is the use of in vitro assays which have been developed to minimize cost and time; along these lines, numerous embryonic tissue culture assays have been conducted with invertebrates, fish, amphibians, avians, and mammals (Wilson and Fraser, '77). One such assay involves the use of Drosophila melanogaster embryonic cells. The effect of a teratogen, in Drosophila embryonic cell cultures, has been shown, at the morphological level, to be a significant inhibition of muscle and neuronal cell differentiation (Bournias-Vardiabasis, '83) and, at the molecular level, an induction of a set of small molecular weight heat shock proteins (Buzin and Bournias-Vardiabasis, '84; Bournias-Vardiabasis et al., '90; Bournias-Vardiabasis et al., '91). The Drosophila in vitro assay serves as a
useful and convenient model from which findings can be extrapolated to human teratogen research.

In recent years, a number of developmental studies have suggested that electromagnetic fields (EMF) might act as a teratogenic agent (Cameron, '93; Chernoff, '92; Cox, '93; Shaw, '93). Presently, a consensus in the health and scientific communities on the potentially deleterious effects of EMF does not exist and a variety of contradictory studies have been published. The claim that EMF pose possible teratogenic or carcinogenic effects is significant because of their ubiquitous presence in our environment. Since exposure to EMF in human populations is universal, it is crucial that further systematic studies are carried out, at the cellular, molecular, and morphological levels, to validate or refute the proposed deleterious effects. The two components of EMF are the electrical field (EF) and magnetic field (MF), but the effect of MF exposure will be investigated here, since in this project, the controlled variable was the magnetic field intensity. The outcomes and applicability of the various magnetic field studies are dependent upon the animal system investigated and the nature and intensity of the magnetic field used (reviewed by Cleary, '93 and Anderson, '93).
Significant epidemiological research has been directed at finding an association between MF and human birth defects (Shaw and Croen, '93) and between MF and human cancers (Savitz, '93). These studies began with the Wertheimer and Leeper ('79) project in which Denver children exposed to 2 to 2.6 mG MF from local power lines had a two to three fold increased risk of dying from cancer when compared to the low exposure children. In the early 1990's, the Electric Power Research Institute (EPRI) surveyed a thousand U.S. homes and found that the median household magnetic field intensity was 0.5 mG and in only 5% of the households were field intensities greater than 2.7 mG (Raloff, '93). The Feychting and Ahlbom ('93) study of 128,000 Swedish children over a twenty-six year period who lived near 220-400 Kv power lines, identified a 2.7 relative risk for leukemia for exposures greater than 2 mG; they failed to show an overall increase in childhood cancers in these children relative to the control population. Feychting and Ahlbom ('94) also found a relative risk of 2.2 for adult myeloid leukemia under the same exposure and environmental conditions as with the children. Savitz et al. ('90) found that the MF exposure from household appliances may have an association with the incidence of childhood cancers and especially from
electric blanket use, which had an odds ratio of 1.7 for leukemia. Lindbohm et al. ('92) found a three-fold increase in the incidence of spontaneous abortions in women that worked with video display terminals (VDTs) of >9 mG compared to those of <4 mG. Yet, Shaw and Croen in their review ('93) concluded that the sum of evidence linking MF exposure from VDTs and spontaneous abortions and other adverse reproductive outcomes to be inconclusive; therefore, they recommended that additional research is necessary.

In a number of studies, “electrical workers”, i.e., electrician, linemen, power station operators, and so forth, were found to have a higher occurrence of leukemia and brain cancers relative to the general population, as reviewed by Savitz ('93). To avoid the weakness of epidemiological research where MF exposures are estimated and other conditions are unknown, Monson ('90) suggests that prospective cohort studies must be performed to truly assign the link between cancers and reproductive outcomes and MF exposure. Hendee and Boteler ('94) found that in 49 of the 53 epidemiological studies that they reviewed correlating EMF exposure and cancer incidence, the odds ratios were below 4, with the majority between 1 and 2. They note that an odds ratio between 1 and 4 represents a "general
relationship" and that the odds ratio, for example, relating cigarette smoking and lung cancer lie in the range of 20 and 30!

Magnetic fields do not appear to cause mutations or DNA damage. Fiorio et al. ('93) did not find an increase in mutation frequency or a decrease in culture viability in their hamster cells exposed to sinusoidal 50-Hz, 2 G MF. Otaka et al. ('92) did not observe an increase in sex-linked recessive lethal mutations in Drosophila melanogaster when exposed to 5 G or 50 G MF. According to the review of genotoxic potential of magnetic fields by McCann et al. ('93), there is no evidence that MF produces genotoxic effects. They found that twenty-two out of twenty-seven studies analyzed showed negative genotoxic effects. They also noted that in those few studies that demonstrated genotoxic effects, the outcome may have been due to coexposure with certain mutagens or ionizing radiation. This field of study is important because carcinogenesis is believed to involve three steps: initiation, promotion, and progression. MF may contribute as a cancer promoter once the DNA has been mutated by chemicals or radiation (Adey, '90). These types of analyses may be the link between laboratory studies and the epidemiological data that has
indicated an increased incidence of cancer due to MF exposure.

A number of "classical" embryonic studies investigating the effect of MF exposure have been carried out. Ubeda et al. ('83) showed that 100-Hz, 4 mG to 1 G pulsed electromagnetic fields (PEMF) was teratogenic to chicken embryos and that these treatments resulted in altered organogenesis and neuronal development. When Martin ('92) exposed chick embryos to 60 Hz, 30 mG MF, he did not find any increase in malformations. In vivo studies by Ho et al. ('92) with Drosophila embryos exposed to 60 Hz MF of 5 to 90 G showed significant cuticular pattern abnormalities and they found a roughly linear increase in abnormalities across the MF intensity gradient. Of most importance to the present study, their data indicated that the embryos were only sensitive to MF exposure during a specific developmental time window. Ma and Chu ('93) exposed intact Drosophila embryos to MF of intensities from 50 to 400 mG and found a significantly higher rate of lethality in the exposed embryos relative to the unexposed ones. In our laboratory, Nguyen et al. ('95) showed that Drosophila embryonic cultures treated with 100 mG or 1 G 60 Hz MF did not have significantly lower numbers of neuronal clusters
and myotubes and intact *Drosophila* embryos treated with MF did not show any developmental defects as assessed in the subsequent adult stage. These results demonstrated that, at least in the *Drosophila* model, MF does not act as a teratogen. Additional work in our lab involving exposure to MF in conjunction with a number of known chemical teratogens also did not produce a decrease in the differentiation index, and suggested that MF were most likely not acting as a coteratogen.

A series of studies examining MF effects at the molecular level have been reported. It has been observed by Phillips et al. ('92) that exposure of CEM-CM3 T-lymphoblastoid cells to a 1G sinusoidal MF at 60 Hz altered the transcription of cellular protooncogens. A 72-Hz, 7.4 G MF treatment of *Sciara coprophilia* salivary gland cells showed a general increase in transcription (Goodman and Henderson, '86) and 60 Hz, 15 G treatments increased translation and especially of those proteins found in the heat shocked cells but not in control cells, suggesting that MF induces a stressed state to the cell. Heat shock protein 70 was not found among the MF exposed cells (Goodman and Henderson, '88). *Drosophila* salivary gland cells that were treated with 60 Hz, 13 G sinusoidal MF
had an increase in transcriptional activity in the 87AD and 93AD regions of chromosome 3R, which are cytological locations of heat shock protein genes and specifically of hsp70 (Goodman et al., '92). Although there is not a strong consensus on the effect of MF, the altered transcriptional activity is a common one and the possibility that MF induces a stressed state in cells is worth further investigation.

It has been repeatedly documented that when a cell comes under stress, a family of polypeptides called the heat shock proteins (hsp) are expressed. Hsp have been observed in a multitude of cell types ranging from bacterial to human (Lindquist and Craig, '88). Under stressed states, it is believed that hsp help cells to survive the stress itself by protecting essential cellular components and by permitting rapid return to normal cellular activity after the stress is removed (Burdon, '87). It has been shown that a preinduction of hsp assists cells to survive subsequent stress treatments (Heikkila et al., '86; Burton et al., '88; Angelidis et al., '90). Several hsp have also been shown to have functions in non-stress conditions and are constitutively expressed. Some hsp have been shown to be developmentally regulated. Specific hsp have been shown to be induced by chemicals, metal ions, hyperthermia,
hypothermia, and anoxia. Seven major heat shock proteins have been identified in Drosophila and these are arranged in three groups: hsp83, hsp70, and the small molecular weight hsp’s, including hsp23 (Bournias-Vardabasis et al., '90).

One significant hsp group that is found across many genera is hsp70; members of this group have been shown to be highly conserved in evolution (Burdon, '87). Hsp70 has been implicated as acting as a “molecular chaperone” and as such, assists in the proper folding and translocation of other polypeptides. This function is important because those treatments that induce the stress response often interfere with protein folding and assembly (Welch, '91) and it has also been shown that hsp70 binds specifically to unfolded polypeptides and not to properly folded ones (Palleros et al., '91). Therefore, hsp70 probably acts by accelerating the restoration of the cell to the non-stressed state. A hsp70 polypeptide has a transient existence and the protein self-degrades in vivo and in vitro (Mitchell et al., '85). The evolved timing of this phenomenon probably coincides with the return to the unstressed state where the elevated levels of hsp70 are no longer needed. Hsp70, has been induced specifically by hyperthermia, hypothermia (Burton et al., '88), and metal ions (i.e., cadmium) (Heikkila et al.,
Hsp70 is not developmentally regulated, but is present in a basal level throughout the fly's lifetime (Eberlein and Mitchell, '87).

The third group of Drosophila hsp's, the small molecular weight hsps, includes the developmentally regulated hsp23, which has been shown to be expressed in high titers between the gastrula and pupal stages and later declines until it is no longer expressed constitutively in 1 week old adults (Eberlein and Mitchell, '87; Arrigo, '87). Since hsp23 is primarily expressed during the early development of the fly, it may possibly serve as a "developmental protector" and act in the assurance of proper development, especially under stressful conditions. It is obviously important that the nervous system develops properly and the above assertion is further credible since hsp23 is expressed predominantly in neuronal cells (Bournias-Vardiaibasis et al., '91).

This thesis dealt with investigating the possible effects of MF on embryonic Drosophila cells at the molecular level. Furthermore, the possible synergistic effects of MF were investigated by exposing Drosophila embryonic cell cultures to MF and to a number of known chemical teratogens. The endpoint for assessing biological activity (or the
stressed state) due to these treatments was the expression of hsp70 and/or hsp23. A monoclonal anti-hsp70 antibody was utilized for hsp70 induction studies and a transgenic hsp23-β galactosidase Drosophila strain was used for the hsp23 induction studies.

Since the induction of the small molecular weight hsp23 is indicative of a teratogenic response, hsp23 induction after exposure to MF would allow classification of MF as a putative teratogen. If exposure to MF did not result in hsp induction alone, but could increase its induction levels when MF are applied in conjunction with known teratogens, then MF could be considered to act as a coteratogen. Here, hsp70 induction was used as an indicator of the general stressed state of the treated cells and to identify whether MF acts along the same mechanisms as other known chemical and physical stressors.

My expectation was that since MF exposure had not resulted in an inhibition of neuronal and/or muscle differentiation (Nguyen et al., '95), it would not cause the induction of hsp23. Furthermore, hsp70 induction would more likely be observed after MF exposure, with or without teratogens, on the basis of previous studies done in this and other biological systems.
At the start of this project, I proposed that MF would be neither teratogenic, nor coteratogenic and that there would not be an induction of hsp23 nor hsp70; that in this system and under the conditions analyzed, MF is not teratogenic and it does not induce a stressed state in the cells.
MATERIALS AND METHODS

Fly stock
Drosophila melanogaster Oregon-R flies, with an hsp23-lacZ gene fusion (stock #700.2, kindly provided by Dr. R. Voellmy, University of Miami, School of Medicine), were raised at 25°C in a 12hr light-12hr dark cycle.

Collections
Embryos were collected for two hours on standard cornmeal medium supplemented with yeast. These embryos were then allowed to develop at room temperature for 3.5 hours, after which at least 95% were in early gastrulation. The embryos were harvested by covering the surface of the medium plate with distilled water and lightly liberating the embryos with a soft brush. The embryos were collected on a fine screen and rinsed with distilled water. Any debris was removed.

Cell culture
The cell culture technique used was modified from Seecof ('79). All glassware and utensils were steam sterilized and used exclusively for tissue culture. The embryos were dechorionated and surface sterilized in a 1:1 mixture of Clorox bleach and 70% ethanol for 1.5 min. They were transferred to a Hirsch funnel connected to a vacuum apparatus and were rinsed three times with sterilized
distilled water and twice with Schneider’s Drosophila medium. The embryos were transferred and homogenized in a 7 ml Dounce homogenizer containing 4 mls of Schneider’s Drosophila medium. The debris was removed by pouring the contents of the homogenizer through a funnel containing a fine screen and into a sterile 15 ml centrifuge tube. The cells were pelleted at 1800 rpm for 4 min and identically resuspended and repelleted three times. The number of cells was determined with a cell counter and the cell solution was diluted with Schneider’s Drosophila medium supplemented with insulin (4 mU per ml) and 22% fetal calf serum to a concentration of 8.0 X 10^5 cells per ml. 1.6 X 10^6 cells were plated on 35mm Nunc tissue culture dishes, with or without gas sterilized Thermanox plastic coverslips. The cells were allowed to adhere for 20 minutes before any further treatments.

**MF Exposure**

Sinusoidally varying MFs at 60 Hz were generated using two Helmholtz coils, each 61 cm in diameter, mounted on a Plexiglas frame, and separated 30.5 cm from one another. Coils were placed horizontally in a Shel-Lab Model 40 incubator maintained at 25°C, thus generating an magnetic field perpendicular to the plane of the culture dishes. A
separate incubator, also maintained at 25°C, was used for control cultures. Coils were driven by house current to provide uniform (0.2%) MF of 100 mG or 1 G within the volume used for placement of culture dishes. Measured background 60 Hz MFs in both incubators were <0.01-0.02 mG. MF intensities were measured at the beginning and at the end of each experiment with a Model 42A milligaussmeter from Monitor Industries (Boulder, CO).

**MF developmental treatment**
The plated tissue culture dishes were divided into six groups: 1) overnight MF exposure [ON], 2) 30 minute MF exposure immediately after plating, followed by 15.5 hours in the control incubator (i.e., no further MF exposure) [MF, ND Fix], 3) 15.5 hours in the control incubator, followed by a 30 minute MF exposure and then 1 hour at 25°C to allow expression of hsp(s) [ND, MF], 4) 30 minute MF exposure, followed by 1 hour at 25°C to allow expression of hsp(s), and then assay [MF, Fix], 5) 16 hours in the control incubator (i.e., unexposed control), and 6) 16 hours in the control incubator, followed by 30 minutes at 37°C and then 30 minutes at 25°C to allow expression of the hsp(s) (i.e., heat shock control). The developmental time schedule is illustrated in Fig. 3. The MF intensity was either 100 mG
or 1 G from a horizontally-oriented Helmholtz coil using the house current of 60 Hz, with an ambient temperature of 25°C. Each experiment, 100 mG and 1 G, was repeated three times. All RT development and recovery occurred in the control incubator at 25°C.

**Teratogen and MF co-treatment**

Three teratogens were studied at two concentrations that were found by Nguyen et al. ('95) to lie within the linear teratogenic dose response (Retinoic acid-all trans: 0.037mM and 0.002mM. Cadmium Sulfate: 0.15mM and 0.003mM. Hydroxyurea: 0.037mM and 0.002mM). The highest teratogen dosages found by Nguyen et al. ('95) were not used here because they yielded too few cells for protein processing and for the hsp23/X-Gal quantification. In the teratogen, MF co-treatment experiments, the dishes were divided into fourteen groups: the two concentrations with or without MF for each teratogen, control (no treatment), and heat shock.

After the plated cells had attached, the medium was removed and replaced with medium containing the teratogen to be tested. In the heat shock, MF without teratogen, and control dishes, the medium was removed and fresh medium was added. After the medium change, the MF groups were placed in the EMF incubator overnight. The no MF groups were
allowed to develop in a control incubator at 25°C overnight. The heat shock dishes were heat shocked the following day for 30 min at 37°C followed by 30 min of recovery at 25°C.

Sham/Sham exposure

One group of untreated cells was left overnight in the control incubator and another in the EMF incubator without current through the Helmholtz coils. This identified incubator-specific effects and determined if there was a difference in experimental technique when handling the two groups of cultures. This test validates that any effects observed in the exposed cultures when compared to the unexposed cultures are the result of the MF treatment alone and are not artificially due to experimental handling errors.

Hsp23-β-galactosidase in situ assay

Hsp23-β-galactosidase transgenic flies were used to assess the hsp23 induction. The assay used is as described in Bournias-Vardiabasis et al. ('91). The synthesized β-galactosidase was visualized by its reaction with X-gal (5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside), which yields a blue colored product. After all appropriate treatments and recoveries, the medium was removed and the coverslips were washed three times with the X-gal Buffer (10mM Sodium
Phosphate pH 7.2, 150mM NaCl, and 1mM MgCl₂). They were fixed in 1% Glutaraldehyde in X-gal Buffer for 15 min, then rinsed three times with X-gal buffer. 70 ml of X-gal Reagent (0.2% X-gal, 3.1mM K₃Fe(CN)₆, 3.1mM K₄Fe(CN)₆•3H₂O, in X-gal Buffer) was applied to each coverslip and then they were incubated overnight in a moist humidified container at 37°C. The next day, the coverslips were rinsed and mounted on slides with glycerol.

**hsp23 quantification**

After the X-gal processing, individual cells where the hsp23 construct was induced would be easily identified since they would be stained blue. The ratio of blue stained neuronal clusters (NCs) to total NCs was determined by counting all of the neuronal clusters in each field and then counting the NCs that were stained for hsp23. Identification of NCs was based on the description of Salvaterra et al. ('87).

Bournias-Vardiabasis et al., ('91), determined that the hsp23 construct was predominantly expressed in the neuronal cells and not appreciably in the other cell types present in the cultures. Six fields per slide were counted. The total NCs and stained NCs for each slide were summed and the ratio of blue stained cells was calculated for that slide. The ratio of each experimental slide was divided by the ratio
for the control slide and these numbers describe the amount of staining attributable to the experimental parameters.

**Protein preparation**

The protein sample processing procedure was modified from Lindquist ('89). After all the treatments and recoveries, the tissue culture dishes were rinsed three times with PBS. With a clean rubber policeman, the cells were scraped into a microcentrifuge tube. This was pelleted at 3000 rpm for 1 min and then the supernatant was decanted. 1 ml of ice-cold 10% Trichloroacetic acid (TCA) was added and the pellet was resuspended. This was kept on ice for 30 min. The cells were pelleted at 1400g for 10 min. The supernatant was decanted and the remaining TCA was removed by wiping the inside of the tube with paper tissue. The pellet was rinsed in 95% ethanol, which was then removed by centrifugation. 200 ml of 300mM PMSF and 800 ml of 95% Ethanol was added and the tube was vortexed. After 20 min at RT, the tube was centrifuged as before. The pellet was dried in a vacuum oven for 10 min. 1 ml of SDS-sample buffer without β-mercaptoethanol or bromophenol blue was added. The protein concentration of the solution was determined with Pierce's BCA Protein Assay. 50 μl of β-mercaptoethanol and 50 μl bromophenol blue were added. Five serial dilutions,
with TBS as diluent, were made so that 1μg, 500ng, 250ng, 100ng, and 50ng of protein would be contained in 30 μl of sample.

**Immunoblotting**

With a S&S Minfold II slot blot apparatus, the protein solutions were applied with slow vacuum onto nitrocellulose presoaked in TTBS (0.1% Tween-20 in TBS). For each experimental group, slots of 1μg, 500ng, 250ng, 100ng, and 50ng of protein were applied. The blot was labeled following the guidelines in the Vector Avidin-Biotin Complex (ABC) Kit and in the Enhanced Chemilluminescent (ECL) Kit. The blot was blocked using 10% nonfat dry milk in TTBS for 1 hr with gentle rocking. The blot was rinsed twice for 1 min with TTBS, then twice for 10 min. The blot was incubated for 1 hr in 7FB *Drosophila* anti-hsp70 (inducible form only) monoclonal rat antibody (Provided by S. Lindquist) diluted 1:4,000 in TTBS. The blot was rinsed as before. It was incubated in polyclonal biotinylated anti-rat secondary antibody diluted 1:10,000 in 1.5% (v/v) normal rabbit serum/TTBS for 1 hr. The blot was rinsed and then incubated in the ABC-complexing reagent for 1 hr. The blot was rinsed and then incubated in the Enhanced Chemilluminescent (ECL, Amersham Corp.) reagent for 1 min. The blot was wrapped in
plastic wrap and exposed to X-ray film for 15 seconds. The film was developed and the intensity of signal was quantified with a Bio-Rad 600 Densitometer. The linear range of optical density along the dilutions was determined and the dilution midway on this range was used as the signal density for data purposes for that experimental group. These values were divided by the control value to determine the relative amount of hsp70 signal.

**Statistical Analysis**

One-way analysis of variance (ANOVA), Tukey-Kramer multiple comparison test was used to determine if the mean (of the three trials per experiment) for each experimental group differed significantly from its control. In the developmental experiment, if a statistically significant difference between an exposure group and the control was calculated, then hsp23/70 was induced by the treatment; if not, then the MF treatments were not teratogenic nor a general stress to the cells. In the teratogen co-treatment experiments, if a statistically significant difference was not found between the MF exposed and unexposed cultures of the same teratogen concentration, then the MF treatments were neither teratogenic, co-teratogenic, nor a general stress to the cells.
RESULTS

Sham/sham exposures were performed on identical cell cultures placed in either the EMF incubator, without current through the Helmholtz coils, or the control incubator and then assessed for the induction of hsp23 and hsp70. Fig. 1 shows a western slot blot using the 7FB Drosophila anti-hsp70 (inducible form only) monoclonal rat antibody on the protein from the sham experiment. The mean of triplicate trials of the hsp23 sham experiment showed 23.6% of the neuronal clusters with X-Gal staining for the control incubator and 22.7% for the EMF incubator; this difference is not statistically significant based on an unpaired t-test. The mean of triplicate trials of the hsp70 sham experiment was an absorbance of 0.0507 for the control incubator and 0.0517 for the EMF incubator; this is a 2.0% difference which was not statistically significant. Therefore, there was not a significant incubator-specific contribution to the hsp23 and hsp70 induction that was observed in the rest of this project and that cultures kept in either incubator were handled similarly.

Photomicrographs of the 100 mG, hsp23/X-Gal developmental experiment are presented in Fig. 4. Table 1 presents the results from the developmental MF exposure
experiments. Neither hsp23 nor hsp70 was apparently induced by MF during any of the developmental stages; the exposure groups did not differ significantly from the control. In the hsp23, 1 G experiment, it appears that the MF reduced the expression of hsp23. The lower values are due to one trial in which the expression was reduced in all conditions, including the heat shock, but not for the control. There is no data for the "MF, Fix" (cell plating followed by 30 min MF treatment then immediate fixation) groups for the hsp23 experiments. The percent of X-Gal stained neuronal clusters was the data criterion and in this group, the cells had not yet differentiated into neuronal clusters so data could not be consistently acquired. Heat shocking the cells induced approximately 3 times as much hsp23/X-Gal and 2.5 times as much hsp70 as the MF exposed groups and the untreated (control) cells.

Fig. 5 shows photomicrographs of hsp23/X-Gal induction in cell cultures treated with teratogens with or without 100 mG MF exposure. Table 2 presents the results from the teratogen cotreatment experiments on the expression of hsp23, with MF intensities of 1 G or 100 mG. For every teratogen and concentration and for either MF intensity, there was no statistically significant difference between
the expression of hsp23 for the teratogen-only treated cells and the cotreated cells; the differences between the relative amounts of hsp23/X-Gal induced in the MF exposed and unexposed groups varied from 1.0% to 8.0%. The 0.037 mM hydroxyurea, over all trials, averaged 1.48 times as much hsp23/X-Gal expression as the control, the 0.037 mM retinoic acid averaged 0.885 times the control, and the 0.15 mM cadmium sulfate averaged 2.77 times the control. The cadmium data are consistent with that reported by Bournias-Vardiabasis et al. (’91) but in this study, the amounts of hsp23/X-Gal induced by the retinoic acid treatments were about half that previously reported. The amount of hsp23/X-Gal expressed by the 0.002 mM hydroxyurea and 0.002 mM retinoic acid averaged about the same as the control and the 0.003 mM cadmium sulfate averaged 1.13 times the control. Heat shocking the cells induced an average of 3.1 times the hsp23/X-Gal as the control. Here, MF did not increase the expression of hsp23 when applied with the teratogens.

Fig. 2 contains Western slot blots using the 7FB Drosophila anti-hsp70 (inducible form only) monoclonal rat antibody on the protein extracted from cells in the 1 G MF teratogen cotreatment experiment. Table 3 presents the
results from the teratogen cotreatment experiments on the expression of hsp70, with MF intensities of 1 G or 100 mG. Hsp70 expression did not differ in the MF cotreatments compared to the teratogen-alone cells under any condition. Hsp70 was not induced by hydroxyurea nor retinoic acid at either concentration. The high concentration of cadmium sulfate was the only treatment that increased the expression of hsp70 (+40%). The heat shocked cells had 1.75 times the expression as the control. The 100 mG experiment was performed only once, because of system and time problems, but the result was consistent with that for the triplicate trials of the 1 G experiments. MF did not increase the expression of hsp70 when applied with teratogens.
DISCUSSION

Previous work in our laboratory (Nguyen et al., '95) demonstrated that *Drosophila* embryonic cell cultures exposed to 100 mG or 1 G 60-Hz sinusoidal magnetic fields in conjunction with chemical teratogens did not result in a marked reduction in the normal developmental profile of neuronal clusters or myotubes when compared to cultures treated with teratogens alone. Additionally, intact *Drosophila* embryos treated *in vivo* to 100 mG or 1 G MF for 24 hours only or for their entire developmental time did not show an increase in developmental abnormalities evident in the adult stage. Based on these results and under those exposure conditions, magnetic fields did not act as a teratogen nor as a coteratogen based on the criteria of Wilson and Fraser ('77).

While Nguyen et al. ('95) investigated possible teratogenic effects of MF's at the morphological level, in this project, I assessed the possible teratogenic effects at the molecular level using similar conditions and treatments. My findings are consistent with their previous work. Hsp23 was not induced by MF alone during any developmental stage nor was it induced when cultures were exposed to MF along with any of the chemical teratogens. Hsp23 has been shown
to be induced when *Drosophila* embryonic cell cultures are exposed to a wide variety of known chemical and physical teratogens (Buzin and Bournias-Vardiabasis, '84; Bournias-Vardiabasis et al., '90; and Bournias-Vardiabasis et al., '91) and therefore, hsp23 induction can be used to implicate previously unidentified teratogens. Here, since hsp23 was shown to not be induced, MF cannot be considered a teratogen. Furthermore, since coexposure of MF and known chemical teratogens did not result in an increased teratogenic response, MF cannot be considered a coteratogen. This assessment further strengthens the work by Nguyen et al. ('95).

The hsp70 assessment studies were carried out to investigate the possibility of magnetic fields causing a generalized stressed state in the cells, which would be evident by an enhanced synthesis of hsp70. Here it was conclusively shown that there was not an increase in the synthesis of hsp70 under any of the conditions applied. Magnetic fields did not stress the cells as previously reported.

The impetus for these two projects was the published works of Goodman et al. ('86, '88, and '92) and Ho et al. ('92). These previous studies do not concur with the
results from our lab. Ho et al. ('92) and Ma and Chu ('93) demonstrated developmental defects in Drosophila embryos exposed to MF, indicating that MF acts as a teratogen. Goodman et al. ('92) provides data most contradictory to the findings of this present study, since when they exposed Drosophila larvae to 60-Hz sinusoidal 13 G MF, salivary gland puffs appeared in the region containing the hsp70 gene. These puffs, indicating regions actively undergoing transcription, were two times as intense as those in the glands that were heat shocked. The MF intensity used was much greater than those used in this study, but the result is striking. The applicability of such high MF exposure research in assessing risk, especially to humans, is not valid because most households and workplaces have an ambient MF intensities of less than 10 mG, although exposure to higher intensities for short periods of time are common (Cameron et al., '93; Raloff, '93). To resolve the difference between the two labs, our lab should reproduce her work in salivary glands with the MF intensities used in her paper and under the conditions in this study. Additionally, embryonic cell cultures should be exposed to the varying intensities and waveforms used by Goodman and
assayed for hsps 23 and 70 and for developmental abnormalities.

These inconsistencies illustrate my greatest reservation in comparing the results from this lab and the works of others—the lack of consistent experimental design. This is the problem with much of the EMF literature. The most credible analyses involve the observation of the effects of changing only one variable at a time; then can adequate inferences be made to the effect of that variable. Ma and Chu ('93) suggested that differences between MF studies could be due to variations in the local geomagnetic field, the AC/DC field alignment, sun spots, solar winds, and magnetospheric variation. These confounding factors enhance the need for coordinated, replicate studies in different laboratories. In fact, when Lacy-Hulbert et al. ('95) and Saffer and Thurston ('95) attempted to exactly duplicate Goodman and Henderson's works on c-myc, they did not find any evidence that MF increased the amount of c-myc transcript as reported previously by Goodman and colleagues (Goodman et al., '89; '90; '92).

The ubiquitous magnetic field poses such a potentially hazardous problem that it necessitates further standardized studies so the risk may be validated and dealt with or laid
to rest as artificial. Guy ('92) stated that a consensus on the bioeffects of EMFs will be achieved if the following four criteria are satisfied: 1) the effects must be reproduced in different independent laboratories, 2) the effects must be reproducible in different species and specifically in primates, 3) the effects must be demonstrated to be hazardous, and 4) the field conditions required to demonstrate a hazardous effect must be identifiable and reproducible.

A number of improvements to this study could have been performed. There should have been a collocation of the Drosophila rearing and tissue culture facilities with the EMF treatment facility. The cells were required to be transported the 15 mile distance between the two labs, which possibly exposed them to additional confounding factors, such as mechanical and thermal stresses. The same system should have been used for both the hsp23 and hsp70 experiments. Both should have been analyzed with western blots and should have been analyzed using the reporter gene technique. Lastly, the Thermanox coverslips onto which the cells were grown were not satisfactory. The cells did not adhere to them as well as to the tissue culture dish itself.
and the X-Gal staining could have easily been done using the whole dish rather than just the coverslip.

In conclusion, neither hsp23 nor hsp 70 were shown to be induced during any developmental stage by MF alone nor were they induced when cultures were cotreated with chemical teratogens. The results of my work do not support the belief that magnetic fields are teratogenic or act as a biological stressor as has been previously reported.
Table 1. Magnetic field exposure to Drosophila cell cultures at differing developmental stages with analysis of the expression of hsp23 or hsp70.\(^{(a)}\)

<table>
<thead>
<tr>
<th>MF Exposure:</th>
<th>hsp23</th>
<th>hsp70</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 mG</td>
<td>1 G</td>
</tr>
<tr>
<td>MF, Overnight</td>
<td>1.00±0.26</td>
<td>0.87±0.14</td>
</tr>
<tr>
<td>30 min, MF; ON no Exp.</td>
<td>1.01±0.28</td>
<td>0.88±0.03</td>
</tr>
<tr>
<td>ON no Exp.; 30 min, MF.</td>
<td>0.93±0.03</td>
<td>0.92±0.11</td>
</tr>
<tr>
<td>30 min, MF; Fix cells.</td>
<td>—(^{(b)})</td>
<td>—(^{(b)})</td>
</tr>
<tr>
<td>Control (No MF)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Heat Shock (37°, 30 min)</td>
<td>2.91±0.10</td>
<td>2.88±0.98</td>
</tr>
</tbody>
</table>

\(^{(a)}\) The experiments were performed in triplicate and their means and s.d were calculated. Values here represent the ratio of the mean ± s.d of hsp induction in the exposure groups to the control, no exposure, group. hsp23 data derived from hsp23-β-Galactosidase/X-Gal assay. hsp70 data derived from immunoblotting with rat monoclonal anti-hsp70 (inducible form only) antibody. One-way Analysis of Variance (ANOVA), Tukey-Kramer Multiple Comparison Test showed that there is no significant difference between the MF exposure groups and the control, no MF group.

\(^{(b)}\) There is no data for the 30 min MF; Fix cells group for the hsp23 experiment because at that developmental stage, neuronal clusters had not yet been formed.
Table 2. Treatment of *Drosophila* cell cultures with various chemical teratogens with or without magnetic field exposure and analysis of the expression of hsp23.

<table>
<thead>
<tr>
<th>MF Exposure:</th>
<th>100 mG</th>
<th>1 G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With MF</td>
<td>No MF</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.037 mM</td>
<td>1.36±0.13</td>
<td>1.36±0.16</td>
</tr>
<tr>
<td>0.002 mM</td>
<td>1.05±0.13</td>
<td>1.08±0.5</td>
</tr>
<tr>
<td>Retinoic Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.037 mM</td>
<td>0.93±0.14</td>
<td>0.94±0.08</td>
</tr>
<tr>
<td>0.002 mM</td>
<td>1.02±0.15</td>
<td>1.01±0.04</td>
</tr>
<tr>
<td>Cadmium Sulfate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15 mM</td>
<td>2.97±0.50</td>
<td>2.94±0.24</td>
</tr>
<tr>
<td>0.003 mM</td>
<td>1.12±0.34</td>
<td>1.12±0.31</td>
</tr>
<tr>
<td>Control (No MF or Teratogen)</td>
<td>n/a</td>
<td>1</td>
</tr>
<tr>
<td>Heat Shock (37°, 30 min)</td>
<td>n/a</td>
<td>3.46±0.25</td>
</tr>
</tbody>
</table>

The experiments were performed in triplicate and their means and s.d were calculated. Values here represent the ratio of the mean ± s.d of hsp23 induction in the exposure groups to the control, no exposure (MF or Teratogen), group. hsp23 data derived from hsp23-β-Galactosidase/X-Gal assay. One-way Analysis of Variance (ANOVA), Tukey-Kramer Multiple Comparison Test showed that there is no significant difference between the MF exposure groups and their corresponding no MF groups.
Table 3. Treatment of Drosophila cell cultures with various chemical teratogens with or without magnetic field exposure and analysis of the expression of hsp70. (d)

<table>
<thead>
<tr>
<th>MF Exposure:</th>
<th>100 mG</th>
<th>1 G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With MF</td>
<td>No MF</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.037 mM</td>
<td>1.03</td>
<td>1.01</td>
</tr>
<tr>
<td>0.002 mM</td>
<td>0.98</td>
<td>0.95</td>
</tr>
<tr>
<td>Retinoic Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.037 mM</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>0.002 mM</td>
<td>0.98</td>
<td>0.98</td>
</tr>
<tr>
<td>Cadmium Sulfate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15 mM</td>
<td>1.44</td>
<td>1.45</td>
</tr>
<tr>
<td>0.003 mM</td>
<td>1.01</td>
<td>1.01</td>
</tr>
<tr>
<td>Control (No MF or Teratogen)</td>
<td>n/a</td>
<td>1</td>
</tr>
<tr>
<td>Heat Shock (37°,30 min)</td>
<td>n/a</td>
<td>1.61</td>
</tr>
</tbody>
</table>

(d)The 100mG experiments were performed only one time. The 1G experiments were performed in triplicate and their means and s.d. were calculated. Values here represent the ratio of the mean ± s.d of hsp70 induction in the exposure groups to the control, no exposure (MF or Teratogen), group. hsp70 data derived from immunoblotting with rat monoclonal anti-hsp70 (inducible form only) antibody. One-way Analysis of Variance (ANOVA), Tukey-Kramer Multiple Comparison Test showed that there is no significant difference between the MF exposure groups and their corresponding no MF groups.
Figure 1. Western slot blot using the 7FB Drosophila anti-hsp70 (inducible form only) monoclonal rat antibody on the protein extracted from sham experiment.\(^{(e)}\)

\(^{(e)}\)The right lane is from cells grown in the EMF incubator without current through the Helmholtz coils and the left is from cells grown in the control incubator. The slots contain 1μg, 500ng, 250ng, 100ng, and 50ng of protein moving down each lane.
Figure 2. Western slot blots using the 7FB Drosophila anti-hsp70 (inducible form only) monoclonal rat antibody on the protein extracted from cells in the 1 G MF teratogen cotreatment experiment.\(^{\text{[f]}}\)

\[\begin{array}{ccc}
\text{HU} & \text{RA} & \text{Cd} \\
\text{Hi/+MF} & & \\
\text{Hi/-MF} & & \\
\text{Lo/+MF} & & \\
\text{Lo/-MF} & & \\
\text{Control} & & \\
\text{HS} & & \\
\end{array}\]

\(^{\text{[f]}}\)The concentrations for the Hi and Low Hydroxyurea (HU) and Retinoic acid (RA) are 0.037 and 0.002mM respectively. The concentrations for the Cadmium Sulfate (Cd) are 0.15 and 0.003mM. Each slot contains 250ng of protein.
Figure 3. Generic developmental experiment time schedule.\(^{(g)}\)

\[\text{Developmental MF Study}\]

\[\text{MF Treatment Timeline}\]

- Overnight
- EMF, Fix
- EMF, ND Fix
- ND, EMF
- Control
- Heat Shock

Note: MF treatments were 100 mG or 1 G. The "Fix" would represent protein processing for the hsp70 experiments and cell fixation for the hsp23 experiments.
Figure 4. Photomicrographs of 100mG MF Developmental, hsp23 experiment: (a) MF overnight. (b) Heat shock.\textsuperscript{[h]}

\textsuperscript{[h]}Blue staining indicates hsp23/β-galactosidase induction. All are on a brightfield scope at 250X magnification using Kodak ASA 400 film with exposure of +1/3 at 1/25\textsuperscript{th} of a second. Photos have been digitally retouched.
Figure 5. Photomicrographs of teratogen treatments with or without 100mG MF, hsp23 experiment: (a) Cd without MF, (b) Cd with MF. The "high" teratogen concentrations are presented here: 0.037 mM Hydroxyurea, 0.037 mM Retinoic Acid, and 0.15 mM Cadmium Sulfate. Blue staining indicates hsp23/β-galactosidase induction. All are on a brightfield scope at 250X magnification using Kodak ASA 400 film with exposure of +1/3 at 1/25th of a second. Photos have been digitally retouched.
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