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Embryo and Gamete Development upon Exposure to CCA Components: CrO_3, CuO, and As_2O_5

by

Nervana Talaat-Elsebaei Mahmoud
Medical and Surgical Bachelor, December 1997, Zagazig University, Egypt

A Dissertation Submitted to the Faculty of Old Dominion University and Eastern Virginia Medical School in Partial Fulfillment of the Requirement for the Degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Embryo and Gamete Development upon Exposure to CCA
Components: CrO_3, CuO, and As_2O_5

Nervana Talaat-Elsabaei Mahmoud
Old Dominion University, 2003
Director: Dr. R. James Swanson

Production of functional gametes and healthy embryos is essential for proliferation of all vertebrates, especially humans. Many compounds have toxic effects on developing gametes and embryos among which are chromium trioxide (CrO_3), cupric oxide (CuO) and arsenic pentaoxide (As_2O_5) as a mixture (CCA) or individually. Controversy surrounding the safety of CCA-treated wood centers primarily on the toxicity of its components and the potential for these metals to be released from the wood.

The aim of this research is to test the hypothesis that CCA components have deleterious effects on embryo development, oocyte maturation and integrity and sperm function.

A two-cell embryo assay was proposed to detect embryotoxicity of the CCA components and their mixtures. Total blastocyst cell numbers, analyzed by fluorescent DNA-binding, were the indicator of embryotoxicity on the subcellular level. Oocytes, in vitro-matured in the presence of CCA components, were analyzed for cell cycle progression. Spindle formation and chromosomal patterns in MI and MII oocytes were analyzed by immunofluorescent staining. A sperm motility index was used to evaluate sperm function in the presence of CCA components. Sperm viability was analyzed using fluorescent staining.

Summarizing our results, embryonic exposure to arsenic, chromium and copper concentrations of as little as 0.5 mg/L have a toxic effect on embryo quality represented by significant reduction in total cell numbers without reduction in the embryonic developmental stages. Increasing the concentration above 0.5 mg/L was accompanied by a dose-dependant decrease in embryonic development in the form of a drop in the number of morula/blastula stage embryos and elevation in fragmented/degenerated embryos. A trace amount of arsenic, chromium or copper inhibits oocyte maturation. The metal

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compounds delay cell-cycle progression and arrest oocytes in meiosis. Aberrant spindle and chromosome misalignments were a common feature in most MI and MII treated oocytes. The sperm motility index and viability showed a reduction in the presence of trace amount of CCA components.

In conclusion, acute-chronic exposure to arsenic, chromium or copper compounds may affect embryo and gamete development and quality on the cellular-subcellular levels.

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TABLES OF CONTENTS

Page

LIST OF TABLES viii

LIST OF FIGURES x

Chapter

I. INTRODUCTION 1
  1.1 Chromated Copper Arsenate (CCA) 1
  1.2 Arsenic, Chromium and Copper Content of CCA 5
  1.3 Health Hazards of Arsenic, Chromium and Copper Combined in CCA 15
  1.4 Hazards of CCA and its Components on Embryos and Gametes 16
  1.5 Oocyte Maturation and Embryonic Development 17
  1.6 Research Techniques and Thesis Contributions 18
  1.7 Specific Aims 22

II. MATERIALS AND METHODS 24
  2.1 Experimental Design 24
  2.2 Animals 25
  2.3 Chemical Preparations 26
  2.4 Toxicity of CCA Components and m-CCA on Embryos 26
  2.5 Toxicity of Individual Components of CCA on Oocytes 31
  2.6 Toxicity of Individual Components of CCA on Sperm 35

III. RESULTS 38
  3.1 Embryo Development and Quality 38
  3.2 Oocyte Maturation and Integrity 62
  3.3 Evaluation of Sperm Function and Viability 81

IV. DISCUSSION 88
  4.1 Impacts on Embryo Development and Quality 88
  4.2 Impacts on Oocyte Maturation and Spindle-Chromosome Behavior 93
  4.3 Impacts on Sperm Function and Viability 96

V. CONCLUSIONS AND FUTURE WORK 100
  5.1 Conclusions 100
  5.2 Future Work 101

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REFERENCES 105

APPENDICES 114

A. RAW DATA FOR EMBYRONIC DEVELOPMENT 114

VITA 118

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LIST OF TABLES

Table | Page

I. Analysis of two-cell mouse embryo development at 48 and 72 hr of culture in the presence of arsenic 41

II. Multiple comparisons of embryonic development in the presence of selected arsenic concentrations at 48 and 72 hr 42

III. Embryo development of two-cell mouse embryo 72 hr post-arsenic treatment 44

IV. Analysis of two-cell mouse embryo development at 48 and 72 hr of culture in the presence of chromium 45

V. Multiple comparisons of embryonic development in the presence of selected chromium concentrations at 48 and 72 hr 45

VI. Embryo development of two-cell mouse embryo 72 hr post-chromium treatment 48

VII. Analysis of two-cell mouse embryo development at 48 and 72 hr of culture in the presence of copper 49

VIII. Multiple comparisons of embryonic development in the presence of selected copper concentrations at 48 and 72 hr 50

IX. Embryo development of two-cell mouse embryo 72 hr post-copper treatment 50

X. Multiple comparisons of embryonic development in the presence of CCA mixture at 48 and 72 hr 54

XI. Embryo development of two-cell mouse embryo 72 hr post-CCA treatment 54

XII. Total cell number of mouse blastocysts grown in vitro in arsenic containing medium 58

XIII. Total cell number of mouse blastocysts grown in vitro in chromium containing medium 59

XIV. Total cell number of mouse blastocysts grown in vitro in copper containing medium 61

XV. Cell cycle progression of mouse oocyte maturation 16 hr post-arsenic treatment 63

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XVI. Cell cycle progression of mouse oocyte maturation 16 hr post-chromium treatment ... 65

XVII. Cell cycle progression of mouse oocyte maturation 16 hr post-copper treatment ... 67

XVIII. Analysis of spindle morphology and chromosome alignment in oocytes 16 hr post-arsenic treatment ... 69

XIX. Analysis of spindle morphology and chromosome alignment in MI oocytes 16 hr post-arsenic treatment ... 70

XX. Analysis of spindle morphology and chromosome alignment in MII oocytes 16 hr post-arsenic treatment ... 70

XXI. Analysis of spindle morphology and chromosome alignment in oocytes 16 hr post-chromium treatment ... 74

XXII. Analysis of spindle morphology and chromosome alignment in MI oocytes 16 hr post-chromium treatment ... 75

XXIII. Analysis of spindle morphology and chromosome alignment in MII oocytes 16 hr post-chromium treatment ... 75

XXIV. Analysis of spindle morphology and chromosome alignment in oocytes 16 hr post-copper treatment ... 77

XXV. Analysis of spindle morphology and chromosome alignment in MI oocyte 16 hr post-copper treatment ... 78

XXVI. Analysis of spindle morphology and chromosome alignment in MII oocyte 16 hr post-copper treatment ... 79

XXVII. Analysis of sperm function 4 hr post-arsenic treatment ... 82

XXVIII. Analysis of sperm function 4 hr post-chromium treatment ... 83

XXIX. Analysis of sperm function 4 hr post-copper treatment ... 84

XXX. Sperm viability in the presence of arsenic ... 85

XXXI. Sperm viability in the presence of chromium ... 85

XXXII. Sperm viability in the presence of copper ... 87

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LIST OF FIGURES

Figure Page

1. Development stage of two-cell mouse embryo cultured in vitro 40
2. Concentration-dependent response of embryo development at 48 and 72 hr of culture in presence and absence of arsenic 43
3. Concentration-dependent response of embryo development at 48 and 72 hr of culture in presence and absence of chromium 47
4. Concentration-dependent response of embryo development at 48 and 72 hr of culture in presence and absence of copper 51
5. Concentration-dependent response of embryo development at 48 and 72 hr of culture in presence and absence of CCA mixtures 55
6. Total cell number of 0.5 As-treated preimplantation embryos 57
7. Distribution of total cell number in mouse blastocysts grown in vitro in the presence and absence of arsenic. 58
8. Distribution of total cell number in mouse blastocysts grown in vitro in the presence and absence of chromium. 59
9. Total cell number of 0.5 Cr-treated preimplantation embryos 60
10. Distribution of total cell number in mouse blastocysts grown in vitro in the presence and absence of copper. 61
11. Morphological stages of oocyte maturation 62
12. Concentration-dependent response of oocyte maturation 16 hr post-arsenic treatment 64
13. Concentration-dependent response of oocyte maturation 16 hr post-chromium treatment 66
14. Concentration-dependent response of oocyte maturation 16 hr post-copper treatment 68
15. Spindle and chromosome behavior in As-exposed meiosis I oocytes 72
16. Spindle and chromosome behavior in As, Cr and Cu MII exposed oocytes 73
17. Spindle and chromosome behavior in Cr-exposed meiosis I oocytes 76
18. Spindle and chromosome behavior in Cu-exposed meiosis I oocytes 80
19. Viability of mouse sperm in the presence of arsenic 86

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CHAPTER I

INTRODUCTION

1.1 Chromated Copper Arsenate (CCA)

1.1.1 CCA Defined

Chromated Copper Arsenate (CCA) is a mixture of metallic salts used as a wood preservative. As the name suggests, this compound contains chromium, copper and arsenic. These metals protect wood from decay due to the action of microbes, fungi, and wood-feeding insects. The arsenic (As) repels insects, the copper (Cu) kills fungi and the chromium (Cr) fixes the copper and arsenic in the wood. Typical uses include treatment of fence posts, decking, playground equipment, and structural lumber used where it will be in contact with concrete or the ground. CCA originated in 1938 and has been manufactured in three formulations over the years, known as CCA-A, CCA-B and CCA-C. Various mixtures of salts can be used to make CCA; therefore, its precise molecular composition may vary from one formulation to another. The original CCA (CCA-A) has been used in the United State since 1938. However, most currently used formulations are CCA Type C which is a mixture of arsenic pentaoxide (34 percent), chromium trioxide (47.5 percent), and cupric oxide (18.5 percent) (Fields, 2001).

CCA-C was first introduced in 1968 and has become commonly used because it is more tightly retained by treated wood than the earlier types of CCA (Cox, 1991). CCA-C, the modern variant of the formula, is the most widely used today. All three formulas contain the same basic ingredients, just in different proportions. Although CCA was developed in the 1930s, it was not until the 1970s that it gained widespread use with the popularity of decks, porches, boardwalks, shelters, fences, and the substitute of wood for metals in children's playsets. For regions that have particular invasive wood eating insects such as termites, CCA-treated wood is also used for framing residential and commercial structures. In 1970, less than 1 million cubic meters (m^3) of CCA-treated

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wood was produced in the United States (U.S). By 1985 that number had increased to about 8 million m^3 and by 1995 to 14 million m^3 (Fields, 2001). In the production of this wood, as much as 250 liters/ m^3 of CCA solution is generally infused into the wood by pressure treatment. Instead of applying the chemical to the surface of the wood, the wood is submerged in a cylindrical tank containing the preservative. The tank is subjected to high pressure to force the CCA into the cells of the wood. This results in final concentrations between 7.800 and 78.000 milligram per kilogram. Common brand names of CCA formulations are Osmose, Wolman, and Rentokil (Fields, 2001).

1.1.2 Safety of CCA

A considerable degree of controversy has surrounded the safety of CCA-treated wood. This controversy has centered primarily on the toxicity of arsenic and chromium and the potential for these metals to be released from the wood. In 1986, the U.S. Environmental Protection Agency (EPA) ruled that CCA pressure-treated wood is safe for interior use, play structures, garden edging, and vegetable stakes (U.S. EPA, 1986). This ruling was based on the tight, "irreversible" binding of the metals to the wood fibers (Dahlgren and Hartford, 1972). However, the metals can be made bioavailable under certain conditions. In several documented circumstances, CCA-treated wood has produced adverse health effects. Peters and colleagues reported that a family was stricken with a variety of illnesses, including bronchitis, pneumonia, gastrointestinal disorders, and severe alopecia after extended burning of CCA-treated wood for heating purposes (Peters et al., 1986a). Peters and colleagues later reported that two workers developed pulmonary, hematologic, and gastrointestinal symptoms after several days of construction of picnic tables using freshly treated CCA lumber in which the metals were inadequately fixed (Peters et al., 1986b).

Data regarding the actual kinetics of release of metals from CCA wood are limited in the peer-reviewed literature, but it appears that several factors, including time, temperature, and humidity contribute to fixation and thus the bioavailability of metals in CCA-treated wood (Gordon et al., 2001). In freshwater environments, minimal leaching of metals from the CCA wood occurs, but it increases under acidic conditions (Warner and Solomon, 1990). In marine water environments, Weis and colleagues have demonstrated that metals are present in the fine fraction of sediments near CCA-treated

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bulkheads in estuaries (Weis and Weis, 1992). These metals were generally confined to within one meter of the bulkheads and resulted in a reduction in the nearby biotic community.

Thus, irreversible binding of the metals to the wood fibers after pressure treatment is not a guarantee, even though it is essential to minimize the potential exposure of wood handlers to chromium, copper, and arsenic. A field study determined that there were evidences that the CCA preservative could leach from wood. In this study the copper, chromium and arsenic content in soils under decks built with CCA-treated wood was measured. The decks ranged in age from 4 months to 15 years. A total of 85 soil samples were collected from under a total of seven decks built with CCA pressure treated lumber. Control soils were acquired at a minimum distance of 5 meters from the decks. The samples were analyzed for copper, chromium and arsenic content by atomic spectroscopy. The average amounts of Cu, Cr, and As in milligram per kilogram (mg/kg) in soils under decks were found to be 75, 43, and 76 compared to 17, 20, and 4, respectively, in control soils and the amount tended to increase with deck age (Stillwell, 1997).

1.1.3 CCA-Treated Wood Playground Equipment

Sensitive individuals will exhibit a different or enhanced response to hazardous chemicals when compared to most persons exposed to the same level of hazardous chemicals in the environment. Reasons may include genetic makeup, age, health and nutritional status, and exposures to other toxic substances. In general, the young with immature and developing organs will be more vulnerable to toxic substances than healthy adults.

Special concerns about exposure of children to toxic chemicals have been prompted by studies of their exposure to CCA caused by playing on pressure-treated wood playground equipment. Children are at particular risk from arsenic-treated wood due to several reasons:

- They are more likely to come into direct contact with the surface.
- Their hands are often sweaty and sticky, which would increase arsenic rub off.
- They are more likely to ingest the arsenic by putting their hands in their mouths.

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- Children receive a higher dose relative to their size (Environmental Working Group, EWG, and the Health Building Network, HBN, 2001).

According to a nationwide sampling overseen by the Environmental Working Group and the Health Building Network, an area of arsenic-treated wood about the size of the hand of a four year child contains an average of 20 times the amount of arsenic allowed in a 6 ounce glass of water by the U.S. EPA. EPA proposed drinking water allows up to 10 micrograms (μg) of arsenic per liter of water (U.S. EPA, 2000). However, the average surface contamination levels in an area about the size of a single four years old handprint was between 174 and 263 μg of arsenic.

It is easy to see how a child playing on structures made of pressure treated wood could easily ingest more than EPA's proposed safe daily dose. Exposures to these levels mean that about one child at every grade school would be expected to develop cancer later in life because of routinely touching arsenic-treated wood on playgrounds and at home (EWG and HBN, 2001).

A Canadian study on soil samples showed that arsenic concentrations at the base of playground equipment were from 1.8 to 23.5 times the concentration in soil 10 meters away from the equipment. Using a cloth (250-500 cm^2) to wipe the structures showed that extractable arsenic was present on all 10 of the play structures studied, in concentrations ranging from 4.8 to 149.3 μg per wipe. The wipe samples were also analyzed for chromium and copper. Concentrations of chromium varied from 4.2 to 555.8 μg per wipe and concentrations of copper varied from 5.0 to 132.2 μg per wipe. The study concluded that inorganic arsenic and chromium are poisonous and carcinogenic when absorbed in excessive amounts and that while small amounts may be harmless, it would be best to avoid any unnecessary exposure to arsenic or chromium (Riedel et al., 1991).

1.1.4 Restrictive CCA Legislation

On February 12, 2002, the EPA announced initiation of a voluntary adoption program by the industry to move consumer use of treated lumber products away from a variety of pressure-treated wood that contains arsenic by December 31, 2003, in favor of new alternative wood preservatives. As of January 1, 2004, the EPA will not allow CCA products to be used to treat wood intended for any residential use. The EPA has not

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concluded that CCA-treated wood poses any unreasonable risk to the public or the environment. Nevertheless, arsenic is a known human carcinogen, thus the agency believes that any reduction in the levels of potential exposure to arsenic is desirable (U.S. EPA, 2002).

1.1.5 Environmental permanence of CCA

After December 31, 2003, wood-processing plants will no longer be able to use CCA to treat wood intended for use in decks, picnic tables, landscaping timbers, gazebos, residential fencing, patios, walkways/boardwalks, and play structures. However, CCA will still be found in wood treated prior to this date and still used in residential settings. In addition, prior built structures containing CCA-treated wood will not be affected by this action. Another important source of CCA release occurs during the disposal process. The dominant disposal method for CCA-treated lumber in the United States is landfill burial. However, the sheer volume of biomass involved would place a considerable burden on the limited landfill capabilities. In addition, the waste decomposes with time, and there are concerns about the toxic metals finding their way into the soil and ground water. Incineration is a second disposal option, but burning CCA-treated wood in conventional incinerators releases dangerous levels of arsenic into the atmosphere. The resultant ash also contains excessive levels of hazardous waste copper and chromium, which require expensive disposal procedures (Fields, 2001).

Even with 2002 the EPA legislation, the biohazard effects of CCA and its components will be a problem for years to come. We cannot completely get rid of CCA from the environment and its health hazards will remain a big concern. CCA will continue to leach from the old wood that is still in service and be released from uncontrolled methods of disposal. Toxic effects will probably be seen for decades.

1.2 Arsenic, Chromium and Copper Content of CCA

As mentioned previously, the controversy about the safety of CCA has centered primarily on their metal components. Following is a brief overview of toxicity, mode of action and health hazards for each component of CCA:

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1.2.1 Arsenic

Arsenic is the most notorious of the chemicals contained in CCA. It is a metalloid element, but when refined, arsenic is tasteless, odorless and colorless. The metalloid, arsenic, is a common environmental contaminant, occurring in both organic complexes and inorganic forms. Inorganic arsenic was found in two forms, the trivalent and the pentavalent arsenic. Trivalent arsenic (arsenite) is substantially more toxic than the pentavalent form (arsenate) (Machado et al., 1999).

Mode of Action

Arsenic has been recognized as a human toxicant for over 2000 years. Tales of the middle ages denoted the element as an acute lethal poison to both humans and pests. An indication of arsenic's broad-spectrum toxicity is the use of arsenic-containing compounds as insecticides, rodenticides, herbicides, and plant growth regulator (WHO, 1981).

Several studies tried to explain the mechanism of arsenic toxicity to living organisms. Exposure to relatively low concentrations of inorganic arsenic has recently been shown to have a dramatic effect on the organization of cytoskeletal elements in cultured cells (Li and Chou, 1992). Inorganic arsenic impairs assembly and disassembly of microtubules, presumably by binding to protein sulphydryl groups, and thus interfering with the mitotic spindle formation and cell division (Leonard and Lauwerys, 1980). Arsenicals further cause chromosomal aberrations, which disrupt cell cycling, thereby reducing the organism's capacity for cellular proliferation (Jha et al., 1992).

The cytotoxicity of arsenic depends to a lesser extent on the form of arsenic. For example, the cytotoxicity of arsenic trioxide is due to its ability to form cyclic adducts with sulphhydryl groups of certain enzymes, resulting in abrupt inhibition of glycolysis and beta oxidation (Muckter et al., 1993). The substitution of pentavalent arsenic for phosphorus in mitochondria uncouples oxidative phosphorylation (Webb, 1966). At low doses, the metabolic effects of either form may be selectively detrimental to the developing embryo and fetus. The cytotoxicity of arsenic is also a consequence of the oxyanions affinity for sulphhydryl groups of histones and nucleic acids, a reaction that may be responsible for inducing the chromosomal damage in both in vitro and in vivo arsenic-exposed cells (Jha et al., 1992). The chromosomal aberrations observed in fetal

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cells particularly following maternal arsenic exposure suggest that this latter mechanism may be involved in the teratogenicity of the arsenic (Nagymajtenyi et al., 1985). Arsenic may generate reactive oxygen species to exert its toxicity, which is implicated in DNA damage, signal transduction and apoptosis. Arsenic also inhibits in vitro cellular DNA repairing enzymes (Li and Rossman, 1989), reduces cytosolic glutathione levels and induces membrane lipid peroxidation (Huang and Lee, 1996), suggesting multiple mechanisms may be involved in arsenic induced cytotoxicity, fetotoxicity and teratogenicity. Arsenic is detoxified by hepatic methylation to organic arsenicals (Buchet and Lauwerys, 1987) and the cofactor, s-adenylmethionine (SAM), is considered the fundamental methyl donor in this pathway. Methionine and protein deficiencies are thought to decrease availability of this cofactor, and have been shown to result in increased tissue retention of inorganic arsenic in vivo (Vahter and Marafante, 1987).

Health Hazards of Arsenic

Due to the untoward exposure of several population groups around the world to arsenic, the adverse health effects of arsenic exposure are well known and well documented. Following is some of its common systemic effects (ATSDR, 2000a):

- **Toxicological Effect**: Mild chronic arsenic poisoning can occur at doses as low as 0.15 mg per day. Serum arsenic level in the adult human normally ranges from 2-3 μg/l. The lethal dose of arsenic for an adult human is between 1 and 2.5 mg/kg of body weight (Cox, 1991).
- **Dermatological Effect**: Skin cancers in the form of basal cell or squamous cell carcinomas are one of the most serious long-term dermatological health hazards from continuous exposure to arsenic. Other serious dermatological hazards from chronic arsenic exposure can include pre-cancerous actinic keratosis (AK) and darkening of the skin (hyperpigmentation) as well as hyperkeratosis of the palms and soles of the feet have also been evidenced.
- **Respiratory Effect**: Continuous arsenic exposure cause irritation and damage to the mucus membranes in nasal passages and airways, including pharyngitis and rhinitis, and can also exacerbate symptoms of asthma. The greatest and most prevalent risk of prolonged arsenic exposure via inhalation is lung cancer (Li et al., 2002).

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- **Carcinogenic Effect**: Chronic oral, dermal or inhalation arsenic exposure can lead to several kinds of cancer. The most common cancers are of the skin, bladder, and lung; the latter being most prevalent in cases of inhalation exposure. Skin cancer can result from dermal or oral exposure, and patients with arsenic-related skin cancer are more prone to other internal cancers (Chen and Lin, 1994). Because of the numerous and extensive carcinogenic risks posed by arsenic exposure, the EPA has ranked it as a Group A carcinogen (U.S. EPA, 2001). Group A carcinogens are considered known human carcinogens. The carcinogenic effect of arsenic may be through increasing intracellular oxidant levels, promoting production of mitogenic transcription factors and antioxidant enzymes (Li et al., 2002).
- **Reproductive/Developmental Effect**: There are serious concerns with arsenic exposure and its potential to cause birth defects in both laboratory animals and humans. A review of the published scientific studies indicates that prenatal arsenic exposure produced offspring with many different kinds of malformations, including cleft palate, limb, skeletal, ophthalmic, urogenital malformation with predominant pattern of fetal neural tube defects, specifically encephaloceles and exencephaly in laboratory animals (Shalat et al., 1996). These results were true for several different species of laboratory animals, and the resultant malformations were dependent on both dosage and timing. Arsenic is known to cross the placental barrier and selectively accumulate in the fetal neuroepithelium during early embryogenesis (Hanlon and Ferm, 1977). Arsenic is also listed as a known teratogen (Shephard, 1998). In one comprehensive study on arsenic's potential for reproductive toxicity the researchers concluded that arsenic should be considered as a probable human reproductive toxin (Shalat et al., 1996). Furthermore, there have been concerns about arsenic as an endocrine disruptor. Arsenic can cause intracellular effects by affecting on the glucocorticoid receptors within the nucleus (Kaltreider et al., 2001).

The mechanistic role of arsenic as a teratogen may be explained by different theories. Inorganic arsenic may impair assembly and disassembly of microtubules, presumably by binding to protein sulphhydryl groups, and thus may interfere with mitotic spindle formation and embryonal cell division (Leonard and Lauwerys, 1980). Increased and unregulated cell death has been proposed as a mechanism of arsenic

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induced teratogenesis (Mottet and Ferm, 1983). Teratogenesis was defined by the Agency for Toxic Substance and Disease Registry U.S. public health service (ATSDR, 1990) as structural defects that affect the development of an organism. Furthermore, arsenic has been shown in diverse in vitro studies to alter transcellular, transcapillary and transplacental transport of various nutrients (Dallaire and Beliveau, 1992), and in vivo to decrease the fetal zinc levels, possibly through induction of maternal metallothionein synthesis (Taubeneck et al., 1994). This latter effect may be particularly significant in that low fetal zinc levels have been shown to be teratogenic in humans (Cherry et al., 1981). Further, deficiencies of select nutrients have been proposed to enhance the teratogenic potential of arsenic (Ferm and Hanlon, 1986).

A series of studies (Nordstrom et al., 1978a; Nordstorm et al., 1978b; Nordstorm et al., 1979) documented the reproductive hazards of arsenic by examining women who worked in a smelter in Sweden and were exposed to arsenic dust. These studies showed that due to their exposure, their babies had a higher than expected incidence of congenital malformations, including lower than normal birth weights and spontaneous abortions. Prolonged low dose human arsenic exposure has also been associated with multiple adverse reproductive outcomes including spontaneous abortion, stillbirth, developmental impairment and congenital malformation (Aschengrau et al., 1989).

In 1997, the National Academy of Science noted that humans might be even more sensitive to the adverse reproductive effects of arsenic than are the standard laboratory species. This conclusion is likely derived from the fact that rodents are more efficient methylators and more completely detoxify inorganic arsenates than humans (Hughes et al., 1994). Moreover, it has been shown that humans are found to be more sensitive to arsenic than most laboratory animals by a factor of as much as 300 times. This heightened human sensitivity to arsenic's toxicity is thought to carry over to teratogenesis (ATDSR, Toxicological profile for arsenic, 2000).

- **Mutagenic Effect**: A mutation is a change in the genetic material in a body cell. Mutations can lead to birth defects, miscarriages, or cancer. The effects of arsenic on genes read like a microcosm of its effects on the entire body as a whole. The deleterious effects of arsenic include DNA damage and a wide variety of genetic

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alterations, which can range from simple gene mutations (DNA base pair changes) to grossly visible changes in chromosome structure or number. Some of these changes may cause genetic damage transmissible to subsequent generations. Moreover, arsenic may cause genetic damage by interfering with the mechanism by which DNA repairs itself (Morton and Dunnette, 1994).

1.2.2 Chromium

Chromium is a metal widely distributed as chromite at a concentration of 250 μg/kg soil. Plants contain between 100-500 μg/kg chromium and foodstuffs contain between 20 and 590 μg/kg. Chromium is an essential trace element for biological systems. Serum chromium level in the adult human normally ranges from less than 0.05 up to 0.5 μg/ml. In the U.S., the health-based guideline for a maximum water chromium concentration of 0.1 mg/l is enforced by the EPA (U.S. EPA, 1998).

Chromium exists in several forms, there is the benign trivalent (III) form that has never been shown to cause any clinical problems and is an essential nutrient for humans in amounts of 50-200 μg per day. Trivalent chromium has been shown to be a cofactor for a low molecular weight protein, which promotes the action of insulin (Jeejeebhoy, 1999). On the other hand, there is the hexavalent chromium (VI) that has been classified as a group A carcinogen (ATSRD, 2000b). Although the form of chromium in CCA is hexavalent, less attention is paid to the chromium in CCA because the treatment process converts it to the more benign trivalent chromium. However, if during the pressure treating process the hexavalent chromium is not fixed completely and converted to the more benign trivalent form, it can leach out of the wood in its original form even if virtually all of the chromium is fixed. The lethal oral dose of hexavalent chromium for adult human is 50 mg/kg of body weight (Cox, 1991).

Mode of Action

Hexavalent chromium is a strong oxidizing agent easily permeating biological membranes. In the cell, hexavalent chromium is reduced to the trivalent form. During the process of reduction, reactive oxygen intermediates are formed and react with DNA. This interaction with DNA is believed to mediate the genotoxic effects of hexavalent chromium (De Flora and Wetterhahn, 1989). In addition, hexavalent chromium

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penetrates the mitochondria and depresses oxygen consumption by inhibiting α-ketoglutarate dehydrogenase, an enzyme that supplies the respiratory complexes with reduced nicotine adenine dinucleotide (Ryberg and Alexander, 1990).

Health Hazards of Chromium

Arsenic often gets the most attention in any discussion of the hazards of CCA and pressure-treated wood. However, the hazards of leachable hexavalent chromium, known to be present in CCA-treated wood at levels as high as 50% of the total chromium, has generally gone unmentioned (Nygren et al., 1992). Hexavalent chromium is as insidious as its partner arsenic. It is also a known teratogen and group A carcinogen. Following are some of the health hazards related to chromium exposure (ATDSR, 2000b).

- **General Effects**: High/moderate or chronic exposure leads to damage in nasal passages, (including nasal septal perforation, nasal ulcers, nasal bleeding), lung damage (with increased risk of non-cancerous lung diseases), asthma attacks, skin irritation, skin ulcers, and kidney problems such as renal proteinuria (ATDSR, 2000b).
- **Carcinogenic Effect**: Exposure to chromium has been known to cause lung cancer since the early 1930s. Based on extensive research on groups of workers in the chrome plating, tanning and welding industries, it is now known that exposure to hexavalent chromium in the workplace increases the risk of lung cancer in exposed persons by a factor of as much as 1500. The EPA has classified hexavalent chromium as a Group A carcinogen (ATDSR, 2000b).
- **Reproductive/Developmental Effect**: Numerous studies performed on laboratory animals using hexavalent chromium demonstrated its teratogenic effects. Landmark studies (Gale, 1978; Gale and Bunch, 1979) found that intravenously administered hexavalent chromium is embryolethal and produces some teratogenic effects in surviving hamster fetuses, including cleft palate and skeletal defects. These studies also found that a dose of 7.5 mg/kg of chromium trioxide was able to produce cleft palates in 85% of the hamster fetuses. Other defects were also elicited including exencephaly, micrognathia, and skeletal defects such as unossified vertebrae, abnormal limbs, and internal defects. The cause may be related to the fact that chromium delays the overall maturation of some fetuses. Chromium trioxide has

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been demonstrated to be both embryolethal and teratogenic when administered intravenously on the 8th day of gestation in hamsters (Iijima et al., 1979). Additional studies (Junaid et al., 1996; Kanojia et al., 1996; Trivedi et al., 1989) have shown that oral doses of hexavalent chromium compounds cause malformations in laboratory animals, including rats and mice. Pre-gestational exposure to hexavalent chromium caused reproductive harm to the offspring of exposed female rats. Rats were exposed to doses of 250, 500 and 750 ppm of potassium dichromate for 20 days immediately prior to fertilization. Chromium was detected in the blood, placenta, and fetuses of these test animals in proportion to the dosage they were given. Severe malformations and birth defects were noted in the offspring of all test animals. Reduced number of implantations, retarded fetal development, embryo- and feto-toxic effects including reduced number of fetuses (live and dead) per dam, and higher incidences of stillbirths and postimplantation loss were noticed in the 500 and 750 ppm-dosed mothers. Therefore, exposure to hexavalent chromium compounds before gestation causes birth defects in laboratory animals, probably due to the bioaccumulation of chromium in the mother and the fetus (Junaid et al., 1996). Moreover, the maternal chromium is reported to pass freely through the placenta to the growing fetus as evidenced from the analysis of bones from 120 human embryos (Kanojia et al., 1996).

These findings clearly show the cause and effect relationship between exposure to hexavalent chromium compounds and birth defects during the time proceeding fertilization and the critical early phases of fetal development. One can easily extrapolate from these studies that periconceptual (prepregnant) and pregnant women run a similar risk of birth defects, if exposed to hexavalent chromium compounds, such as those found in CCA-treated wood products, during this critical period of fetal development.

1.2.3 Copper

Copper is an essential nutrient and humans have a natural, efficient homeostatic mechanism for regulating the body's levels of copper ions over a wide range of dietary intake. Copper also occurs naturally in all plants and animals and at high concentrations in mussels and oysters. Copper is a heavy metal that is toxic in the unbound form. Almost all of the copper in the body is bound to proteins, thereby reducing the

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concentration of unbound copper ions to almost zero (Arancibia et al, 2003). Most diets contain enough copper (2-5 mg) to prevent deficiency and not enough to cause toxicity. The World Health Organization suggests that 10-12 mg per day may be the upper safe limit for consumption (ATDSR, 2002). In the U.S., the health-based guideline for a maximum water copper concentration of 1.3 mg/l is enforced by the EPA.

Trace amounts of copper are required in the synthesis of hemoglobin and several human enzymes (Cohen, 1979). Copper is an essential component of a number of metalloenzymes involved in catalyzing oxidative metabolic reactions. The normal human serum level is in the range of 114 μg/dl (85-236 μg/dl) of which 95% is carried by the alpha-globulin copper oxidase ceruloplasmin. The remainder is bound to albumin or amino acids. In the body, copper shifts between the cuprous (Cu^{+}) and the cupric (Cu^{++}) forms, though the majority of the body's copper is in the Cu^{++} form. The ability of copper to easily accept and donate electrons explains its important role in oxidation-reduction reactions and the scavenging of free radicals (ATDSR, 2002).

Mode of Action

Copper, except in extremely high doses, is considered nontoxic to humans. Copper is considered the least problematic metal in the CCA compound (Fields, 2001). The incidence of copper toxicosis in general population is remarkably low. However, chronic copper toxicosis is likely to be of greater concern and may result in death in extreme cases. Copper poisoning can develop under certain conditions, depending on factors such as genetic, age, diet and efficiency of intestinal absorption and biliary secretion. Many of the toxicant effects of copper are related to its oxidative damage to membranes or macromolecules. As a metal active in oxidation-reduction, copper ions are able to catalyze the formation of hydroxyl radicals. The resulting oxyradicals have the potential to damage cellular lipids, nucleic acids, proteins, and carbohydrates, resulting in wide-ranging impairment in cellular function and integrity. The exposure of DNA to these hydroxyl radicals in the presence of copper results in oxidative damage to DNA. In addition, the exposure of DNA to hydrogen peroxide in the presence of Cu (II) results in oxidative damage to the DNA in the form of strand breaks and base oxidation (Bremner, 1998).

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A common consequence of copper induced production of reactive oxygen species is increased lipid peroxidation. This resulted in peroxidation of mitochondrial membrane lipids presented with changes in mitochondrial function in the form of reducing cellular charge, increasing mitochondrial leakage of calcium into cytosol, or exposing the cell to increased amounts of superoxide generated by disruption of normal electron flow (Bremner, 1998). The toxicity of copper at the cellular level is also due to its capacity to inhibit sulfhydryl groups of glutathione, cysteine, homocysteine, methionine, coenzyme-A and pantetheine. Several important enzymes, i.e., glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and glutathione oxidase, are inhibited by copper ions (Olivares and Uauy, 1996).

Health Hazards of Copper

- **General Effect**: The adult human body burden of copper is typically 80 to 150 mg. If copper in excess of this amount is ingested, it is excreted. If very large doses of copper are consumed and homeostatic mechanisms break down, signs of acute toxicity include vomiting, diarrhea, jaundice, anemia, and urinary problems will occur (ATSDR, 2002).
- **Respiratory Effects**: In humans, copper is a respiratory irritant. Factory workers exposed to copper dust experienced mucosal irritation of mouth, eyes, and nose (Askergren and Mellgren, 1975; Suciu et al.,1981).
- **Hepatological Effect**: A key feature of the development of copper toxicosis is the hepatic accumulation of copper and hepatotoxicty. Under normal circumstances, much of the copper in the liver occurs in the cytosol. With copper toxicity excess copper accumulate in hepatic cell nucleus and lysosomes. Copper is an essential component of several important enzymes including superoxide dismutase, a free radical scavenger and cytochrome-c oxidase involved in mitochondrial respiratory metabolism as an electron transport protein (Rodrigues et al., 2003). Copper overload impairs hepatic mitochondrial respiration, primarily through a decrease in cytochrome c oxidase activity. DNA has also been reported to be a target of metal-induced damage in the liver, which may manifest as malignant transformation. Reduced cellular ATP levels, lysosomal fragility, impaired cellular calcium homeostasis, and

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damage to DNA may all contribute to hepatocellular injury in copper overload. Copper also exerts its effects by inhibiting the metabolic enzymes and by damaging the subcellular membrane structures (Chvapil et al., 1972). It inhibits the Na^+/K^+-pump leading to an increase of $[Na^+]$. As a consequence the operation mode of the Na^+/Ca^{2+}- exchanger reverses and $[Ca^{2+}]$ rises (Benders et al., 1999). Moreover, accumulation of copper in hepatic nuclei destabilizes DNA (Bryan and Frieden, 1967) and inhibits RNA polymerase activity (Novello and Stripe, 1969), manifested in liver fibrosis and malignant transformation (Britton, 1996).

- **Reproductive/Development Effect**: The reaction of the mammalian embryo to copper represents a biological enigma. On one hand, there is evidence that deficiency of copper during gestation is detrimental to embryonic survival in rat and chick embryos. On the other hand, the presence of small amounts of excess copper in the form of an intrauterine device composed primarily of metallic copper has a detrimental effect on mammalian pre-embryonic development, preventing implantation and blastocele development (Chang et al., 1970). Intravenous injection of copper salts into pregnant hamsters on the 8th day of gestation caused an increase in the embryonic resorption as well as the appearance of developmental malformations in surviving offspring. Malformations of the heart appeared to be a specific result of copper toxicity (Ferm and Hanlon, 1974). Delayed growth and development were observed in the offspring of rats exposed to copper at 130 mg/kg/day in the form of copper sulfate in their diet for 7 weeks prior to mating and during gestation (Haddad et al., 1991). Sexual impotence was reported in 16% of examined factory workers exposed to copper dust (Suciu et al., 1981).

1.3 Health Hazards of Arsenic, Chromium and Copper Combined in CCA

The combination of known toxins and carcinogens into a single chemical formula may result in potent toxin, carcinogen and teratogen. CCA may present a greater acute toxicity hazard than exposure of the constituent compounds separately (Mason and Edward, 1989). Exposures to CCA-treated wood components or to CCA-contaminated environment actually represent combined exposures to the three metals, arsenic, chromium, and copper. Biological effects associated with a co-exposure to a mixture of

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metals are recognized to significantly differ from effects caused by an exposure to each metal separately. The presence of chromium and copper may affect toxicokinetics (e.g., absorption, tissue distribution/retention, biliary and urinary excretion) of arsenic and *vice versa*. As bulk chemical agents are used for the CCA treatment, other minor chemical contaminants are also of concern in their effect on the subsequent disposition of arsenic with co-ingestion by exposed children.

A study found that the acute toxicity of CCA to daphniamagna and to a species of alga, *Selenastrum capricortum*, was greater than might be predicted from the toxicity of copper, although copper was the most toxic of CCA's three metals when the test organisms were exposed to these metals individually (Solomon and Warner, 1989). This suggests that the metals act jointly to cause toxicity. Additional experiments using the metals in pairs indicated that copper and chromium have a synergistic toxic effect (Mason and Edward, 1989). The same study found that combined acute exposure to hexavalent chromium, arsenate, and copper causes a marked decrease in fetal weight, increase in fetal resorption, and abnormal formation in rats, while none of the metals is teratogenic when administered in separate preparations intraperitonially. Co-exposures to arsenic are known to cause a profound accumulation of copper by the kidney cortex (Ademuyiwa et al., 1996). Although, copper is a relatively nontoxic metal, possible adverse consequences associated with its accumulation in the kidney should be considered under these exposure conditions.

From the previous evidence, toxicological, teratogenic and reproductive hazard are strongly implicating the arsenic and chromium elements of CCA as the causative agents. This could be due to the fact that arsenic and chromate cross the fetal placenta barrier in pregnant women. The California Hazard Evaluation System and Information Service (HESIS, 2001) have recommend that pregnant or nursing women minimize their exposure to this compound. They even go on to state a similar point about men's reproductive health.

1.4 Hazards of CCA and its Components on Embryos and Gametes

CCA compounds are found in many places in the child's environment during the most critical developmental period. The resulting long-term exposure may cause chronic

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toxicity within sensitive developing organs like those found in the reproductive system. Both male and female gametes are very sensitive cells. Chronic toxicity could cause such severe change that the damage to the germ cells could result in permanent decrease in number or increase in abnormal morphology producing intractable infertility.

Reproductive Toxicity as defined by Agency for Toxic Substance and Disease Registry U.S. public health service is the occurrence of adverse effects on the reproductive system that may result from exposure to a chemical. The toxicity may be directed to the reproductive organs and/or the related endocrine system. The manifestation of such toxicity may be noted as alterations in sexual behavior, fertility, pregnancy outcomes, or modifications in other functions that are dependent on the integrity of this system (ATSDR, 2002). CCA toxicity on the reproductive system may be demonstrated as abnormality in the gametes in the form of decrease in number or increase in abnormal morphology and/or abnormality in embryonic and fetal development.

1.5 Oocyte Maturation and Embryonic Development

Oocytes are long-lived postmitotic cells (Zamboni and Thompson, 1972). They are formed during intrauterine life, and their number does not increase after birth. The precursors of oocytes, the primordial germ cells, originate from the endoderm of the yolk sac. These germ cells migrate to the presumptive gonads and convert to actively dividing oogonia. Before birth, the cessation of mitotic process and the initiation of meiosis lead to the formation of the primary oocytes. Once formed, the primary oocyte persists in prophase of the first meiotic division until the time of puberty. At puberty, one of these primary oocytes will grow every month until it becomes a fully-grown oocyte. Just prior to ovulation, the fully-grown oocyte in the Graafian follicle resumes meiosis and completes the first meiotic division ended by the emission of the first polar body (PB). Once the ovulation has occurred, the second meiotic division begins. It then stops at metaphase II with chromosomes aligned on the metaphase II spindle (Knobil and D. Neil, 1993). At this time, the oocyte is called mature or metaphase II oocyte. Although the exact cellular mechanisms responsorial for this meiotic arrest remain uncertain, it is generally presumed that a granulosa cell-derived putative meiosis inhibitor is in play.

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This hypothesis is based on the observation that denuded (granulosa-free) oocytes are capable of spontaneously completing meiotic maturation in vitro. If sperm penetration occurs, the mature metaphase II oocyte undergoes a second meiotic division, after which the second polar body is eliminated. The resulted one-cell embryo will then undergo a rapid mitotic division passing in the following preimplantation stages: two-cell embryo, four-cell embryo, eight-cell embryo, morula then blastocyst (Knobil and D. Neil, 1993).

1.6 Research Techniques and Thesis Contributions

In this research, a number of techniques will be used. For each of these techniques, we present a brief overview of their previous usage and our contribution to use them in this proposed study. Mouse embryos and gametes were chosen as the animal model in this work because of their high genetic similarity to humans. In addition, their sensitivity to identify the toxicological effect and dose of many chemicals that proved to cause malformation, congenital defects and carcinogenic in human was approved in many reproductive studies (Ackerman et al.,1984; Eichenlaub-Ritter and Boll, 1989).

1.6.1 Mouse Embryo Assay

Two-cell mouse embryo assay was initiated in our embryonic laboratory since 1980 as a quality control screening assay for media, supplements, cultureware, and related equipment used in developing human *In Vitro* Fertilization (IVF) program for Jones Institute in Norfolk, Virginia (Ackerman et al., 1984). Culture of two-cell mouse embryos to the blastocyst stage has been the most commonly used quality control standard for human IVF (Dandekar and Glass, 1990). Two-cell embryos collected from fallopian tube of hormonally stimulated mice were able to detect a toxin in the cultured media in an exposure dependant manner. Preimplantation embryo culture models provided insights into the vulnerability of reproduction to metal toxicities (Hanna et al., 1997).

1.6.2 Embryo Quality

Following fertilization, the embryo, enclosed in an acellular envelope of the zona pellucida, undergoes a number of cleavage divisions ending with blastocyst formation (Hardy et al., 1989). Blastulation is a morphogenetic event in early embryonic

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development common to all mammalian species (De La Fuente and King, 1997). The eutherian blastocyst is composed of two distinct cell types: an outer epithelial layer of trophectoderm (TE) responsible for blastocoel fluid accumulation and specialized for implantation, and an inner cell mass (ICM). Following the implantation, the TE only gives rise to components of placenta and extraembryonic membranes whereas the ICM forms all three germ layers of the fetus as well as the complementary contribution to the extraembryonic membranes (Van Soom et al., 2001). Allocation of cells to these two primary lineages occurs at the preceding morula stage mainly during the fourth cleavage division. Total cell number of the blastocyst has been previously reported to be a good indicator of embryo quality and developmental rate. The total embryo cell numbers appeared to be dependant on the culture medium used (Van Soom et al., 1997). Therefore, the environment clearly plays a role during early embryonic development on the number of cells in an embryo (Knijn et al., 2003). Culture conditions affect blastocyst formation rate (Viuff et al., 2001), reducing embryo quality by decreasing the number of embryonic cells allocated to the ICM, and by increasing the incidence of apoptosis in individual blastomeres (Brison and Schultz, 1997; Hardy, 1997). Nuclear staining of whole-mounted blastocysts for determination of cell number has been improved in efficiency and ease of handling with the introduction of chromatin-specific fluorochromes (Thouas, 2001). Monochromatic staining methods using nuclear-specific dyes have been used to stain for total blastocyst cell numbers and are still in use today (Fong and Bongso, 1999). Fluorescent DNA-binding dye has provided a rapid and accurate method for counting the number of cells in the preimplantation mouse embryos (Ebert et al., 1985).

To detect and compare the relative toxicities of different concentrations of tested metal compounds (arsenic pentaoxide, chromium trioxide and cupric oxide) on embryo development and quality, we examined both embryonic cell proliferation and differentiation by analysis of the rate of blastocyst formation and embryonic cell numbers at the end of the culture period.

1.6.3 In Vitro Maturation of Oocytes

Oocyte maturation is defined as the reinitiating and completion of the first meiotic division, subsequent progression to metaphase II, and stimulating nuclear and

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cytoplasmic processes, which become essential for fertilization and early embryo development. Oocytes are arrested in prophase I of meiosis during the fetal period. Completion of the first meiotic division takes place when oocytes have undergone extensive growth in cellular interaction with the granulosa and theca cells. The oocyte undergoes asymmetric cytokinesis and extrudes the first polar body containing a haploid chromosome complement. The first meiotic division is thus completed and the second meiotic division is initiated, but oocytes remain arrested in metaphase II until contact is made with a spermatozoon. The initiation of maturation in fully-grown oocytes, which are present in antral follicles, is based on the mid-cyclic onset of the luteinizing hormone (LH) surge or the exogenous administration of human chorionic gonadotropin (hCG). Mechanisms of oocyte maturation in vivo and in vitro are still under investigation. In vitro animal models provided insight into the importance of substances affecting oocyte maturation and its inhibition, such as cAMP, calcium, cell-cycle proteins, growth factors, gonadotropin-releasing hormone (GnRH), gonadotropins, purines and steroids.

In vitro maturing mouse oocytes have been employed in many studies (Eichenlaub-Ritter et al., 1986; Eichenlaub-Ritter et al., 1988a), to investigate factors affecting oocytes developments during in vitro maturation to metaphase II. Mouse oocytes isolated from large antral follicles of ovary of hormonally unstimulated animals (Eichenlaub-Ritter and Boll, 1989) were cultured in absence or presence of aneugens or selected metabolites at defined concentrations. In this way, the threshold concentration of each element can be identified. Most importantly, these oocytes maturing in vitro were processed for immunofluorescence to examine their spindle formation and chromosomal behavior.

The differences in the mechanisms of spindle formation and cell cycle control during mitosis and meiosis and in the characteristic pattern and cell biochemistry of germ cells have an impact on the predisposition to errors in chromosome segregation in response to certain drugs and chemicals. When germ cell chromosomes of in vivo exposed rodents to aneugens were analyzed cytogenetically, most of them were displaying non-disjunction, and meiotic delay (Eichenlaub-Ritter et al., 1988a). The formation of a functional spindle, completion of the first meiotic division and high fidelity of chromosome segregation are essential features of healthy oocytes (Eichenlaub-

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Ritter et al., 1986). Spindle morphology and chromosome alignments were used as one indicator for the capacity of the oocyte to form a chromosomally balanced embryo. Chemicals such as chloral hydrate (Eichenlaub-Ritter and Betzendahl, 1995) were proved to be toxic to the developing oocytes. Their toxic effect was through affecting on oocytes meiotic maturation and fidelity of chromosome segregation in mammalian oocytes maturing in vitro.

Therefore, these techniques were used in this research to detect the toxicity of various concentrations of the tested metal compounds on oocyte maturation and spindle-chromosome behavior.

1.6.4 Sperm Motility Bioassay

The sperm motility bioassay was used in several studies and became an invaluable quality-control test in some laboratories. This test has been used before and has showed a greater sensitivity to embryotoxic factors (Bavister, 1974; Bavister and Andrews, 1988). The degree of sensitivity is greatly increased when protein or other chelating agents (e.g., amino acids) are excluded from the culture media (Esterhuizen et al., 1994). A sperm motility index (SMI) of spermatozoa obtained from the cauda epididymis of adult male (hamster or mouse) was the parameter used for comparison between the treated and untreated sperm samples in all previous studies.

In this study, the sperm motility assay was used to detect the toxicity of arsenic, chromium and copper compounds on sperm function. Not only SMI was compared between different tested compound concentrations, but also motility percentage and forward progressive movement score were compared between samples.

1.6.5 Sperm Viability

One of the most important parameters in the evaluation of fertility is the analysis of sperm viability. The assessment of sperm viability is one of the basic elements of semen analysis and is particularly important in samples, where many sperm are immotile but not dead, to distinguish between immotile dead sperm and immotile live sperm (Pintado et al., 2000). Several methods have been used to distinguish between viable and non-viable sperm cells, such as the nigrosin-eosin stain (Hancock, 1951) and fluorescent probes (Garner et al., 1986). Some would argue that these methods are unreliable because some

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sperm cells showed partial staining (eosin-nigrosin) or because the method may overestimate the number of non-viable spermatozoa (fluorescent probes). Fluorescent staining with propidium iodide (PI) is a rapid and reliable assay for determining the percentage of live and dead spermatozoa in a semen sample (Huo et al., 2002).

Sperm viability in the presence of tested compounds was analyzed, using fluorescent staining with PI, to distinguish between immotile dead sperm and immotile live sperm and to detect if the toxicity were through spermiostatic or spermicidal effect.

1.7 Specific Aims

Gamete and embryo deterioration in function and development with exposure to toxic compounds is a significant problem. Many compounds and various materials have been proven toxic to body systems specially the reproductive one. CCA mixture and its individual components comprise four of these potentially toxic compounds that many studies have referred to their harmful effects on various body systems. No previous studies have been found that investigated the hazardous effects of the CCA compound and its individual components on preimplantation embryos or gametes. The lack of contribution in this topic was our main motivation to investigate and study embryo and gamete toxicity in the presence of chromium trioxide (CrO_3), cupric oxide (CuO) and arsenic pentaoxide (As_2O_5) components of CCA.

To test the hypothesis that CCA components have detrimental effects on embryo development, oocyte maturation and integrity and sperm function, three major aims were designed to assess the validity of this hypothesis:

Aim 1: To determine the effect of CCA (single components, mixtures and synergism-potentiation) on preimplantation embryo development (growth and differentiation) at the cellular and subcellular level. Hypothesis: CCA components reduce embryo development through deleterious effects on cell division and differentiation.

Aim 2: To determine the developmental and genetic effect of individual components of CCA on in vitro matured germinal vesicle (GV) oocytes. Hypothesis: CCA components have toxic effects on cell cycle progression, spindle formation and chromosomal alignment.

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Aim 3: To determine the effect of CCA components on sperm function and viability. Hypothesis: CCA components lead to sperm dysfunction and lethal changes.

In addition, the following items were identified:

- The toxic concentration (TC_{50}) for each tested metal component of CCA for embryo development.
- The toxic concentration (TC_{50}) for each tested metal component of CCA for oocyte maturation.
- The toxic concentration (TC_{50}) for each tested metal component of CCA for sperm function.

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CHAPTER II

MATERIALS AND METHODS

2.1 Experimental Design

Determining embryo and gamete toxicity from exposure to CCA and its components provides the understanding critical for determining the risks in both acute and chronic contact. This novel research protocol can test the toxicity of any compound on embryos and gametes and in this manner was used to evaluate the toxicity of CCA and it's components. To test the hypothesis that CCA components have detrimental effects on embryo development, oocyte maturation and sperm function, three major experimental groups were employed:

- Group A: Testing the toxicity of individual components of CCA and the co-mingled CCA compound (mixed CCA; m-CCA) on embryos included three main experiments, a brief description of them follows:

  Experiment 1: Toxicity of individual components of CCA

  - Testing the dose-dependent toxicity of individual components of the CCA compound (chromium trioxide, cupric oxide and arsenic pentaoxide) on two-cell mouse embryo development.
  - Determining the toxic concentration (TC_{50}) and the effect of each CCA component on two-cell mouse embryo development.

  Experiment 2: Toxicity of the CCA compound

  - Detecting the toxicity-potentiating effects of mixing the previously determined nontoxic concentration of individual components of CCA on two-cell mouse embryos.

  Experiment 3: Embryo staining and total cell counting

  - Determining the total cell numbers of blastocyst stage embryos cultured in the nontoxic concentration of individual components of CCA.

- Group B: Testing the toxicity of individual components of CCA on oocytes included two main experiments:

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Experiment 1: Analysis of cell cycle progression

- Testing the dose-dependant toxicity of individual components of the CCA on oocyte maturation.
- Determining the toxic concentration (TC_{50}) and the effect of each CCA component on oocyte maturation.

Experiment 2: Cytogenetic analysis

- Analyzing spindle morphology and chromosome behavior in the presence of individual components of CCA.

- Group C: Testing the toxicity of individual components of CCA on sperm included two experiments:

  Experiment 1: Sperm motility assay

  - Testing the dose-dependant toxicity of individual components of the CCA compound on sperm.
  - Determining the toxic concentration (TC_{50}) and the effect of each CCA component on sperm motility

  Experiment 2: Sperm viability

  - Monitoring the viability of the sperm in the presence of CCA components.

2.2 Animals

Animals used in this study were B6CBAF1/J female mice (Jackson Laboratory, Bar Harbor, ME, USA) and CD1 male mice (Charles River Laboratories, Wilmington, MA, USA). All animals used in this study were kept in the same environmental conditions, in 14-hr light/10-hr dark, temperature controlled (21-23°C) room. They were provided with laboratory rodent chow and water ad libitum.

2.2.1 Justification of Animal Use

Mouse embryos and gametes were chosen as the animal model in this study for several reasons:

- Mouse embryo culture is sensitive to toxins (Ackerman et al., 1984).
- Mouse embryo culture is commonly used to predict success for human IVF. Results obtained will have a history of application to human embryo and gamete function.

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- Many reproductive studies have used the mouse model resulting in a large body of literature.
- Superovulation is possible in the mouse model, allowing collection of a large number of embryos and gametes per female mouse.

All experiments and animal procedures conform to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) (Assurance 98-040). Euthanasia was by cervical dislocation, approved by the Panel of Euthanasia of American Veterinary Medical Association.

2.3 Chemical Preparations

Chromium (Cr), copper (Cu), and arsenic (As) are used in this study as abbreviations for chromium trioxide (Sigma, St. Louis, MO, USA), cupric oxide (Fluka, St. Louis, MO, USA) and arsenic pentaoxide (Sigma). Stock solutions of 100x (100 mg/L water) arsenic pentaoxide (As_2O_5) and chromium trioxide (CrO_3) were prepared and stored at room temperature. The stock solution of cupric oxide (CuO) at 100x (100 mg/L water) resulted in fine precipitations. Cupric oxide was completely dissolved by adding few drops of 1 M HCl and adjusting the pH of the solution to 7.4 with 1M NaOH. The final concentration of 100x stock solution of cupric oxide was 86 mg/L.

2.4 Toxicity of CCA Components and m-CCA on Embryos

2.4.1 Determining the Toxicity of Individual CCA Components on Embryos

Two-cell mouse embryos were cultured in media containing different concentrations of each component of CCA to create dose-response curve. The beginning concentration for each component was 0.001 mg/L and was increased until 100% embryolethality was achieved. Embryo development was analyzed at 48 and 72 hr of culture.

Media Preparation

Embryos were collected and cultured in modified Krebs bicarbonate-buffered (mKBB) supplemented with 0.4% (4 mg/mL) bovine serum albumin (BSA fraction V; Sigma). Medium was prepared, as described by Ackerman (Ackerman et al., 1983), checked for a pH value between 7.6 and 7.8, filter sterilized through 0.22 μm filter unit

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(Nalge Co., Rochester, NY, USA) and refrigerated at 4°C. Culture medium beyond 4 weeks age was discarded. All media were pre-tested before use to prove their support of normal embryo development. Acceptable pre-tested cultured medium supported development of two-cell mouse embryos to the morula or blastocyst stage equal to or greater than 75% after 72 hr culture (Ackerman et al., 1984).

Each experiment included a negative control group. Negative control embryos were cultured in 0.4% BSA mKBB medium.

Superovulation and Mating

Female mice, 1-6 months old, were induced to superovulate by an intraperitoneal (IP) injection with 0.1 mL (5 IU) pregnant mare serum gonadotropin (PMSG; Sigma; 2000 IU/ 40 mL phosphate-buffered saline (PBS) stored at –20°C in 1 mL aliquots) followed 48 hr later by an IP injection of 0.1 mL (5 IU) human chorionic gonadotropin (hCG; Sigma; 5000 IU/100 mL PBS, stored at –20°C in 1 mL aliquots) (Hogan et al., 1994). Immediately post-hCG injection, each female mouse was placed in an individual cage with one proven fertile male CD-1 mouse. Approximately, 16 hr post-hCG injection, female mice were inspected for a coitus positive vaginal mucous plug.

Embryo Collection

Plug-positive female mice were sequentially euthanized by cervical dislocation approximately 42 hr post-hCG injection. The abdominal wall was aseptically opened and oviducts were excised and placed into 2 mL equilibrated mKBB culture medium in sterile 35-mm polystyrene Nunclon culture dish (Thomas Scientific, Swedesboro, NJ, USA). Using a Zeiss dissecting microscope (Eastern Microscope Co., Raleigh, NC, USA), two-cell embryos were irrigated from the oviducts with a 30-gauge needle inserted into the fimbriated end and connected to 1-mL tuberculin syringe (B-D Becton Dickinson, Lincoln Park, NY, USA) filled with mKBB medium (Swanson and Leavitt, 1992). Only morphologically normal two-cell embryos, as judged by oval, bilateral symmetry of two blastomeres with narrow perivitelline space and presence of two small polar bodies were harvested in 10 μl aliquots and aseptically dispensed into various cultures.

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Embryo Culture

Individual two-cell embryos were cultured in per well in a 96-well Falcon culture plate (B-D). Each well contained a final volume of 200 μL including equilibrated (minimum 30 minutes) 0.4% BSA mKBB medium plus the desired concentration of the test compound CrO_3, CuO or As_2O_5. Each compound was tested in sequentially increasing freshly prepared concentrations with two or more replications separately for at least 2 experiments. In each experiment, different concentrations of each compound were added to 0.4% BSA mKBB culture medium shortly before use. Media equilibration were then took place for at least 30 min before each experiment. Embryos were cultured at 37°C in 5% CO_2 in 100% humidified air for 72 hr.

Embryo Monitoring and Analysis

Embryos were morphologically evaluated at 48 and 72 hr of culture using an Olympus inverted microscope (Olympus, America Inc., Melville, NY, USA) fitted with an OLRC unit (Olympus Relief Contrast). Images were taken with a QImaging digital camera (Quantitative Imaging Co., Burnaby, British Columbia, Canada) using Q Capture 2 software version 1.1.

Developing embryos were categorized as 2-cell, 3-4 cell, 6-8 cell, morula, blastocyst, degenerated (condensed and granulated blastomeres), or fragmented (numerous and uneven-sized blastomeres or cytoplasmic blebs).

Defining Embryotoxicity of CCA Components

For each test compound, the following parameters were defined relative to dose and toxic effect on embryo development:

- Toxic Concentration (TC_{50}): The concentration that inhibits 50% of embryo development to both morula and blastocyst stages at 48 and 72 hr of culture respectively when compared with the negative control group.
- Nontoxic Concentration: The highest concentration of compound exhibiting no statistical difference on embryo development when compared with the negative control.

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- Subtoxic Concentration: The concentration at which embryonic development exhibiting slight insignificant reduction when compared with the negative control group.

Statistical Analysis

As a nonparametric equivalent to one-way analysis of variance (ANOVA), the Kruskal-Wallis ANOVA test was performed within each experiment to determine significant differences among the treated groups. Treated groups were compared with each other and with the control group using the Mann-Whitney test with Bonferroni adjustment, a nonparametric equivalent to an independent t-test, for pair-wise multiple comparisons. Comparing the number (percentage) of embryo development in different embryonic stage was performed using Chi-Square test.

2.4.2 Determining the Toxicity of the m-CCA on Embryos

Toxic and nontoxic concentrations of Cr, Cu and As were estimated from the previous experiment. To detect synergistic embryotoxic effects of mixing the three CCA components, nontoxic-subtoxic concentrations were chosen for each one and diluted with mKBB culture media for the experimental m-CCA solution.

Five serial dilutions of m-CCA compounds were prepared, each was containing different nontoxic-subtoxic concentrations of the individual CCA components.

- CCA1 contained 1.7 mg/L chromium, 6.08 mg/L copper and 0.3 mg/L arsenic in a final volume of 8 mL medium.
- CCA2 (3/4 of CCA1) contained 1.275 mg/L chromium, 4.56 mg/L copper and 0.225 mg/L arsenic in a final volume of 8 mL medium
- CCA3 (2/4 of CCA1) contained 0.85 mg/L chromium, 3.04 mg/L copper and 0.15 mg/L arsenic in a final volume of 8 mL medium.
- CCA4 (1/4 of CCA1) contained 0.425 mg/L chromium, 1.52 mg/L copper and 0.075 mg/L arsenic in a final volume of 8 mL medium.
- CCA5 (1/10 of CCA1) contained 0.17 mg/L chromium, 0.608 mg/L copper and 0.03 mg/L arsenic in a final volume of 8 mL medium.

Embryo collection, culture, monitoring and statistical analysis were performed as described for the proceeding experiments.

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2.4.3 Embryo Staining and Total Cell Counting

To detect the quality of the treated embryos, total cell numbers of blastocyst stage embryos were used as an indicator of embryotoxicity on the subcellular level. In vivo produced two-cell mouse embryos were cultured in the presence of nontoxic concentrations of individual CCA compounds. The total cell numbers were determined in embryos reaching blastocyst stage after 72 hr of culture. The non embryo-toxic concentration for each component of CCA was determined from the previous experiments. The nontoxic concentration for each of the 3 compounds was surprisingly the same at a value of 0.5 mg/L.

Embryo Staining

The staining protocol used in this study was a modification of Thouas method (Thouas, 2001), in which, Zona-intact (expanding, or partially hatched) blastocysts were incubated in 500 μL of solution 1 [BSA-HEPES buffered M2 medium (Hogan et al., 1994) with 1% Triton X-100 (Sigma)] in 9-cavity Pyrex brand plate (Fisher, Pittsburgh, PA, USA) for up to 10 s or until trophectoderm visibly shrank as monitored visually under a Ziess dissecting stereomicroscope. The Triton X-100 treatment increases the intensity of fluorescence of DNA-dye complex due to loss or clearing of the cytoplasm without disruption of nuclear integrity. Blastocysts were then immediately transferred into 500 μL of solution 2 (fixative solution of 100% ethanol with 25 μg/mL bisbenzimide (Hoechst 33342; Sigma)) and stored at 4°C overnight in a humidified chamber. Hoechst is a membrane-permeable, fluorescent DNA stain with low cytotoxicity that intercalates with specific regions of the DNA. On the second day, fixed and stained blastocysts were transferred from solution 2 directly into 15 μL droplets of glycerol (Sigma) on a glass microscope slide. Blastocysts were gently flatted by applying slight pressure to a cover slip. This compression allowed accurate determination of the number of cells in comprising the blastocyst since all the nuclei could be observed in essentially a single focal plane. Coverslips were sealed with nail varnish for preservation. Slides were stored at 4°C and evaluated within 1 week of preparation.

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Total Cell Count

Cell counting was performed directly from images obtained on a Nikon epifluorescence-fluorescence microscope (Nikon, Melville, NY, USA) with excitation filter UV 330-380. Photographs were taken by a Spot camera (Diagnostic Instruments, Sterling Heights, MI, USA) using Spot software version 3.0.

Statistical Analysis

The total cell numbers for blastocysts were compared between treated groups and control groups using one-way ANOVA.

2.5 Toxicity of Individual Components of CCA on Oocytes

To determine the effect of each component of CCA on oocyte development, in vitro maturation of germinal vesicle (GV) stage mouse oocytes was performed in the presence of different concentration of each component of CCA. Cell cycle progression and cytogenetic analysis were used as sensitive indicators for oocyte toxicity on the cellular and subcellular level. Each component of the CCA compound was tested separately in 2 or more replicates.

2.5.1 Analysis of Cell Cycle Progression

Oocyte Isolation

Female mice were injected with 0.1 mL (5 IU) PMSG to stimulate follicular development. At 46-48 hr post-injection, female mice were euthanized by cervical dislocation. The abdomen was opened aseptically and ovaries with their fat pads were dissected and transferred into 35-mm petri-dish (Thomas Scientific) with 3 mL of pre-warmed M2 medium (Sigma). The fat pads were dissected away and ovaries were transferred to another 35-mm petri-dish. Large antral follicles were punctured using a 30-gauge, ½ inch sterile needle attached to a 1 mL sterile syringe. Oocytes were stripped of the surrounding cumulus cells by gentile aspiration/expulsion through a glass hand operated micropipette (internal diameter about 90 μm). Nude oocytes (without cumulus cell layers) found prior to the stripping process were discarded. Oocyte stripping had to be short and gentle to insure viable cells. Oocytes with a GV and a diameter of 70-80 μm

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were collected and washed in freshly prepared Waymouth medium (Gibco, Grand Island, NY, USA) supplemented with 5% freshly thawed fetal calf serum (FCS; Gibco).

Oocytes Culture

Each group of ten oocytes was transferred to a 4-well Nunclon multidish containing a final volume of 1 mL preincubated 5% FCS-Waymouth medium plus the desired test compound concentration. Oocytes were cultured for 16-17 hr at 37°C in 100% humidified, 5% CO_2 / 95% air. Control oocytes cultured in 5% FCS-Waymouth medium (untreated) were run with each experiment. Experimental and control oocytes were always freed by lots, from the ovaries of one to two mice during a period of not more than 20 min and were cultured in individual wells according to their collection time lots. This time lot segregation ensured that development was synchronous in the population of oocytes analyzed in each individual experiment.

Cell Cycle Progression

Cell cycle progression was analyzed with a compound microscope 16-17 hr after in vitro culture. Evaluation included the following progression of the number of oocytes moving from least to greatest development. (1) oocytes meiotically incompetent or G_2 arrested with an intact germinal vesicle (GV), (2) oocytes having resumed maturation and resolved their nucleus (germinal vesicle breakdown, GVBD, MI-arrested) and (3) oocytes having progressed to second meiosis after undergoing cytokinesis and extraction of the first polar body (PB, metaphase II, MII arrested). In vitro maturation was considered normal when at least 65% of oocytes in the control group extruded a first polar body within 16 hr of culture (Eichenlaub and Betzendahl, 1995).

Images for each oocyte maturation stage were taken by an Olympus inverted microscope with a QImaging digital camera (Quantitative Imaging Corporation) using Q Capture 2 software version 1.1.

Defining Oocyte Toxicity of CCA Components

For each CCA component, the following parameters were defined relative to dose and toxic effect and dose on oocyte in vitro maturation:

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- Toxic Concentration (TC_{50}): The concentration that inhibits 50% of GV oocytes from maturing to the metaphase II stage after 16 hr of culture when compared with the control group.
- Nontoxic Concentration: The highest concentration of the compound exhibiting no statistical difference on oocyte maturation when compared with the control group.

2.5.2 Cytogenetic Analysis

Exposed oocytes which had resumed maturation and undergone GVBD and had either (1) failed to emit a polar body (MI) or (2) had emitted the first polar body (MII), were used for indirect anti-tubulin immunofluorescence.

Preparation of Slides for Immunofluorescence

A circle with a diameter of about 1 cm was drawn on the upper surface on each previously ethanol-cleaned, frosted slide (VWR International Inc., West Chester, PA, USA) using a Pap pen (Research Products International Co., Mount Prospect, IL, USA). Slides were coated with poly-L-lysine (VWR), washed with distilled water and air-dried.

Preparation of Oocytes for Immunofluorescence

Tubulin, actin and chromatin were stained and observed by immunostaining and fluorescent microscopy (Eichenlaub-Ritter et al., 1988a; Soewarto et al., 1995). Nine-cavity Pyrex brand plates were used through the staining steps. Oocytes with or without PB were stained in separate wells. Denuded oocytes were fixed and extracted for 30 min in a water bath at 37°C in 200 µL microtubule-stabilizing buffer (phosphate-buffered saline, supplemented with 0.1 M PIPES, 5 mM MgC12, 0.5% Triton X-100, 2% formaldehyde and 2.5 mM EGTA) which preserves microtubules and chromosomes (Eichenlaub et al., 1986; Eichenlaub and boll, 1989; Soewarto et al., 1995). Oocytes were washed extensively and blocked overnight in a moist chamber at 4°C in 200 µL wash medium (Blocking solution; phosphate-buffered saline, supplemented with 0.02% NaN_3, 0.01% Triton X-100, 0.2% non-fat dry milk, 2% rabbit serum, 2% bovine serum albumin and 0.1 M glycine; Liu and Keefe, 2002). Non-specific binding of primary and secondary antibodies was prevented by incubation in blocking solution containing 2% rabbit serum before the experimental antibody incubation steps.

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To visualize the meiotic spindles, oocytes were washed in 200 µL PBS, then incubated with 100 µL of the primary antibody, mouse monoclonal anti-α-tubulin antibody (1:150 PBS; Sigma) for 45–60 min in a 37°C water bath. Oocytes were then incubated in 200 µL blocking solution for 1 hr followed by incubation in 100 µL of the secondary antibody, fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:200 PBS; Sigma) in a 37°C water bath for 1 hr. After PBS washing, oocytes were incubated in darkness for 15-20 min in 100 µL 4'-6-diamidino-2'-phenylindole (DAPI, 10 µg/mL PBS; Sigma) for staining the chromosomes. Finally, oocytes were washed twice in PBS then mounted onto a specially prepared slide. Twenty µL of DABCO anti-fade medium (1,4-diazobicyclo-octane, 4 mg/mL in 25% glycerol/PBS, pH 7.4; Sigma) solution was used on each coverslip. Coverslips were sealed with nail varnish. Slides were stored at 4°C and visualized within 1 month of preparation.

Immunofluorescent Microscopy

The cells were evaluated using a Nikon Eclipse E600 fluorescent microscope with an epifluorescent attachment (Nikon) using a combination of two filter sets, (1) DAPI filter (BA 515) for chromosome visualization and (2) FITC filter (BA 420) for spindle visualization. Images were taken by a Spot RT digital camera (Diagnostic instruments) using Spot RT software version 3.2.

Analysis of Spindle Morphology and Chromosome Alignment

For each immunostained oocyte, spindle shape and chromosome alignment were examined. The number of oocytes with normal or aberrant spindles and the number of oocytes with aligned or unaligned chromosomes were recorded for each treated and control group.

Statistical Analysis

Cell cycle progression data and cytogenetic data were analyzed using a Chi-Square test. The Chi-Square test was performed to determine whether there were significant differences among the treated groups. The treated groups were then compared with the control using the Chi-Square with Yates correction.

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2.6 Toxicity of Individual Components of CCA on Sperm

2.6.1 Sperm Motility Assay

Sperm Preparation

Spermatozoa were obtained from 3-6 month old CD1 proven fertile male mice. The males were sacrificed by cervical dislocation. The peritoneal cavity was opened and the cauda epididymis and vas deferens were removed into a 1000 μL drop of Earl's balanced salt solution (EBSS; Sigma) covered with equilibrated embryo-tested mineral oil (Sigma). The vas deferens was irrigated and the epididymis was multiply transected and its contents were gently expelled. The sperm suspension was incubated for 10 min at 37°C in 100% humidified 5% CO_2 / 95% air to allow the sperm dispersion before assessment (Summers et al., 1995). The primary suspension was evaluated before use. Sperm concentration and motility were determined using a Makler™ chamber (Diagnostic instruments). Sperm suspensions with a motility less than 70% were rejected. Using a sterile tip fitted with an Eppendorf pipetter (Fisher), eighty-three μL of well-stirred homogenous primary sperm suspension was delivered to inseminate each culture dish.

Preparation of Culture Dishes for Assay

Polystyrene Nunclon 35-mm petri-dishes were used throughout the assay. EBSS medium was equilibrated 30 min, oil was equilibrated one day before each experiment. Serum, protein and amino acid-free EBSS medium was used to increase the sensitivity of the assay (Esterhuizen et al., 1994). On the day of the experiment, culture dishes were prepared, each containing 417 μL of equilibrated media with the desired test compound concentration covered with oil. The test dishes were inseminated with 83 μL of the primary sperm suspension (final sperm concentration, 1-2 x 10^6/mL) and incubated at 37°C in 100% humidified, 5% CO_2 / 95% air for 4 hr. Each treatment was in triplicate.

Sperm Motility Evaluation

After 4 hr of incubation, dishes were removed from the incubator and sperm motility was evaluated. Two parameters of sperm motility were evaluated: (1) percentage of motile and (2) the quality of sperm motility. The percentage of motile sperm was

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estimated using a Makler™ chamber. The quality of motility or forward progression quality (FPQ) was assessed subjectively, using a standard scale (Bavister, 1974): 0-no movement; 1-twitching with no forward progressive movement (fpm); 2-slow fpm; 3-good purposeful fpm; 4-rapid fpm without activation and 5-hyperactivation. Half scores were also used when 50% of the motile sperm showed different score from the remaining 50%. For calculating the final test scores, the two motility parameters were combined to yield a sperm motility index (SMI):

SMI= percentage of motile sperm • $(FPQ)^2$.

Defining Toxicity of CCA Component on SMI

For each CCA component, the following parameters were defined relative to the dose and the toxic effect on sperm motility:

- Toxic Concentration (TC_{50}): The concentration that causes 50% reduction in SMI after 4 hr incubation with CCA components compared with the control group.
- Nontoxic Concentration: The highest concentration of the compound exhibiting no statistical difference on SMI when compared with the control group.

Statistical Analysis

For each group, the mean and standard deviation of the SMI were calculated. The mean of the SMI for each treated group was compared with the control group and other treated groups using ANOVA with Tukey's procedure for multiple comparisons. To know if the SMI significant difference between groups was due to difference in the percentage of motile sperm or FPQ score, these two parameters were analyzed and compared for each group using AVOVA with Tukey's procedure.

2.6.2 Sperm Viability

From the previous experiment, the lowest toxic concentration for each CCA component on sperm was determined. The lowest toxic-tested-concentration for all of three compounds was 0.5 mg/L. Sperm preparation and incubation in the presence of this concentration (0.5 mg/L) were done as described above. After 4 hr incubation, 25 μL of each sperm sample was taken through the staining procedure.

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Sperm Staining

Aliquots of the sperm suspensions were stained with propidium iodide (PI; Sigma) as described in (Pintado et al., 2000). PI is a membrane-impermeable dye excluded from viable cells but entering dead or dying cells. PI was used for identification of dead cells. PI stained the cell by binding to DNA through intercalating into nucleic acid bases. A stock solution of 20x PI (100 μg/mL water) was prepared and stored at -20°C. A volume of 47.5 μL of sperm suspension was incubated with 2.5 μL of 1x (5 μg/mL) PI staining solution at room temperature for 5 min in total darkness. To avoid spermatozoa sticking to the glass and producing staining artifacts, 5 μL of bovine serum albumin (100 mg/mL EBSS) was added to the stained sperm suspension. Ten μL of sperm suspension was mounted on a microscopic slide, coversliped and sealed with nail varnish.

Fluorescent Microscopy

Spermatozoal slides were observed using an Olympus reflected fluorescent microscope (Olympus) with a TRITC filter (BA 590). Slides were observed within 20 minute of preparation to avoid staining artifacts which could result in overestimation of non-viable cells. Sperm were observed only in the central area of the slide. Spermatozoa found to be completely or partially stained were regarded as non-viable. Non-viable spermatozoa showed a bright red-orange fluorescence when stained with PI. Images were taken using an Olympus laser scanning microscope (Olympus) model FV-300 equipped with Fluoview software version 3.047a.

Assessment of Sperm Viability

For each preparation, 100 spermatozoa were counted in four different fields. The percentage of viable cells was calculated from the following equation:

Percentage of viable cells = unstained immotile live cells / total immotile cells (stained + unstained).

Statistical Analysis

For each preparation, the percentage of stained cells was transformed using arcsine transformation to obtain a normal distribution. In all experiments, statistical analysis between control and treated groups was determined using the student's *t* test.

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CHAPTER III

RESULTS

3.1 Embryo Development and Quality

3.1.1 Embryo Development in the Presence of Individual CCA Components and their Mixture

Two-cell embryos collected from sexually mature female mice were cultured in vitro in well-defined culture medium (mKBB supplemented with 0.4% BSA). Embryonic development at 48 and 72 hr of in vitro cultured was observed in presence and absence of different concentrations of individual CCA components and their co-mingled CCA compound (mixed CCA; m-CCA). Embryos in each developmental stage were assigned a developmental score. The developmental embryonic scores ranged from –1 to 4. These scores correspond to the following developmental stages: -1 for fragmented or degenerated embryos; 0 for 2-cell embryos; 1 for 3-4 cell embryos; 2 for 5-8 cell embryos; 3 for morula and 4 for blastula (blastocyst). Each CCA component (arsenic pentaoxide, chromium trioxide and cupric oxide) was tested for its embryonic effect. The tested compounds were run in different concentrations (treated groups). For each test compound, embryo developmental scores of control and treated groups were ranked. The mean rank of developmental scores for control and treated groups was calculated and statistically analyzed using non-parametric statistics equivalent to one-way ANOVA (Kruskal-Wallis test). To detect the embryonic developmental effect and the toxicity threshold of different concentrations of the tested compound, the mean rank of embryonic development for each concentration was compared with the control as well as the other concentrations. The more advanced the developmental stage, the higher the score. A higher score produced higher mean rank which indicated better embryonic development.

Pairwise comparisons of each concentration with control and with other treated groups were performed by post-hoc multiple comparison tests (Mann-Whitney). Multiple comparisons usually require some adjustment of the criterion for significance ($P < 0.05$) in order to decrease type I error. The criterion for any pairwise comparison is:

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0.05 / [N (N-1)/2] where N is the number of the groups known as Bonferroni adjustment. To decrease the number of multiple comparisons and increase the significance of the result, selected concentrations for each tested compound were chosen. Different concentrations of the tested compound were excluded and replaced by only one concentration if their mean ranks were in the same range of the chosen concentration with no statistically significant difference between them.

Preimplantation embryos advanced into five distinct morphological development stages: 2-cell, 3-4 cell, 5-8 cell, morula and blastocyst (Figure 1A-E). Embryos failing development progressed to the fragmented-degenerated stage (Figure 1F). As a second test of embryo development and to localize and magnify the toxic effect of each tested compound, the six embryonic stages were regrouped into three combined stages at the 72 hr culture period. The first category (premorula stage) includes 2-cell, 3-4 cell and 5-8 cell embryos. The second category (morula-blastocyst stage) includes morula and blastocysts embryos. The third category (fragmented-degenerated stage) includes fragmented and degenerated embryos. The proportion (percentage) of tested compound-treated embryos in different development stages was recorded and compared with the control group at 72 hr of in vitro culture using the Chi-Square test. Generally, when two-cell mouse embryos were cultured in 0.4% BSA mKBB, the majority was competent to develop and reached morula-blastocyst stage within 72 hr of culture. Only a few, possibly morphologically abnormal 2-cell embryos, were incompetent to completely develop and remained in the premorula stage or progressed to the fragmented-degenerated stage.

Curve estimation (Cubic model) was used to explain the percentage of variability in embryo development as a function of concentration for each tested compound (dose-response curve). Best-fit curve (theoretical curve) represents the best curve that is able to explain the behavior of the observed curve (actual data) with regression coefficient (R^2) = 1. The closer the R^2 value to 1 the more powerful the theoretical curve explains the behavior of the observed curve. For each curve, the regression equation and the regression coefficient were given at 48 and 72 hr.

Throughout the result section, results were considered statistically significant if $P < 0.05$ and highly significant if $P < 0.0001$. For multiple comparisons with Bonferroni

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adjustment, results were considered statistically significant if $P < 0.05 / [N (N-1)/2]$ and highly significant if $P < 0.005 / [N (N-1)/2]$.

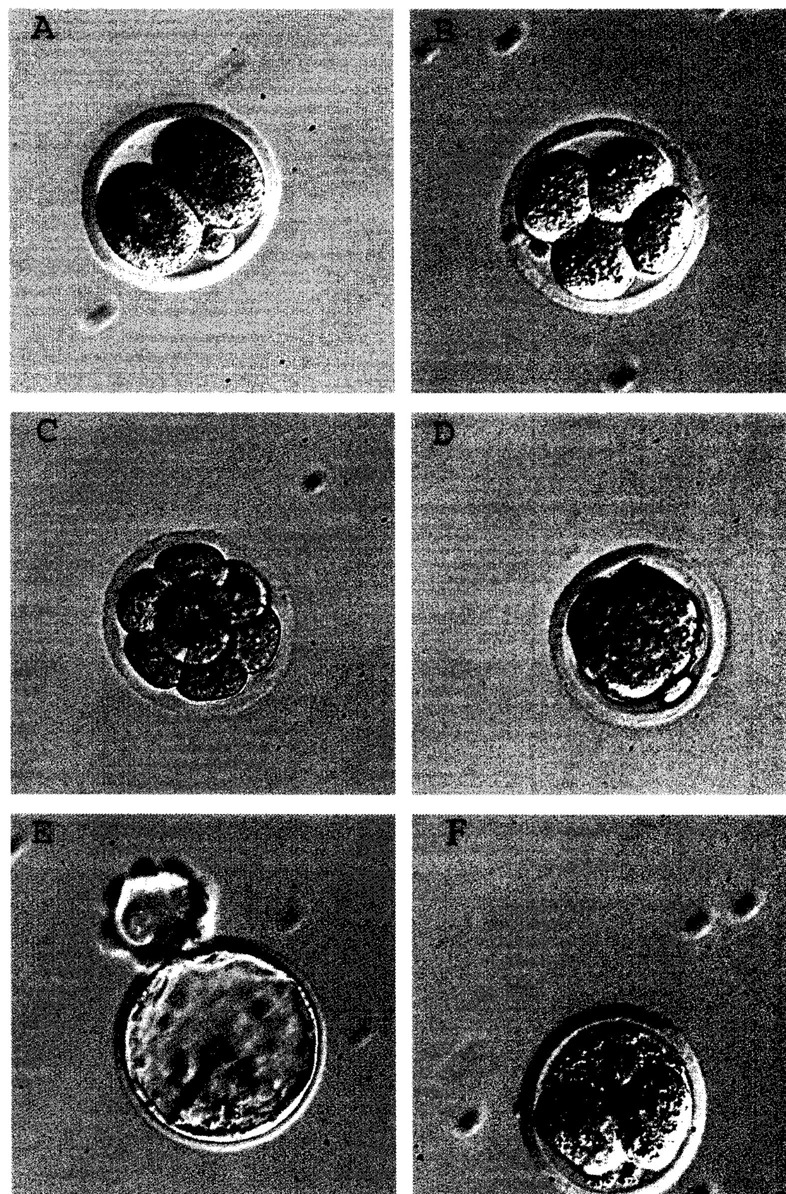

Figure 1. Development stage of two-cell mouse embryo cultured in vitro. **(A)** 2-cell embryo. **(B)** 4-cell embryo. **(C)** 8-cell embryo. **(D)** Morula. **(E)** Blastocyst. **(F)** Fragmented embryo. Embryos were observed under Olympus relief contrast optics.

Embryonic Development in the Presence of Arsenic

To detect the toxic effect of arsenic on preimplantation embryos, mean rank of embryonic development for As-treated groups was assessed and compared with the

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control group at 48 and 72 hr of culture. In the presence of 0.5 mg/L or less of arsenic in culture medium, embryonic development did not appear to be significantly different compared with the control group. In addition, no significant difference was found in embryonic development among 0.1, 0.3 and 0.5 mg/L As (Table I). Increasing arsenic concentration above 0.5 mg/L appeared to have a significant suppressing effect on embryo development. Consequently, 0.5 mg/L As was considered as a cut point and 0.1 and 0.3 mg/L As concentrations were excluded from multiple comparisons and represented by 0.5 mg/L As.

Table I. Analysis of two-cell mouse embryo development at 48 and 72 hr of culture in the presence of arsenic

Concentration [mg/L]	Embryo number	Mean rank of embryonic development	
		48 hr	72 hr
Control	30	196.9	186.0
0.1	30	190.4	177.0
0.3	30	205.2	186.0
0.5	30	194.1[a]	186.0[d]
0.7	30	139.1[**, a]	136.5[**, d]
0.9	30	119.7[**, b]	136.5[**, e]
1.1	30	88.6[**, b, c]	100.5[**, e, f]
1.3	30	48.8[**, c]	60.0[**, f]
1.5	30	36.7[**]	51.0

** Highly significantly different from control, $P < 0.0001$.
The same superscript within columns denotes significant difference, $P < 0.05$.

At 48 hr, embryonic development of the control group had the highest value compared with the treated groups. At 72 hr, both control and 0.5 As-treated groups exhibited the highest mean rank of embryonic development with no significant difference ($P = 1.00$) between them (Table II). All the remaining As-treated groups showed significant reduction in their embryonic development compared with the control group at 48 and 72 hr ($P < 0.00024$; Table II). This reduction highly increased with the increase of arsenic concentration in culture medium, reaching 2/3 of the control value at 1.5 mg/L arsenic concentration. Pairwise comparisons between different concentrations were significantly different ($P < 0.00024$), indicating that the reduction of embryo

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development had a concentration-dependant response to arsenic presence in the culture medium (Table II).

Table II. Multiple comparisons of embryonic development in the presence of selected arsenic concentrations at 48 and 72 hr

Concentration [mg/L]	Embryo number	Mean rank of embryonic development	
		48 hr	72 hr
Control	30	168.2	155.0
0.5	30	166.4 [a, b, c, d, e]	155.0 [n, o, p, q, r]
0.7	30	125.3 [**, a, f, g, h]	116.5 [**, n, s, t]
0.9	30	111.8 [**, b, i, j]	116.5 [**, o, u, v]
1.1	30	84.0 [**, c, f, k, l]	88.5 [**, p, w, x]
1.3	30	47.4 [**, d, g, i, k, m]	57.0 [**, q, s, u, w, y]
1.5	30	35.5 [**, e, h, j, l, m]	50.0 [**, r, t, v, x, y]

** Highly significantly different from control, $P < 0.00024$.
Pairs of values compared together indicated by same superscript.
a – y pairwise comparisons are highly significantly different, $P < 0.00024$.

The high mean rank of control and 0.5 As-treated group at 48 and 72 hr resulted from the fact that 100% of their embryos were in morula-blastocyst stage with high development score (Table III). With increasing arsenic concentration in the culture medium above 0.5 mg/L, the percentage of embryos at morula-blastocyst stage declined in concentration-dependant manner reaching 0% at 1.5 mg/L As concentration. At the same time, the percentage of fragmented-degenerated embryos increased reaching 100% at 1.5 mg/L As concentration.

The pattern of embryonic development in the presence of arsenic (dose-response curve) was compared at 48 and 72 hr (Figure 2). The theoretical curve (best-fit curve, represented by a solid line) was able to explain the behavior of the observed curve (actual data points, represented by a dotted line) in 98% (R^2) at 48 hr and 97% at 72 hr. Both 48 and 72 hr best-fit curves showed an obvious sharply declining trend in embryonic development in the presence of arsenic. This decline in embryonic development increased with rising arsenic concentration in the culture medium reaching its maximum at 1.5 mg/L As.

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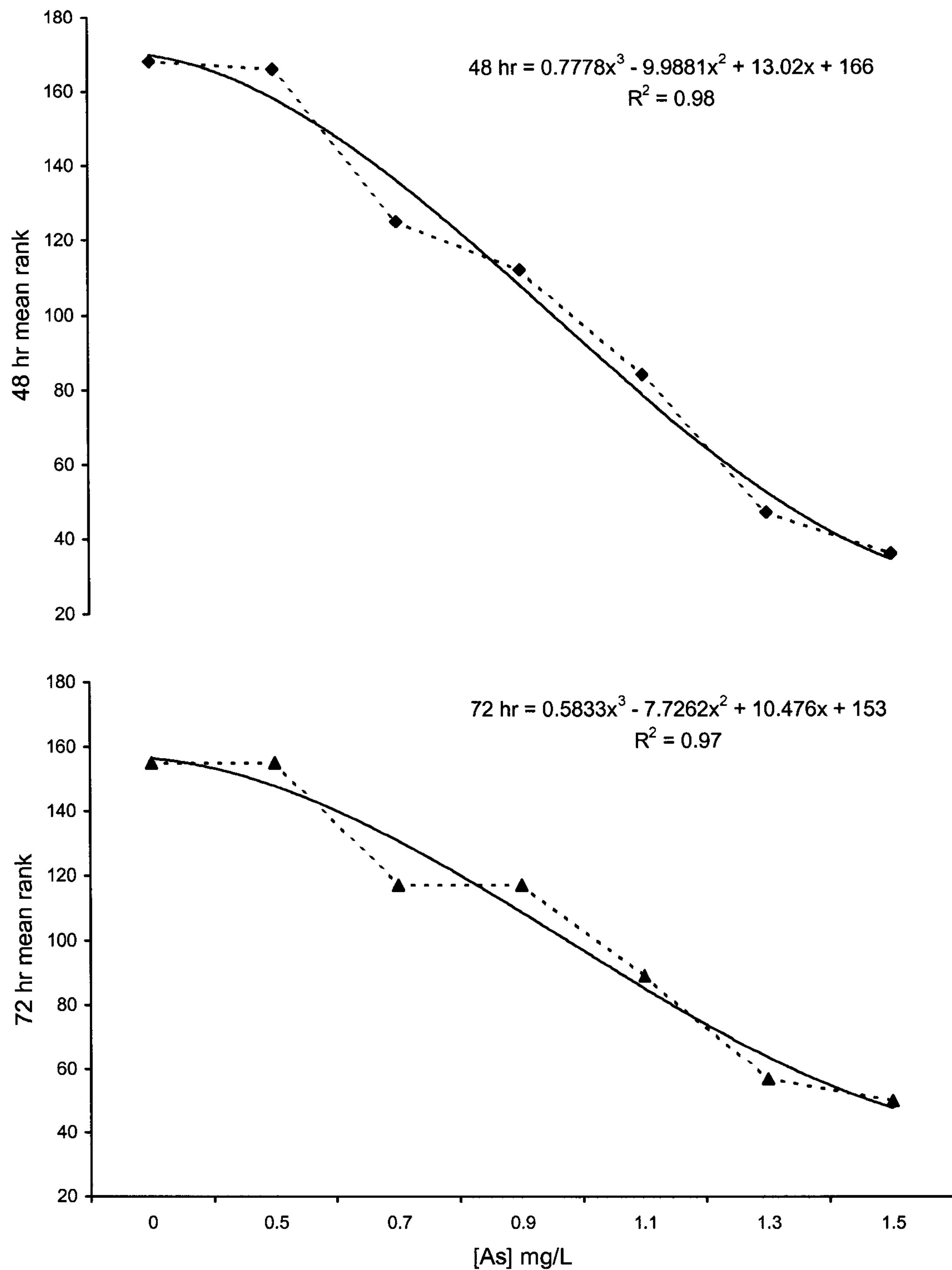

Figure 2. Concentration-dependent response of embryo development at 48 and 72 hr of culture in presence and absence of arsenic. Dotted curves represent the actual data points; continuous solid curves represent the best-fit curve. R^2 represents the regression coefficient.

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Arsenic TC_{50} on embryo development was expected to be in the range of 1.1 mg/L. At 48 and 72 hr, the mean rank of 1.1 mg/L As-treated embryos was about half the control value (84 versus 168.2, 88.5 versus 155 respectively) (Table II). In addition, only 36.7% of 1.1 mg/L As-treated embryos were able to reach the morula-blastocyst stage while the remaining 63.3% were in the fragmented-degenerated stage (Table III). This indicates that arsenic has an inhibitory toxic effect on embryo development and increases embryo fatality.

Table III. Embryo development of two-cell mouse embryo 72 hr post-arsenic treatment

Concentration [mg/L]	Embryo number	Number (%) of embryo		
		Premorula	Morula-Blastocyst	Fragmented-Degenerated
Control	30	0	30 (100)	0 (0)
0.5	30	0	30 (100)	0 (0)
0.7	30	0	19 (63.3)**	11 (36.7)
0.9	30	0	19 (63.3)**	11 (36.7)
1.1	30	0	11 (36.7)**	19 (63.3)
1.3	30	0	2 (6.7)**	28 (93.3)
1.5	30	0	0 (0)	30 (100)

**Highly significantly different from control, $P < 0.0001$.

Embryonic Development in the Presence of Chromium

Embryo development of two-cell mouse embryo at 48 and 72 hr in presence and absence of different concentrations of chromium was tested (Table IV). To decrease the number of comparisons between the control and the treated groups and between the treated groups with each other, all the tested Cr concentrations were divided into four groups; 0.1-0.5 mg/L; 0.7-1.2 mg/L; 1.5-1.9 mg/L; and 2-3 mg/L group. Within each group, significant difference in the mean rank was tested. If all values within the group did not significantly differ, one value was chosen to represent the group. Therefore, 0.5; 1.2; 1.5; 1.9; 2.5; and 3 mg/L Cr were used for multiple comparisons (Table V).

The mean rank of embryonic development of the control was the highest at 48 hr compared with the treated groups. At 72 hr, 0.5 mg/L Cr-treated group had higher value, statistically insignificant ($P = 0.437$), compared with the control. Although embryonic development showed a concentration-dependant decreasing pattern in respond to the

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presence of chromium in the culture medium, only 2.5 and 3 mg/L showed significant reduction in embryonic development compared with the control group ($P < 0.0024$ at 72 hr for 2.5 mg/L Cr; $P < 0.0024$ at 48 &72 hr for 3mg/L Cr; Table V).

Table IV. Analysis of two-cell mouse embryo development at 48 and 72 hr of culture in the presence of chromium

Concentration [mg/L]	Embryo number	Mean rank of embryonic development	
		48 hr	72 hr
Control	50	152.0	150.1
0.1	6	144.3	153.7
0.2	18	140.0	159.1
0.3	21	129.1	143.6
0.5	18	147.3	163.9
0.7	22	156.7	163.1
0.9	21	162.4	164.9
1.1	25	167.9 [a]	167.3 [c]
1.2	17	138.5 [a]	144.6 [c]
1.5	34	136.2 [a]	126.9 [c]
1.7	17	121.9 [a]	104.6 [c]
1.9	6	123.0 [a]	83.8 [c]
2.0	10	58.3 [b]	44.1
2.5	7	56.2 [*, b]	47.4 [*]
3.0	9	16.2 [*, b]	21.8 [*]

* Significantly different from control, $P < 0.05$.
The same superscript within columns denotes significant difference, $P < 0.05$.

Table V. Multiple comparisons of embryonic development in the presence of selected chromium concentrations at 48 and 72 hr

Concentration [mg/L]	Embryo number	Mean rank of embryonic development	
		48 hr	72 hr
Control	50	77.9	78.3
0.5	18	76.1	85.5 [b, c]
1.2	17	71.8	76.1
1.5	34	71.0	68.0
1.9	6	64.8 [a]	47.0
2.5	7	33.9	28.1 [*, b]
3.0	9	11.7 [*, a]	14.8 [*, c]

* Significantly different from control, $P < 0.0024$.
Pairs of values compared together indicated by same superscript.
a – c pairwise comparisons are highly significantly different, $P < 0.00024$.

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The number (percentage) of embryos at different development stages at 72 hr of culture was analyzed (Table VI). Control group and 0.5 mg/L Cr-treated group have the highest percentage (82%, 88.9% respectively) of morula-blastocyst stage embryos compared with other treated groups. Although, the percentage of 0.5 mg/L Cr morula-blastocyst stage embryos showed a higher value compared with the control group, no significant difference was found between these two groups ($P = 0.645$; Table VI). In addition, 80% of control morula-blastocyst stage embryos were in blastocyst stage compared with 77.8% for 0.5 mg/L Cr-treated embryos (Table A.4). Increasing chromium concentration was accompanied by a drop in the number of morula-blastocyst stage embryos and an elevation in the number of premorula arrested and fragmented-degenerated embryos. The presence of 3 mg/L chromium in the culture medium caused complete inhibition of embryo development. At this concentration, none of the cultured two-cell embryos was able to reach the morula-blastocyst stage with 66.7% of them were in the fragmented-degenerated stage and the remaining 33.3% were arrested at the premorula stage after 72 hr of culture (Table VI).

To determine the development capability of the Cr-treated premorula stage embryos, embryos were cultured for an additional 24 hr (total 96 hr of culture) and their development was checked. Even after prolonged culture, premorula stage embryos failed to develop to morula-blastocyst stage and all of them became degenerated. This indicated that even though some embryos were initially able to develop to premorula stage in chromium presence; they were unable to complete their development normally even with the extended culture and progressed to fragmented-degenerated stage.

From table V and VI, chromium TC_{50} was determined to be around 1.9 mg/L Cr. At this concentration, only 50% of cultured embryos were able to reach morula-blastocyst stage while the other 50% failed (33.3% were at premorula stage, 16.7% at fragmented-degenerated stage).

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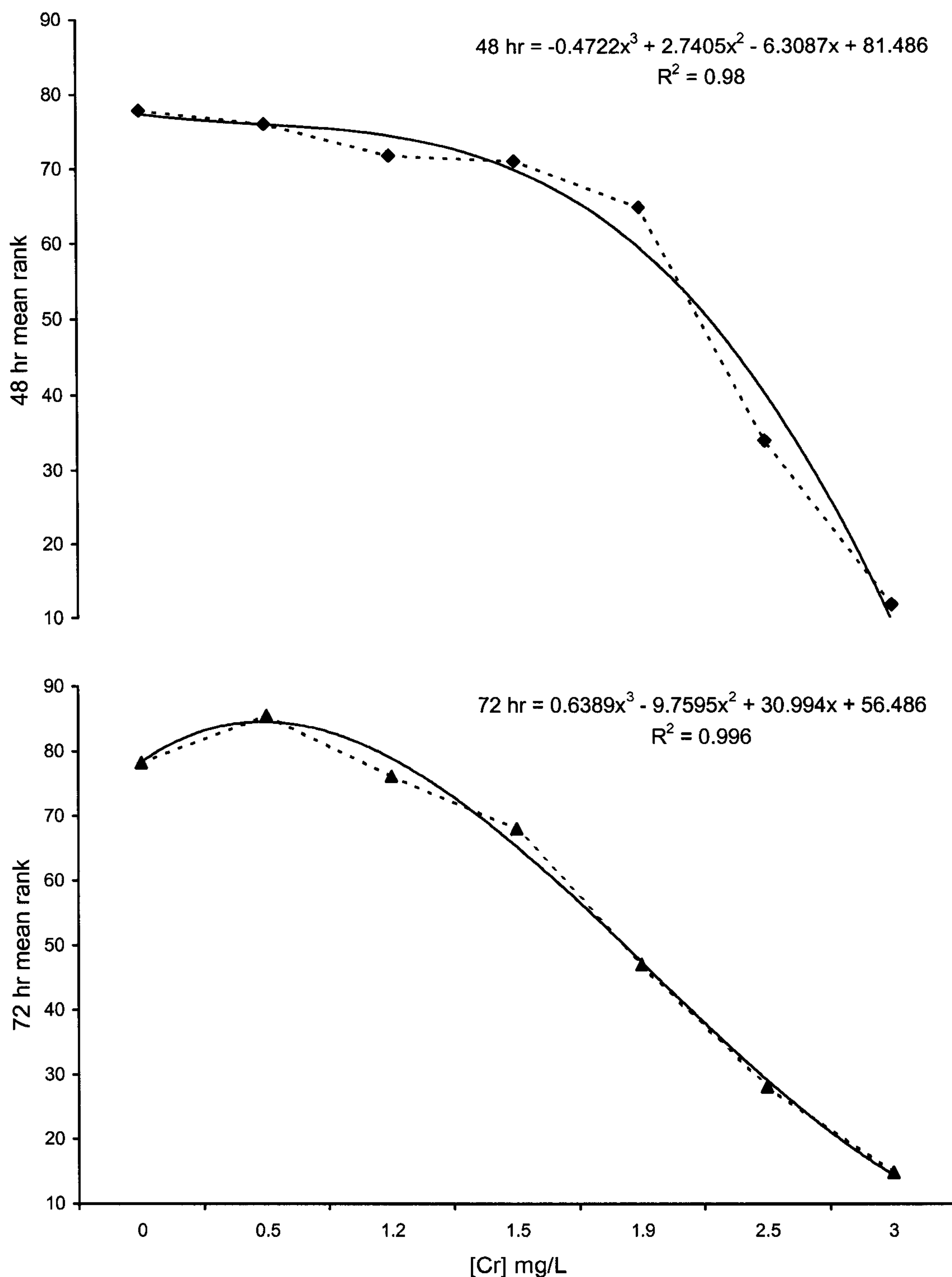

Figure 3. Concentration-dependent response of embryo development at 48 and 72 hr of culture in presence and absence of chromium. Dotted curves represent the actual data points; continuous solid curves represent the best-fit curve. R^2 represents the regression coefficient.

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Table VI. Embryo development of two-cell mouse embryo 72 hr post-chromium treatment

Concentration [mg/L]	Embryo number	Number (%) of embryo		
		Premorula	Morula-Blastocyst	Fragmented-Degenerated
Control	50	2 (4.0)	41 (82.0)	7 (14.0)
0.5	18	0 (0)	16 (88.9)	2 (11.1)
1.2	17	3 (17.6)	14 (82.4)	0 (0)
1.5	34	9 (26.5)**	24 (70.6)	1 (2.9)
1.9	6	2 (33.3)	3 (50.0)	1 (16.7)
2.5	7	4 (57.1)**	1 (14.3)**	2 (28.6)
3.0	9	3 (33.3)**	0 (0)	6 (66.7)**

** Highly significantly different from control, $P < 0.0001$.

The behavioral pattern of embryo development at 48 and 72 hr was analyzed using curve estimation (Figure 3). At 48 hr, embryonic development showed slight reduction in the presence of chromium, this reduction became greater at 1.9 mg/L chromium concentration reaching the lowest value at 3 mg/L Cr. At 72 hr, the behavior of embryonic development was slightly different. Embryonic development showed obvious elevation (ascending limb of the curve) at 0.5 mg/L Cr then gradually dropping (descending limb), reaching their minimum value at 3 mg/L Cr. The theoretical curve was able to explain 98%-99% of the variability on embryonic development as a function of chromium concentration level at 48 and 72 hr of culture respectively (Figure 3).

Embryonic Development in the Presence of Copper

The presence of copper in concentration up to 5 mg/L in embryo culture medium did not show any detrimental effect on embryo development. The mean rank of embryonic development for these Cu-treated embryos at 48 and 72 hr was in the same range of the control value and even higher at certain concentrations (Table VII). Embryonic development in the presence of 1 mg/L and 5 mg/L copper concentrations were significantly different ($P < 0.05$) from each other at 48 hr. However, at 72 hr no significant difference was found between them. In addition, embryonic development at 5 mg/L Cu and 6 mg/L Cu did not show any significant difference at 48 and 72 hr. Therefore, for multiple comparisons, 0.1, 1, 5 and 6 mg/L copper concentrations were represented by 1 and 5 mg/L Cu (Table VIII). Even though the presence of 1 mg/L

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copper in the culture medium appeared to have a better effect on embryo development at 48 and 72 hr with higher mean rank compared with the control group, no significance difference was found between these two groups ($P = 0.161$ at 48 hr; $P = 0.571$ at 72 hr). Increasing copper concentration above 5 mg/L showed a significant suppressing effect on embryo development, this suppressing effect reached it's maximum with the presence of 10 mg/L copper concentration in the culture medium (Table VIII).

Table VII. Analysis of two-cell mouse embryo development at 48 and 72 hr of culture in the presence of copper

Concentration [mg/L]	Embryo number	Mean rank of embryonic development	
		48 hr	72 hr
Control	28	128.9	122.6
0.1	11	147.7[a]	124.0
1.0	16	142.4[a, b]	126.2
5.0	30	108.1**, [a, b]	124.5
6.0	20	96.3**, [a]	114.8**
7.0	22	61.5**	59.6**
8.0	19	46.5**	35.5**
9.0	21	33.6**	31.5**
10.0	10	18.0**	31.5**

** Highly significantly different from control, $P < 0.0001$.
The same superscript within columns denotes significant difference, $P < 0.05$.

Pairwise comparisons of embryonic development mean rank between groups were significantly different indicating that the reduction in embryonic development has a concentration-dependant response to copper's presence in the culture medium (Table VIII).

The percentage of embryos reaching the morula-blastocyst stage in 1 mg/L or 5 mg/L copper-treated groups was higher than the control group. However, no significant difference was found between them ($P = 0.235$; $P = 0.51$ respectively; Table IX). In the presence of 7 mg/L copper in the culture medium, only 13.6% of embryos were able to reach the morula-blastocyst stage while 50% were still in the premorula stage and the remaining 36.4% were in the fragmented-degenerated stage. All embryos cultured at 8 or 10 mg/L copper had completely failed to reach the morula-blastocyst stage with 89.5% and 100% of them were in the fragmented-degenerated stage respectively.

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Table VIII. Multiple comparisons of embryonic development in the presence of selected copper concentrations at 48 and 72 hr

Concentration [mg/L]	Embryo number	Mean rank of embryonic development	
		48 hr	72 hr
Control	28	93.3	86.0
1.0	16	100.0 [a, b, c, d]	88.5 [j, k, l]
5.0	30	75.1 [**, a, e, f, g]	87.4 [m, n, o]
7.0	22	40.1 [**, b, e, h]	38.9 [**, j, m, p]
8.0	19	28.9 [**, c, f, i]	22.1 [**, k, n, p]
10.0	10	10.0 [**, d, g, h, i]	19.5 [**, l, o]

** Highly significantly different from control, $P < 0.0003$.
Pairs of values compared together indicated by same superscript.
a – p pairwise comparisons are highly significantly different, $P < 0.0003$.
g pairwise comparison is significantly different, $P < 0.003$.

Table IX. Embryo development of two-cell mouse embryo 72 hr post-copper treatment

Concentration [mg/L]	Embryo number	Number (%) of embryo		
		Premorula	Morula-Blastocyst	Fragmented-Degenerated
Control	28	0 (0)	26 (92.9)	2 (7.1)
1.0	16	1 (6.3)	15 (93.7)	0 (0)
5.0	30	0 (0)	29 (96.7)	1 (3.3)
7.0	22	11 (50.0)	3 (13.6)**	8 (36.4)**
8.0	19	2 (10.5)	0 (0)	17 (89.5)**
10.0	10	0 (0)	0 (0)	10 (100)**

** Highly significantly different from control, $P < 0.0001$.

There was no single copper concentration that fit exactly the TC_{50} definition. In the presence of 5 mg/L Cu in the culture medium, significant reduction in embryonic development was seen at 48 hr compared with the control. However, at 72 hr 5 mg/L Cu-treated embryos did not show any significant difference in their embryonic development or in the percentage of morula-blastocyst embryos compared with the control group. At 7 mg/L Cu, a sudden toxic embryonic effect was obtained with great reduction in embryonic mean rank, produced by decreasing morula-blastocyst embryo numbers and increasing fragmented-degenerated numbers. Therefore, 6-7 mg/L Cu was considered as the range for TC_{50}.

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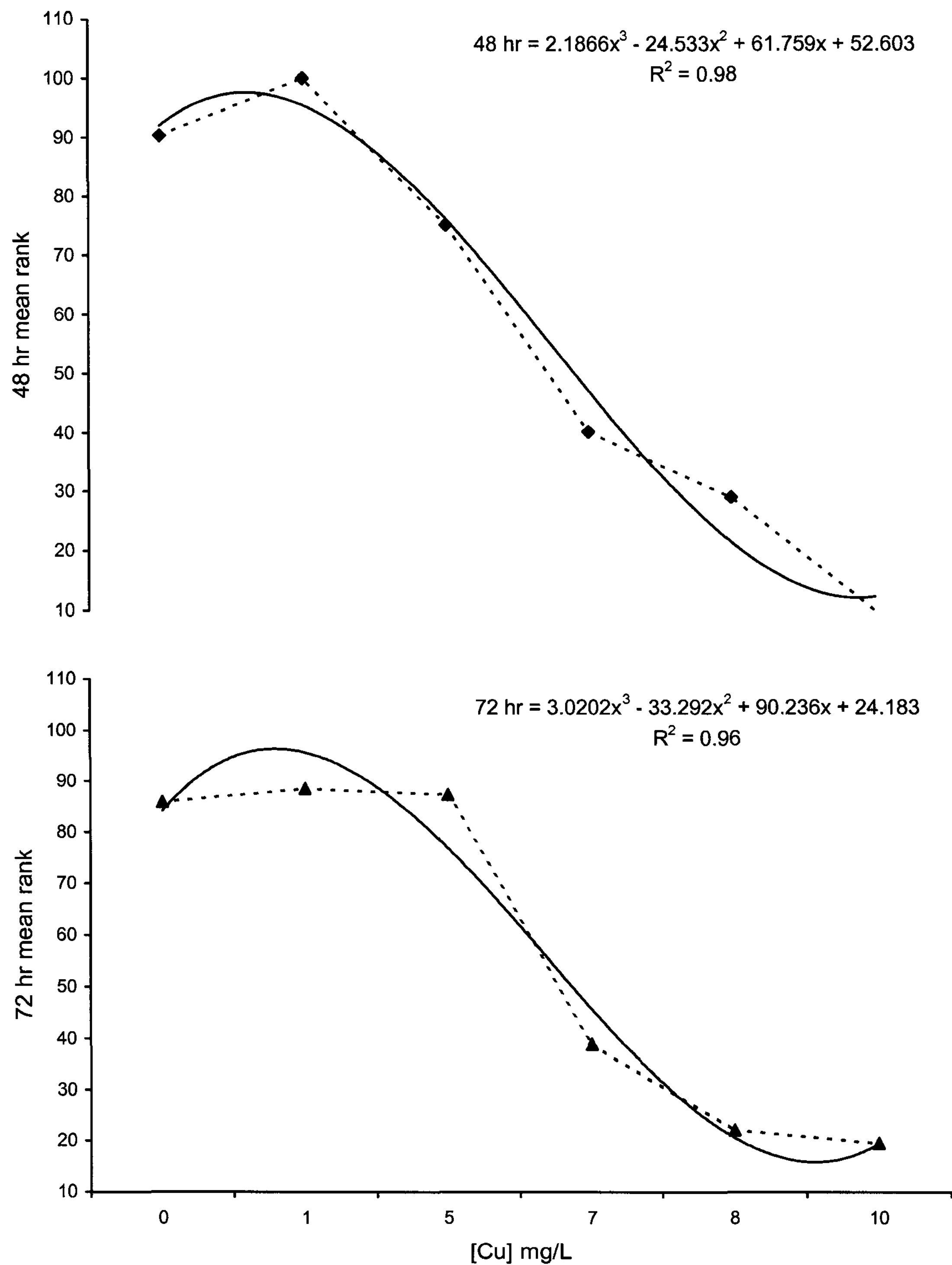

Figure 4. Concentration-dependent response of embryo development at 48 and 72 hr of culture in presence and absence of copper. Dotted curves represent the actual data points; continuous solid curves represent the best-fit curve. R^2 represents the regression coefficient.

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The variability of embryo development in the presence of copper was analyzed at 48 and 72 hr. At 48 and 72 hr, embryonic development showed a rising trend (ascending part of the curve) at 1 mg/L copper concentration. This trend changed to falling at 5-7 mg/L copper concentrations reaching the lowest value at 10 mg/L (Figure 4). The best fit curve was able to explain almost all the embryonic behavior in the presence of copper, with R^2 equals to 0.98 at 48 hr and 0.96 at 72 hr.

Embryonic Development in the Presence of CCA Mixture

The results from the previous experiments helped to determine the nontoxic-subtoxic concentration for each CCA component. The mixtures of these concentrations (m-CCA) were tested to detect their synergistic toxic effect on embryo development that result from their presence together. Five mixtures were tested, m-CCA1 represented the mixture with the highest concentration while m-CCA5 was the one with the lowest concentration (1/10 concentration of m-CCA1). All m-CCA mixtures showed a significant suppressing effect on embryo development compared with the control group except m-CCA5. In the presence of m-CCA5, there was no significant reduction in embryonic development compared with the control group (Table X).

At 48 hr, m-CCA5-treated embryos showed a significantly higher rate of embryonic development than the control group. However, at 72 hr no statistically significant difference was found between the m-CCA5 and the control group ($P = 0.06$). The slightly lower rate of the control group embryonic development compared with m-CCA5 was directly related to the slightly higher percentage of the fragmented-degenerated embryos with negative scores in the control group compared to the m-CCA5. At 48 hr, 4% of control had fragmented-degenerated embryos versus 2.4% for m-CCA5. In addition, 95.2% of the m-CCA5 embryos were at the blastocyst stage resulting in a higher development score while 0% embryos reached the blastocyst stage in the control group (Table A.7). At 72 hr, 10% of control embryos were in the fragmented-degenerated stage versus 2.4% for the m-CCA5. Moreover, 97.6% of m-CCA5 were in blastocyst stage versus 90% for control (Table A.8). Most of the m-CCA5 blastocysts were morphologically abnormal and were characterized by one or more of the following features: unincorporated blastomeres, multiple cavities (possibly intracellular vacuoles), no discrete ICM visible or low numbers of mural TE cells.

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The m-CCA1 group showed a significantly lower mean rank of embryo development compared with the control group. Moreover, the m-CCA1 embryonic development mean rank exhibited the lowest value compared with other treated groups. Diminishing m-CCA1 embryonic development was due to increasing the number of premorula and fragmented-degenerated embryos (with low or negative score) and decreasing the number of morula-blastocyst stage embryos (with high score). By increasing the mixture concentration, the number of embryos reaching the morula-blastocyst stage was significantly reduced in a concentration-dependant manner reaching 4.9% in the m-CCA1. Correspondingly, the number of premorula and fragmented-degenerated embryos increased, reaching 12.2% and 82.9% respectively (Table XI) at m-CCA1. Pairwise comparisons of embryonic development mean rank between each m-CCA group were significantly difference and decreased with increasing m-CCA concentrations. Clearly, embryonic development has a clear concentration-dependant response to m-CCA presence in the culture medium (Table X).

The variability in embryonic development in the presence of CCA mixtures was analyzed at 48 and 72 hr. At 48 hr, the presence of low concentration of CCA, CCA5, did not show any significant toxic effect on embryo development. Increasing the CCA concentration above CCA5 resulted in a step reduction in embryonic development until the CCA2 value and then a slight elevation occurred at CCA1 (Figure 5). This elevation of CCA1 compared with CCA2 was directly correlated to higher percentage of CCA1 premorula stage embryos were at the 8-cell stage compared with CCA2 (Table A.7).

At 72 hr the embryonic development pattern was similar to 48 hr. The rising lower part of the 72 hr curve resulted from a higher mean rank value for CCA1 and CCA2 verses CCA3. Lower mean rank of CCA3 at 72 hr was resulted from 50% of the premorula stage embryos being in the 2-cell stage with a score of zero (Table A.8). The picture was different for CCA2 and CCA1. In CCA2, half of the premorula stage embryos were at the 4-cell stage and the remaining were in the 8-cell stage. In CCA1 premorula stage embryos, 70% of them were at 4-cell stage and the remaining at 8-cell stage (Table A.8). In addition, no significant difference was found between their embryonic development mean rank (Table X).

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Table X. Multiple comparisons of embryonic development in the presence of CCA mixture at 48 and 72 hr

Concentration [mg/L]	Mean rank of embryonic development	
	48 hr	72 hr
Control	197.1	237.8
m-CCA1	110.4**, a, b	113.7**, i
m-CCA2	65.4**, c, d, e	115.9**, j
m-CCA3	130.4**, c, f, g	97.0**, k, l
m-CCA4	169.7 a, d, f, h	117.8**, k, m
m-CCA5	280.9**, b, e, g, h	251.5 i, j, l, m

The m-CCAi is a chromated copper arsenate mixture arranged from the highest to the lowest concentration.
** Highly significantly different from control, $P < 0.0003$.
Pairs of values compared together indicated by same superscript.
a – m pairwise comparisons are highly significantly different, $P < 0.0003$.

Table XI. Embryo development of two-cell mouse embryo 72 hr post-CCA treatment

Concentration [mg/L]	Embryo number	Number (%) of Embryo		
		Premorula	Morula-Blastocyst	Fragmented-Degenerated
Control	50	0 (0)	45 (90.0)	5 (10.0)
m-CCA1	82	10 (12.2)	4 (4.9)**	68 (82.9)**
m-CCA2	41	6 (14.6)	2 (4.9)**	33 (80.5)**
m-CCA3	43	4 (9.3)	6 (14.0)**	33 (76.7)**
m-CCA4	41	1 (2.4)	12 (29.3)**	28 (68.3)**
m-CCA5	41	0 (0)	40 (97.6)	1 (2.4)

** Highly significant different from control, $P < 0.0001$.

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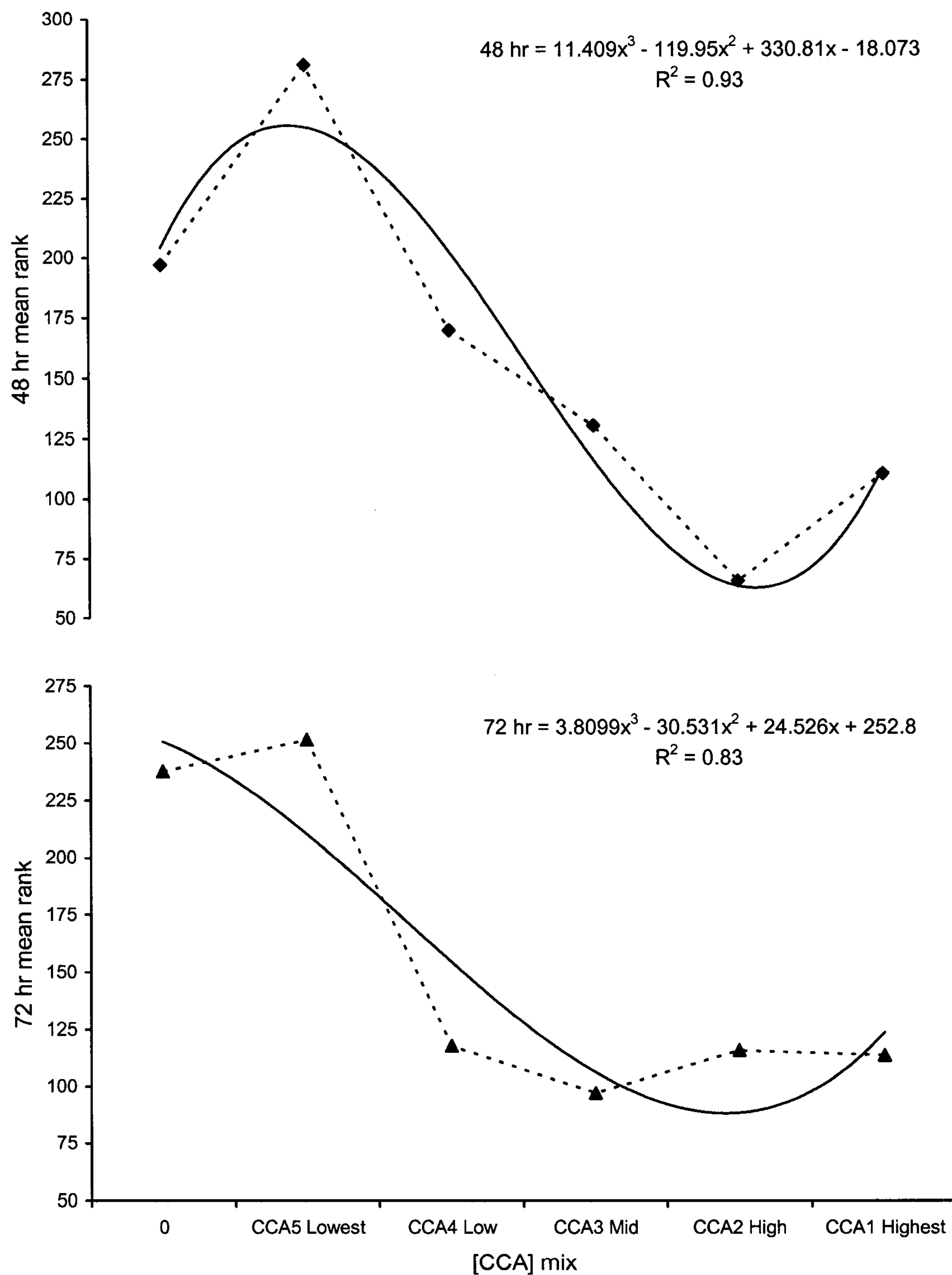

Figure 5. Concentration-dependent response of embryo development at 48 and 72 hr of culture in presence and absence of CCA mixtures. Dotted curves represent the actual data points; continuous solid curves represent the best-fit curve. R^2 represents the regression coefficient.

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3.1.2 Embryo Quality

The non embryo-toxic concentration for each CCA component was determined in the previous section. The nontoxic concentration for each of the 3 tested compounds was surprisingly the same at a value of about 0.5 mg/L. Therefore, in vitro grown mouse blastocysts cultured in the presence and absence of 0.5 mg/L of individual components of CCA were taken, stained with Hoechst dye and observed under fluorescent optics (Figure 6A, A'). Blue color in all images represents the chromatin in the blastomere nuclei that were stained by membrane-permeable, DNA binding Hoechst stain. Nomarski images were taken concurrently (Figure 6B, B') and were superimposed on the fluorescent pictures, so that each embryo could be visualized with its nuclear material (Figure 6C, C'). When in vitro cultured blastocysts were analyzed for total cell number, most of them had a total cell mean number of 79.4 ± 4 (Table XII). The range of total cell distribution for the majority of them (75%) located between 70-90 cells per embryo. In cases of hatching or hatched blastocysts, total cell numbers increased above the normal range reaching around 95 ~ 100 cell per embryo (Figure 6A). Embryonic total cell numbers have previously been reported to be a good indicator of embryo quality and development rate (Hardy et al., 1989). The higher the cell numbers the better the embryo quality.

Embryo Quality in the Presence of Arsenic

Two-cell mouse embryos reaching the blastocyst stage after 72 hr of culture in the presence of 0.5 mg/L arsenic in their culture medium were analyzed to count their total cell numbers. Although most of them displayed normal morphology compared with the control group (Figure 6B', B; using Nomarski differential interference contrast optics), their total cell numbers (52.9 ± 3.8) showed significant reduction ($P < 0.0001$) compared with the control group (Table XII; Figure 6A', A). This reduction was about 33.4% compared with control.

In most of the 0.5 mg/L As-exposed blastocysts, the total cell numbers were located in the lower ranges (<40 and 40-69) compared with the control group (6.7% at < 40 range versus 0%; 86.7% at 40-69 range versus 12.5% respectively). Only one embryo had total cell numbers in the normal range (70-90) (Figure 7).

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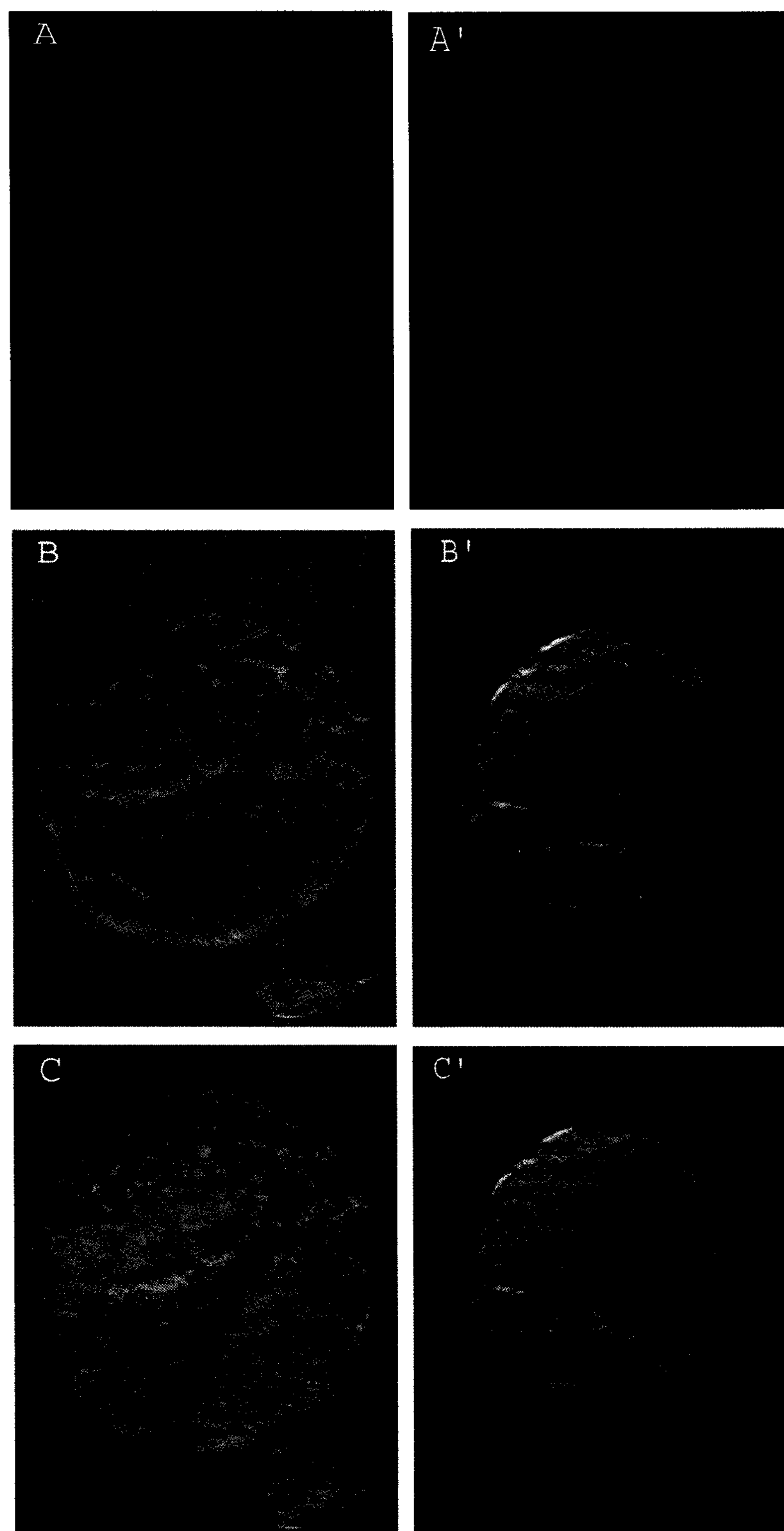

Figure 6. Total cell number of 0.5 As-treated preimplantation embryos. Left panel represents control group. Right panel represents As-treated group. **(A, A')** Under fluorescent optics. **(B, B')** Under Nomarski optics. **(C, C')** Superimposed pictures for **(A, B; A', B')**.

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Table XII. Total cell number of mouse blastocysts grown in vitro in arsenic containing medium

Concentration [mg/L]	Embryo Number	Total cell number (Mean ± SEM)
Control	16	79.4 ± 4.0
0.5	15	52.9 ± 3.8**

** Highly significantly different from control, $P < 0.0001$.

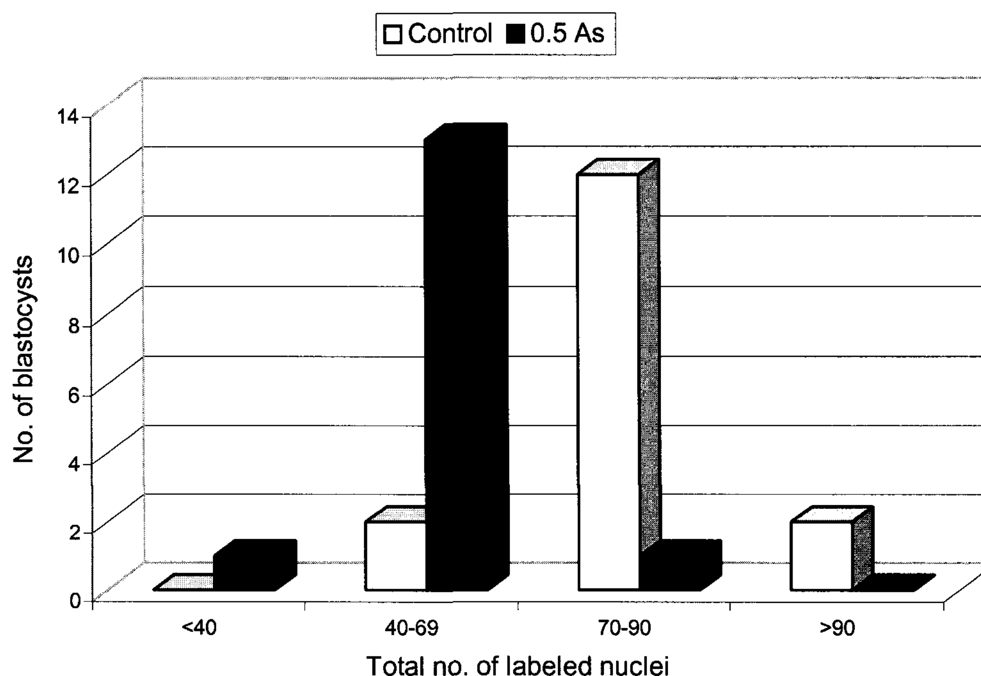

Figure 7. Distribution of total cell number in mouse blastocysts grown in vitro in the presence and absence of arsenic.

Embryo Quality in the Presence of Chromium

Although the presence of 0.5 mg/L chromium in the culture medium appeared to be safe for embryo development (Table V and VI), morphologically normal Cr-treated blastocyst developed embryos showed highly significant ($P < 0.0001$) lower total cell numbers (46.7 ± 2.1) compared with the control group (79.4 ± 4.0) (Table XIII; Figure 9A', A). The reduction in total cell numbers was 41.2% compared with control.

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Nearly all (92.8%) Cr-exposed blastocysts had total cell numbers located in the 40-69 range, the remaining (7.2%) were located in the < 40 range (Figure 8). All the Cr-exposed blastocyst embryos exhibited normal external morphology compared with the control group (Figure 9B', B) with only a slight reduction in their size when compared with control.

Table XIII. Total cell number of mouse blastocysts grown in vitro in chromium containing medium

Concentration [mg/L]	Embryo Number	Total cell number (Mean ± SEM)
Control	16	79.4 ± 4.0
0.5	14	46.7 ± 2.1**

** Highly significantly different from control, $P < 0.0001$.

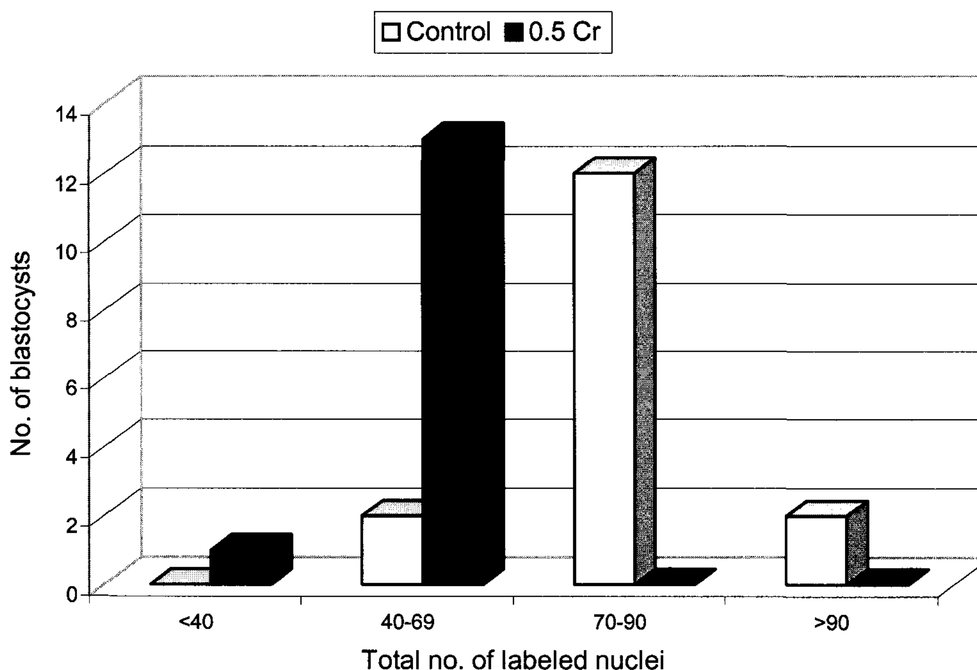

Figure 8. Distribution of total cell number in mouse blastocysts grown in vitro in the presence and absence of chromium.

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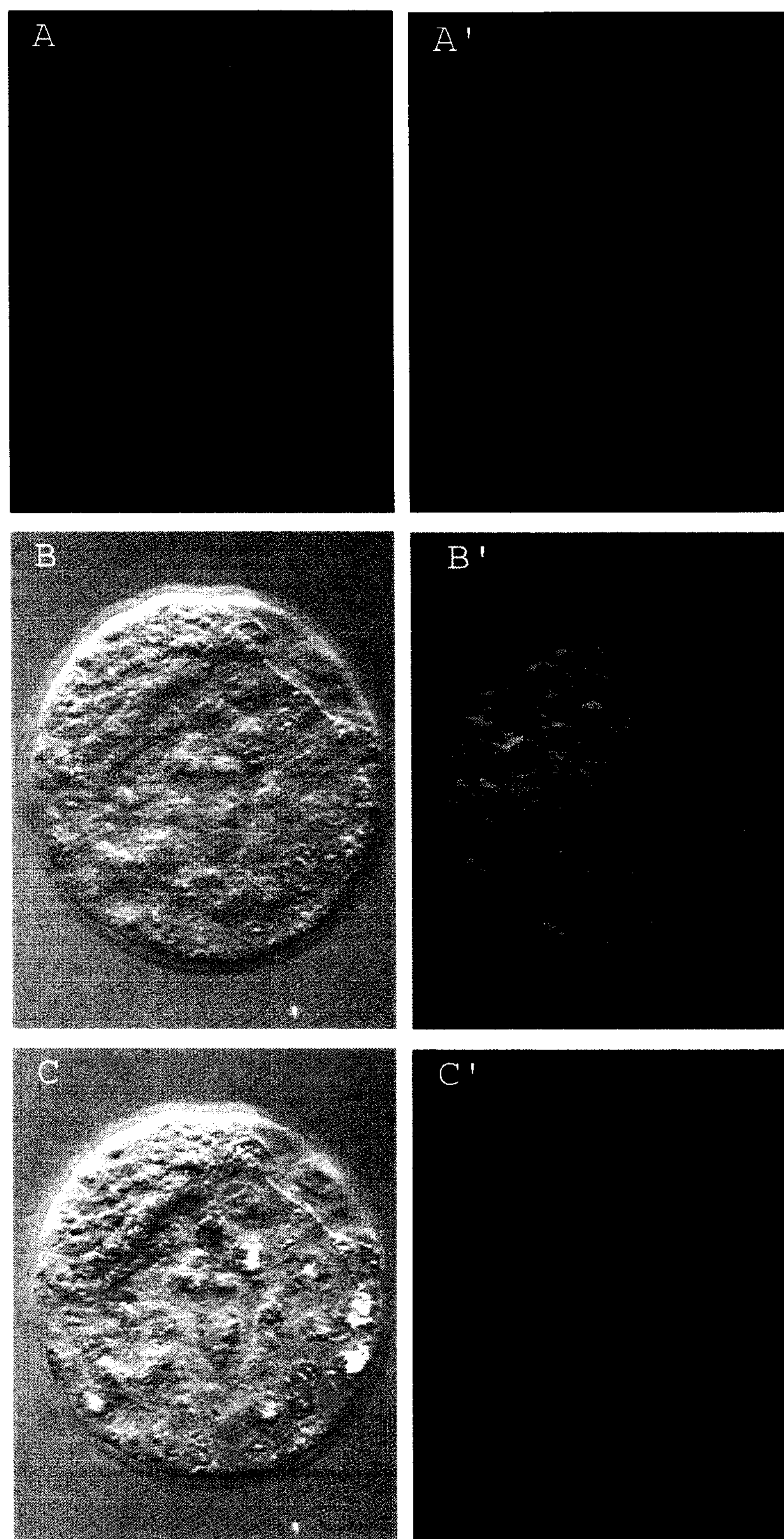

Figure 9. Total cell number of 0.5 Cr-treated preimplantation embryos. Left panel represents control group. Right panel represents Cr-treated group. **(A, A')** Under fluorescent optics. **(B, B')** Under Nomarski optics. **(C, C')** Superimposed pictures for **(A, B; A', B')**.

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Embryo Quality in the Presence of Copper

Total cell numbers in 0.5 mg/L Cu-exposed blastocyst embryos were significantly lower (63.5 ± 3.3) compared with the control group (Table XIV). The reduction in total cell numbers in the Cu-exposed blastocysts was 20% compared with control. In addition, around 37.5% of these blastocysts had total cell numbers located in the normal range while the remaining 62.5% were in the 40-69 range (Figure 10). Even though, copper still appeared to have the least problematic effect on embryo development and quality when compared with the other CCA components.

Table XIV. Total cell number of mouse blastocysts grown in vitro in copper containing medium

Concentration [mg/L]	Embryo Number	Total cell number (Mean ± SEM)
Control	16	79.4 ± 4.0
0.5	16	63.5 ± 3.3**

** Highly significantly different from control, $P < 0.005$.

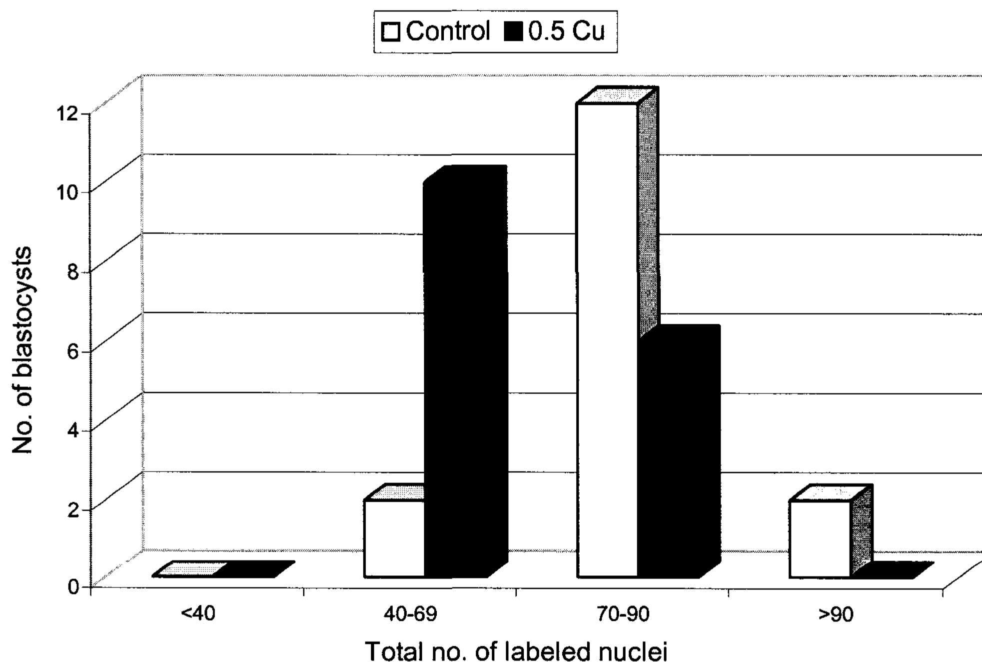

Figure 10. Distribution of total cell number in mouse blastocysts grown in vitro in the presence and absence of copper.

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3.2 Oocyte Maturation and Integrity

3.2.1 Cell Cycle Progression of In Vitro Maturing Mouse Oocytes

When germinal vesicle (GV) oocytes from sexually mature B6CBAF1/J female mice were isolated from antral follicles and cultured in 5% FCS-Waymouth medium (Figure 11A), most of them spontaneously resumed maturation and underwent germinal vesicles breakdown (GVBD) within 2 hr of culture (Figure 11B). Most of the control oocytes possessed a polar body (PB) after 16 hr of culture and arrested in meiosis II with condensed metaphase II chromosome (Figure 11C). Only a few oocytes, possibly derived from preantral follicles, were incompetent to mature and remained in the dictyate stage with intact GV or arrested in prophase I after GVBD (MI arrested oocytes) or became degenerated (Figure 11D).

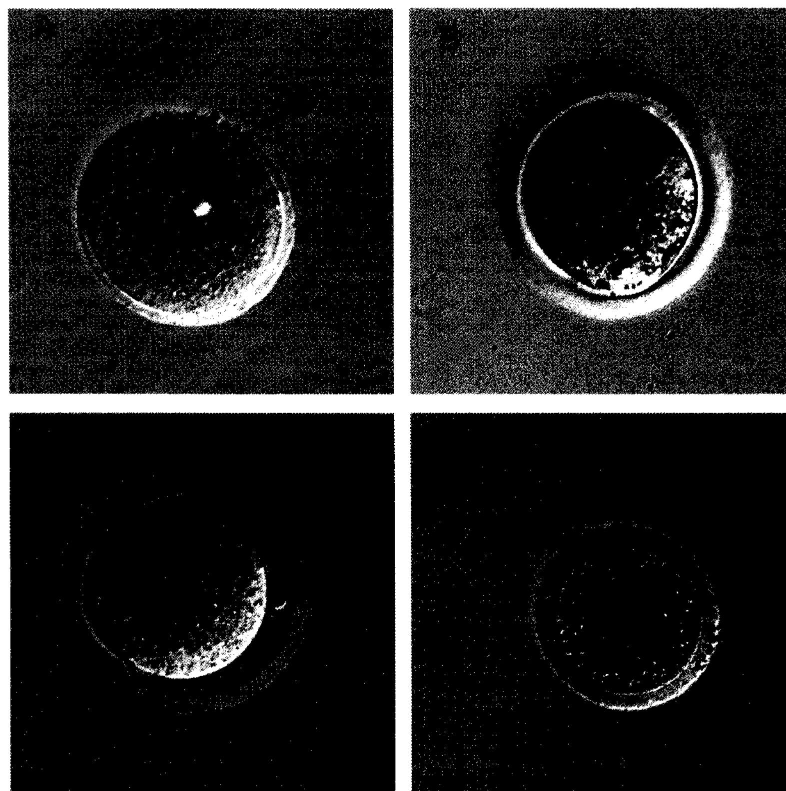

Figure 11. Morphological stages of oocyte maturation. **(A)** GV. **(B)** GVBD. **(C)** PB. **(D)** Fragmented oocyte. Oocytes were observed under Olympus relief contrast optics.

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Cell Cycle Progression of Oocytes in the Presence of Arsenic

Exposure of mouse oocytes to arsenic during in vitro maturation had a dramatic effect on maturation kinetics (Table XV). In the presence of arsenic, maturation rates, indicated from the number of metaphase II oocytes, were significantly much lower compared with the control value ($P < 0.0001$) and were decreasing with increasing arsenic concentration in culture medium. Correspondingly, the number of meiosis I arrested oocytes after GVBD gradually increased with rising arsenic concentration in cultured medium and was highly significantly different from control ($P < 0.0001$ for all As-treated groups except for 0.1 mg/L As; $P < 0.05$). At the same time, the number of maturation incompetent GV oocytes elevated in all As-treated groups compared with the control, where a significant difference was found only at 0.1 and 0.7 mg/L As. Most of the As-treated groups showed a significantly higher number of fragmented oocytes compared with the control group (Table XV).

Table XV. Cell cycle progression of mouse oocyte maturation 16 hr post-arsenic treatment

Concentration [mg/L]	Oocytes number	Number (%) of oocytes			
		GV	MI	MII	Fragmented
Control	292	15 (5.1)	42 (14.4)	231 (79.1)	4 (1.4)
0.05	96	6 (6.3)	30 (31.3)**	56 (58.3)**	4 (4.1)
0.10	138	21 (15.2)**	31 (22.5)*	78 (56.5)**	8 (5.8)*
0.70	151	29 (19.2)**	56 (37.1)**	59 (39.1)**	7 (4.6)*
1.40	126	11 (8.7)	62 (49.2)**	33 (26.2)**	20 (15.9)**

* Significantly different from control, $P < 0.05$.
** Highly significantly different from control, $P < 0.0001$.

There was no single arsenic concentration that showed a safe, nontoxic, effect on oocyte maturation (Table XV) compared with the control group. All arsenic concentrations even the lowest tested one, 0.05 mg/L As, showed toxic inhibitory effect on oocyte maturation. In the presence of 0.7 mg/L As, the percentage of As-exposed oocytes at metaphase II stage was highly reduced and reached half the value of the control group (79.1% of control oocytes reached MII stages versus 39.1% for 0.7 mg/L As-treated oocytes).

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Moreover, at this concentration the number of fragmented, GV incompetent and MI arrested oocytes increased significantly, ~ 2.5-3.5 fold compared with control (Table XV). Therefore, 0.7 mg/L arsenic concentration appeared to be the TC_{50} for oocyte maturation.

There was a dose-response reduction in the ability of oocytes to complete the first meiosis in the presence of arsenic in the maturation medium (Figure 12). There was diminution in number of oocytes with PB after 16 hr of maturation when 0.05 mg/L As was applied. The decline became most dramatic with 1.4 mg/L As and exhibited intermediate values between these concentrations. At the same time, the percentage of GV, GVBD and fragmented oocytes gradually increased with rising arsenic concentrations in the medium reaching a maximum value at 1.4 mg/L arsenic concentration (Figure 12).

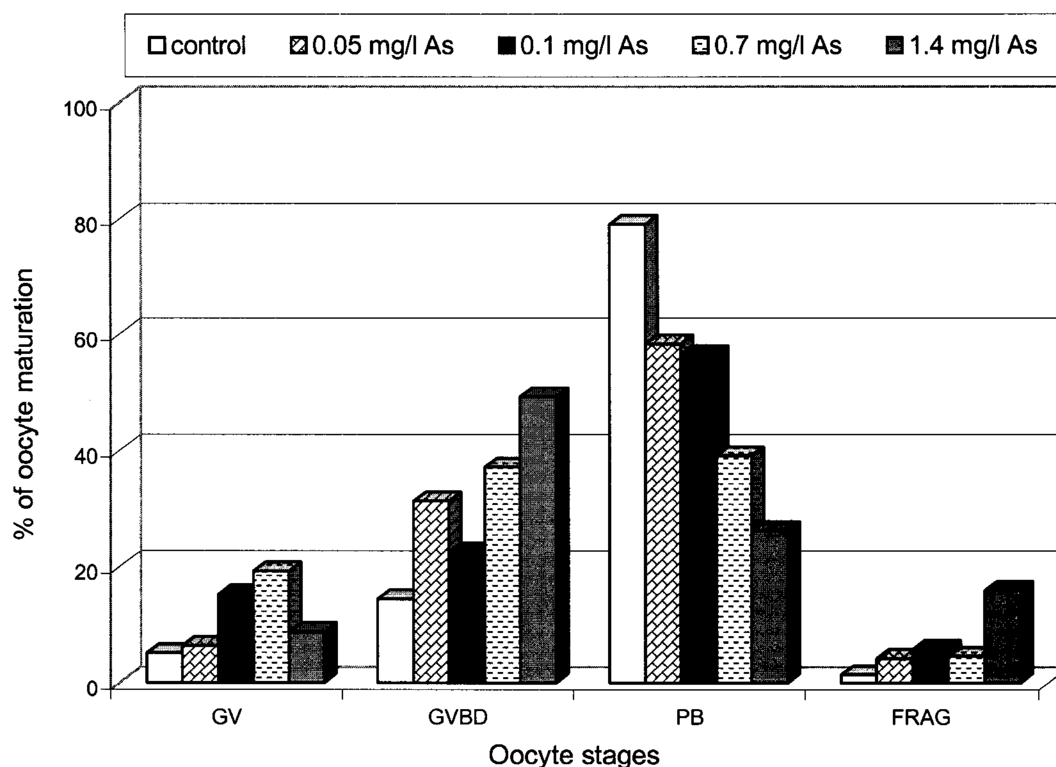

Figure 12. Concentration-dependent response of oocyte maturation 16 hr post-arsenic treatment. Percentage of oocytes with GV, GVBD, PB and FRAG is shown.

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Cell cycle progression of Oocytes in the Presence of Chromium

The presence of chromium in oocyte medium showed an inhibitory effect on their maturation. Number of oocytes developed to MII significantly decreased with the presence of 0.1 mg/L chromium and reached maximum reduction at 2.5 mg/L Cr ($P <$ 0.0001; Table XVI). At the same time, the number of fragmented oocytes significantly increased compared with control ($P < 0.0001$ at 0.5 and 2.5 mg/L Cr; $P < 0.05$ at 0.1 mg/L Cr). At 2.5 mg/L Cr, 20.9% of cultured oocytes were fragmented compared with 0% for control. In addition, the rate of GV oocytes consistently increased approximately to 4 times the value of the control (Table XVI).

In the presence of the 2.5 mg/L Cr in the oocyte maturation medium, all oocytes failed to emit the first PB and to develop to MII compared with control (0% versus 74.8% respectively). Some (53.7%) arrested at meiosis I after GVBD, others remained at GV stage (25.4%) or became fragmented (20.9%). When these oocytes were cultured for a longer time (19 hr), none of them was able to reach the MII stage, suggesting that this maturation block was irreversible.

Table XVI. Cell cycle progression of mouse oocyte maturation 16 hr post-chromium treatment

Concentration [mg/L]	Oocytes number	Number (%) of oocytes			
		GV	MI	MII	Fragmented
Control	103	6 (5.8)	20 (19.4)	77 (74.8)	0 (0)
0.1	82	5 (6.1)	31 (37.8)*	43 (52.4)**	3 (3.7)*
0.5	91	10 (11.0)	55 (60.4)**	21 (23.1)**	5 (5.5)**
2.5	67	17 (25.4)**	36 (53.7)**	0 (0)**	14 (20.9)**

* Significantly different from control, $P < 0.05$.
** Highly significantly different from control, $P < 0.0001$.

There was a clear concentration-dependant response in the percentage of oocytes matured to MII in the presence of chromium in the culture medium. The number of Cr-exposed oocytes reaching MII exhibited an inverse pattern with increasing chromium concentration, complete inhibition appeared at 2.5 mg/L Cr. Correspondingly, the

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percentage of GV, GVBD, and fragmented oocytes gradually increased with rising chromium concentration (Figure 13).

From Table XVI and Figure 13, the TC_{50} for chromium on oocyte maturation was expected to be between 0.1 and 0.5 mg/L. There was no concentration found to have safe-nontoxic effect on oocyte maturation. Finally, 2.5 mg/L Cr appeared to be severely toxic with 100% inhibition of oocyte maturation to MII.

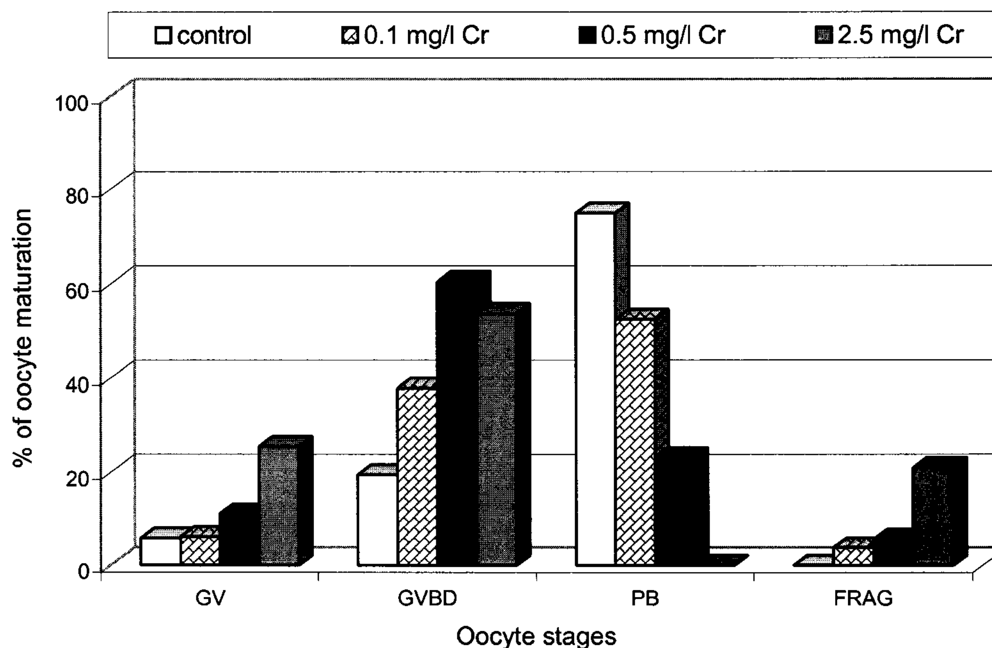

Figure 13. Concentration-dependent response of oocyte maturation 16 hr post-chromium treatment. Percentage of oocytes with GV, GVBD, PB and FRAG is shown.

Cell Cycle Progression of Oocytes in the Presence of Copper

In the presence of trace amount of copper in the culture medium (0.05 mg/L Cu), maturation rate significantly reduced ($P < 0.0001$) with only 52.4% of all oocytes was able to reach MII. This reduction significantly increased with rising copper concentrations and highly manifested at 5 mg/L Cu (at this concentration, only 1.6% of oocytes reached MII, versus 67.3% for control).

Furthermore, the number of MI arrested and fragmented oocytes significantly augmented with elevating copper concentration, reaching to the highest value at 5 mg/L

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Cu (Table XVII). In the presence of this concentration, 83.8% of in vitro matured oocytes arrested at meiosis I and 6.5% of oocytes were fragmented. Succinctly, copper presence at certain concentrations in oocyte maturation medium induced meiosis I arrest.

Table XVII. Cell cycle progression of mouse oocyte maturation 16 hr post-copper treatment

Concentration mg/L]	Oocytes number	Number (%) of oocytes			
		GV	MI	MII	Fragmented
Control	107	8 (7.5)	27 (25.2)	72 (67.3)	0 (0)
0.05	105	3 (2.9)	40 (38.1)*	55 (52.4)*	7 (6.7)*
0.10	128	11 (8.6)	61 (47.6)**	50 (39.1)**	6 (4.7)*
0.50	96	7 (7.3)	30 (31.3)	55 (57.2)	4 (4.2)*
2.50	90	9 (10.0)	36 (40.0)*	41 (45.6)*	4 (4.4)*
5.00	62	5 (8.1)	52 (83.8)**	1 (1.6)**	4 (6.5)*

* Significantly different from control, $P < 0.05$.
** Highly significantly different from control, $P < 0.0001$.

From data obtained in (Table XVII), 0.1 mg/L Cu was considered the TC_{50} for oocyte maturation. In the presence of this concentration only 39.1 % of cultured oocytes reached the MII stage with more than 60% (47.7% MI arrested oocytes + 8.6% GV meiotically incompetent oocytes + 4.7% fragmented oocytes) failed to reach their second meiosis.

Unexpectedly, in the presence of 0.5 mg/L Cu, oocyte maturation rate showed a higher value compared with 0.05 and 0.1 mg/L Cu. Moreover, no significant difference was found between the percentage of MII in 0.5 mg/L copper and the control. Further analysis of this case is required. By increasing the concentration above 0.5 mg/L Cu, oocyte maturation returned to the decline pattern and reached a minimum value at 5 mg/L Cu (Table XVII and Figure 14).

Figure 14 represents the percentage of GV, GVBD, MII and fragmented oocytes in the presence and absence of copper in the cultured medium. Slight significant reduction in MII was observed at 0.05 mg/L Cu but the reduction became dominant at 5 mg/L Cu and exhibited intermediate values between these concentrations.

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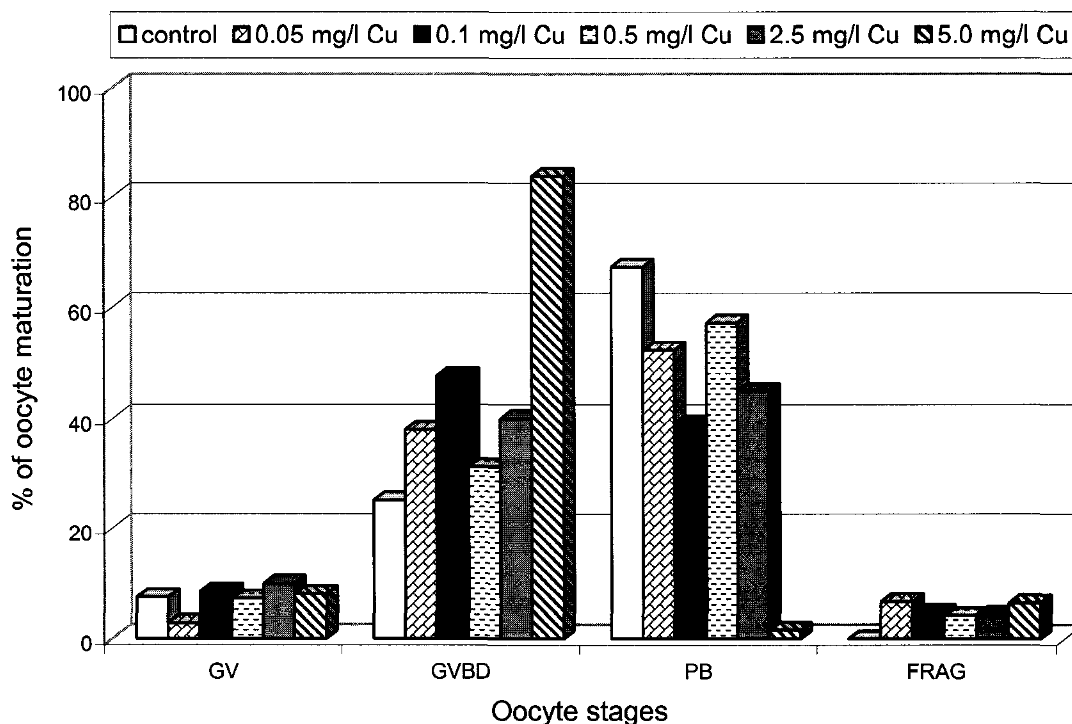

Figure 14. Concentration-dependent response of oocyte maturation 16 hr post-copper treatment. Percentage of oocytes with GV, GVBD, PB and FRAG is shown.

3.2.2 Spindle Morphology and Chromosome Alignment of In Vitro Maturing Oocytes.

Spindle and chromosome behavior in MI and MII in vitro-matured oocytes in the presence and absence of individual components of CCA were analyzed using fluorescent microscopy. Most control groups exhibited normal spindle morphology. For control and treated groups, both MI and MII oocytes were evaluated separately in order to detect if the abnormality in spindle morphology and chromosome alignment was confined to MI or MII maturation stage. Metaphase II represents the end point for oocytes development and maturation before fertilization. Therefore, spindle structure and chromosomal behavior were compared at this stage between control and treated groups.

To detect if the cause of meiosis I blocking of the treated oocytes related to spindle and chromosome abnormalities, MI-arrested treated oocytes were analyzed and were compared at this stage between control and treated groups.

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Spindle Morphology and Chromosome Alignment in As-exposed Oocytes

Aberrant spindle shape and unaligned chromosomes were highly presented in all As-exposed oocytes (Table XVIII). At low arsenic concentration, 0.05 mg/L, the extent of the disturbance in spindle formation and chromosomal alignment was very high and highly significant ($P < 0.0001$) compared with the control group (91.1% versus 20.6% for spindles; 80.4% versus 8.4% for chromosomes; Table XVIII). Raising the As-concentration above 0.05 mg/L did not appear to increase the rate of spindle and chromosome abnormality but the percentage of aberrant spindles and misaligned chromosomes continued to be significantly higher than in the control group ($P < 0.0001$; Table XVIII). At the chromosomal level, 1.4 mg/L arsenic-treated oocytes showed the highest significant rate of chromosome misalignment compared with the control group and treated groups (Table XVIII).

Table XVIII. Analysis of spindle morphology and chromosome alignment in oocytes 16 hr post-arsenic treatment

Concentration [mg/L]	Oocyte number	Spindle		Chromosome	
		Normal (%)	Aberrant (%)	Aligned (%)	Unaligned (%)
Control	107	85 (79.4)	22 (20.6)	98 (91.6)	9 (8.40)
0.05	56	5 (8.90)	51 (91.1)**	11 (19.6)	45 (80.4)**, a
0.10	61	8 (13.1)	53 (86.9)**	24 (39.3)	37 (60.7)**, a, b
0.70	106	21 (19.8)	85 (80.2)**	30 (28.3)	75 (70.7)**, c
1.40	52	6 (11.5)	46 (88.5)**	8 (15.4)	44 (84.6)**, b, c

** Highly significantly different from control, $P < 0.0001$.
Pairs of values compared together indicated by same superscript.
a and b pairwise comparisons are significantly different, $P < 0.05$.
c pairwise comparison is highly significantly different, $P < 0.0001$.

Disturbance in spindle formation and chromosome alignment was characterized in both MI arrested and MII As-treated oocytes after 16 hr of culture. However, most of the spindle and chromosome abnormalities of the control group were confined to MI arrested oocytes (56.3% aberrant spindle at MI versus 5.3% at MII; 18.8% unaligned chromosomes at MI versus 4% at MII; Table XIX and XX). Although, 0.05 mg/L arsenic MI arrested oocytes had the highest rate of spindle abnormalities compared with treated

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groups, there was no statistically significant difference between 0.05 mg/L As and other treated groups (Table XIX).

Table XIX. Analysis of spindle morphology and chromosome alignment in MI oocytes 16 hr post-arsenic treatment

Concentration [mg/L]	Oocyte number	Spindle		Chromosome	
		Normal (%)	Aberrant (%)	Aligned (%)	Unaligned (%)
Control	32	14 (43.7)	18 (56.3)	26 (81.2)	6 (18.8)
0.05	39	3 (7.70)	36 (92.3)**	7 (17.9)	32 (82.1)**
0.10	22	3 (13.6)	19 (86.4)*	3 (13.6)	19 (86.4)**
0.70	63	8 (12.7)	55 (87.3)**	13 (20.6)	50 (79.4)**
1.40	33	4 (12.1)	29 (87.9)**	5 (15.2)	28 (84.8)**

* Significantly different from control, $P < 0.05$.
** Highly significantly different from control, $P < 0.0001$.

Metaphase II oocytes, which escaped arsenic-induced meiosis I arrest, had been separately analyzed for spindle and chromosome behavior. Unexpectedly, there was significant elevation in the number of aberrant spindles and chromosomes compared with the control group ($P < 0.0001$). Moreover, no statistically significant differences were found between 0.05 mg/L and 1.4 mg/L As with regard to the abnormalities in spindle morphology and chromosome alignment. However, 1.4 mg/L As showed the highest significant rate of chromosome abnormalities compared with treated groups (Table XX)

Table XX. Analysis of spindle morphology and chromosome alignment in MII oocytes 16 hr post-arsenic treatment

Concentration [mg/L]	Oocyte number	Spindle		Chromosome	
		Normal (%)	Aberrant (%)	Aligned (%)	Unaligned (%)
Control	75	71 (94.7)	4 (5.30)	72 (96.0)	3 (4.0)
0.05	17	2 (11.8)	15 (88.2)**, a	4 (23.5)	13 (76.5)**
0.10	39	6 (15.4)	33 (84.6)**	20 (51.3)	19 (48.7)**, c
0.70	35	13 (35.3)	22 (64.7)**, a, b	16 (45.7)	19 (54.3)**, d
1.40	19	2 (10.5)	17 (89.5)**, b	3 (15.8)	16 (84.2)**, c, d

** Highly significantly different from control, $P < 0.0001$.
Pairs of values compared together indicated by same superscript.
a – d pairwise comparison are significantly different, $P < 0.05$.

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Generally, in both 0.05 mg/L As MI arrested oocytes and MII oocytes a constant gap (~10-12%) in the rate of aberrant spindle and misaligned chromosome was found. This gap tends to decrease with increasing arsenic concentration, reaching about 3-5% at 1.4 mg/L As (Table XIX and XX). This indicates that arsenic may have a predominant toxic effect on spindle morphology rather than on chromosome alignment.

Immunofluorescent images of spindles and chromosomes, in which spindles were stained with FITC-conjugated second antibody (shown in green) and chromosomes were stained with DAPI (shown in blue), showed that metaphase I arrested As-exposed oocytes had irregular, asymmetrically shaped spindles with misaligned chromosomes compared with typical anastral, barrel-shaped spindles with condensed chromosomes aligned at spindle equator of control group (Figure 15A, A'). Depending on arsenic concentration, abnormalities in spindles and chromosomes differed. Most of the 0.05 mg/L MI arrested As-exposed oocytes possessed aberrant irregular shaped multipole spindles with individual displaced chromosomes (Figure 15B, B'; arrows point to two displaced chromosomes). At 0.1 mg/L As, most oocytes had ball-like spindles, very short spindles with round poles, and unordered mass-like chromosomes (Figure 15C, C'). The interpolar space of these spindles appeared much shorter compared with control (Figure 15C). In the presence of 0.7 mg/L or 1.4 mg/L arsenic, some of the treated oocytes possessed asymmetrical spindles with one small pointed fusiform tipping end and one broad pole (Rhombus-shaped spindle) with misaligned chromosomes (Figure 15D, D'; arrows point to three displaced chromosomes). Others showed slightly hypertrophied, enlarged, rectangular shaped spindles with unordered chromosomal masses (Figure 15E, E'). Interestingly, chromosome misalignments in As-exposed oocytes were found over both intact and disrupted MI spindles, suggesting that MI spindles can be formed without alignment of chromosomes. Therefore, chromosome abnormalities alone cannot determine the integrity of spindle at MI oocytes. On the other hand, disrupted spindles generally indicated chromosome misalignments or defects.

On further maturation, most control oocytes emitted a polar body after 9-11 hr and possessed metaphase II spindles by 12 hr of maturation. Even after 16 hr culture, most controls had square anastral spindles with flat poles and well-aligned chromosomes (Figure 16A, A'). In contrast to the advanced development of controls, most As-treated

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oocytes remained blocked in metaphase I with aberrant spindles and misaligned chromosomes (Table XIX). Moreover, most of the As-exposed oocytes reaching MII exhibited abnormal spindles with chromosomes misaligned and disrupted along the spindle or with completely disrupted spindle. Some of them, possessed asymmetrical irregular spindles with fusiform pole and misaligned chromosomes (Figure 16B, B'; arrows point to four displaced chromosomes). Others had round poles, small ball-like spindles with mass-like chromosomes (Figure 16C, C' and 16D, D'). In the cases of severely disrupted spindles, chromosomes scattered all over the spindles were seen in 1.4 mg/L arsenic-treated oocytes (Figure 16E, E'; arrows point to some of the displaced chromosomes).

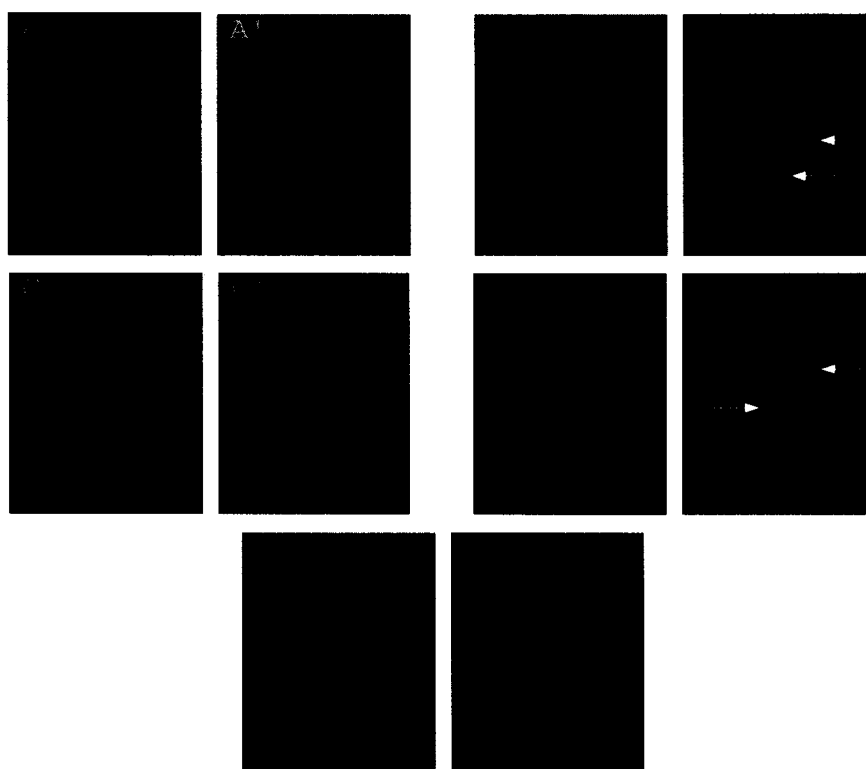

Figure 15. Spindle and chromosome behavior in As-exposed meiosis I oocytes. **(A, A')** Control. **(B, B')** 0.05 mg/L As. **(C, C')** 0.1 mg/L As. **(D, D')** 0.7 mg/L As. **(E, E')** 1.4 mg/L As. **A-E** Meiotic I spindles stained by anti-tubulin; **A'-E'** DAPI-stained chromosomes. Arrows indicate chromosome misalignment.

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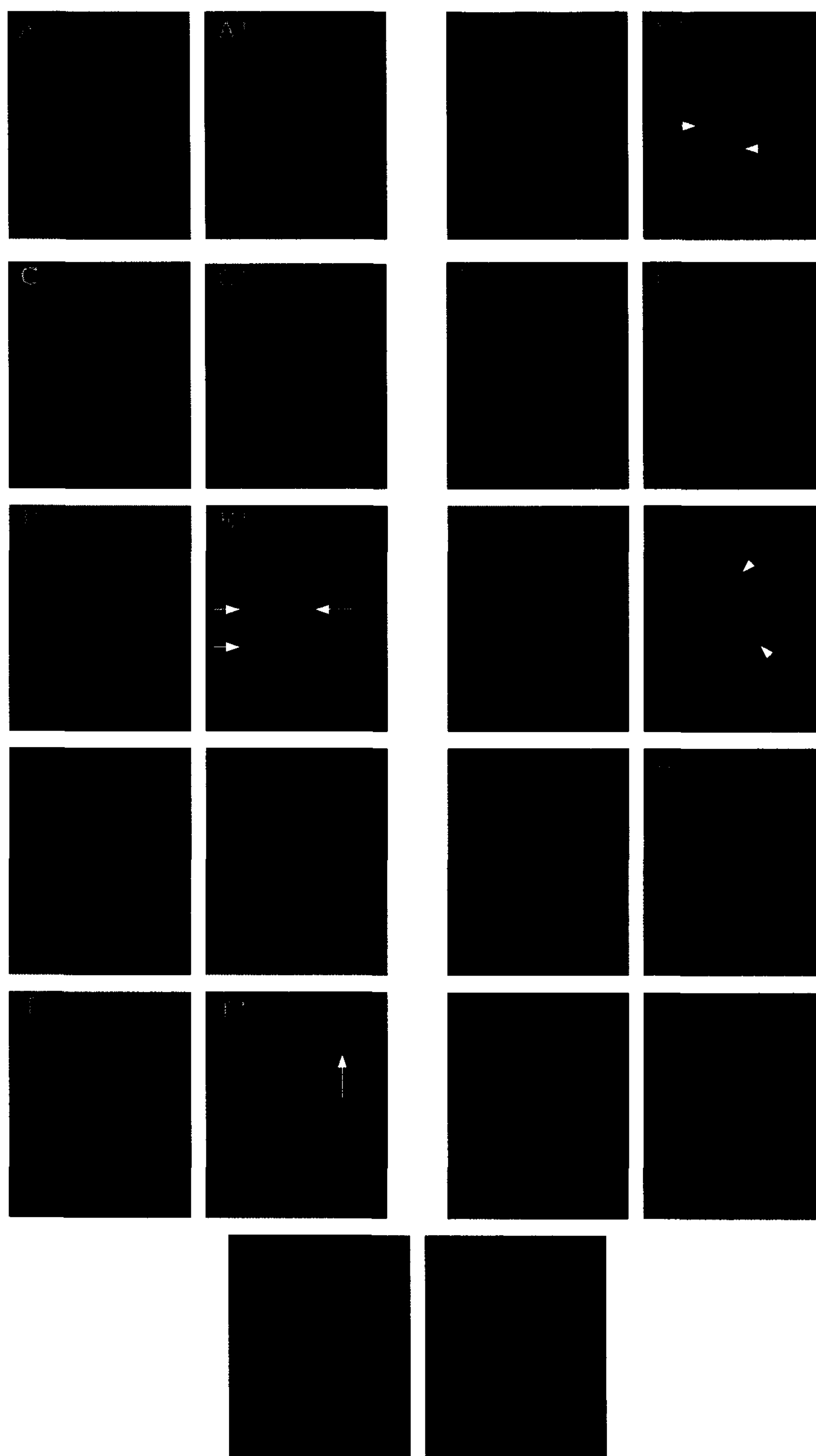

Figure 16. Spindle and chromosome behavior in As, Cr and Cu MII exposed oocytes. **(A, A')** Control. **(B, B')** 0.05 mg/L As. **(C, C')** 0.1 mg/L As. **(D, D')** 0.7 mg/L As. **(E, E')** 1.4 mg/L As. **(F, F')** 0.1 mg/L Cr. **(G, G')** 0.5 mg/L Cr. **(H, H')** 0.05 mg/L Cu. **(I, I')** 0.1 mg/L Cu. **(J, J')** 0.5 mg/L Cu. **(K, K')** 2.5 mg/L Cu.

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Spindle Morphology and Chromosome Alignment in Cr-exposed Oocytes

Exposure of oocytes to chromium in maturation medium for 16 hr culture appeared to have a highly significant ($P < 0.001$) potent toxic effect on spindle morphology and chromosome alignment compared with the control group. The percentage of aberrant spindles and misaligned chromosomes significantly increased in a dose-dependant manner with elevating chromium concentration in the culture medium ($P < 0.0001$; Table XXI). All 0.5 mg/L Cr-exposed oocytes had significantly ($P < 0.0001$) aberrant spindles with 80% of them having misaligned chromosomes compared with the control group. At 2.5 mg/L Cr, all chromium-exposed oocytes significantly exhibited spindle and chromosome abnormalities compared with the control group (Tables XXI).

Table XXI. Analysis of spindle morphology and chromosome alignment in oocytes 16 hr post-chromium treatment

Concentration [mg/L]	Oocyte number	Spindle		Chromosome	
		Normal (%)	Aberrant (%)	Aligned (%)	Unaligned (%)
Control	101	76 (75.2)	25 (24.8)	87 (86.1)	14 (13.9)
0.1	62	32 (51.6)	30 (48.4)$^{**, a, b}$	19 (30.6)	43 (69.4)**
0.5	25	0 (0)	25 (100)$^{**, a}$	5 (20.0)	20 (80.0)**
2.5	8	0 (0)	8 (100)$^{**, b}$	0 (0)	8 (100)**

** Highly significantly different from control, $P < 0.0001$.
Pairs of values compared together indicated by same superscript.
a – b pairwise comparisons are highly significantly different, $P < 0.005$.

Most of the spindle and chromosome abnormalities localized and were displayed in MI arrested oocytes, especially at 0.5 and 2.5 mg/L chromium concentration. At 2.5 mg/L Cr, all MI arrested oocytes exhibited highly significant aberrant spindles with misaligned chromosomes compared with the control group ($P < 0.0001$; Table XXII). This explained why no oocytes in the presence of 2.5 mg/L Cr concentration were able to develop to MII.

Even 0.1 mg/L Cr MII oocytes, which escaped Cr-induced meiosis I arrest, displayed significantly higher rates of aberrant spindles with unaligned chromosomes compared with control (50% versus 17.4% for spindle; 69.9% versus 4.3% for chromosome; Table XXIII).

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Both MI and MII 0.1 mg/L Cr-treated oocytes exhibited higher rates of chromosomal abnormalities compared with spindle abnormalities (76.9% versus 50% for MI oocytes; Table XXII; 69.9% versus 50% for MII oocytes; Table XXIII). Therefore, chromosome abnormalities alone cannot determine the integrity of the spindles at MI or MII oocytes, indicating that chromosomal abnormalities can be found over both intact and disrupted spindles.

Table XXII. Analysis of spindle morphology and chromosome alignment in MI oocytes 16 hr post-chromium treatment

Concentration [mg/L]	Oocyte number	Spindle		Chromosome	
		Normal (%)	Aberrant (%)	Aligned (%)	Unaligned (%)
Control	32	19 (59.4)	13 (40.6)	21 (65.6)	11 (34.4)
0.1	26	13 (50.0)	13 (50.0)[a, b]	6 (23.1)	20 (76.9)**
0.5	25	0 (0)	25 (100)**, [a]	5 (20.0)	20 (80.0)**
2.5	8	0 (0)	8 (100)**, [b]	0 (0)	8 (100)**

** Highly significantly different from control, $P < 0.0001$.
Pairs of values compared together indicated by same superscript.
a pairwise comparison is highly significantly different, $P < 0.0001$.
b pairwise comparison is significantly different, $P < 0.05$.

Table XXIII. Analysis of spindle morphology and chromosome alignment in MII oocytes 16 hr post-chromium treatment

Concentration [mg/L]	Oocyte number	Spindle		Chromosome	
		Normal (%)	Aberrant (%)	Aligned (%)	Unaligned (%)
Control	69	57 (82.6)	12 (17.4)	66 (95.7)	3 (4.30)
0.1	36	18 (50.0)	18 (50.0)**	13 (36.1)	23 (69.9)**

** Highly significantly different from control, $P < 0.0001$.

Immunofluorescent analysis of MI and MII Cr-treated oocytes confirmed that chromium severely interferes with spindle integrity and chromosome alignment (Figure 16 and 17). At 0.1 mg/L Cr, most of the treated oocytes (MI and MII) possessed irregular oval shaped spindles (Figure 16F and 17B) with chromosomes located outside the equator (Figure 16F' and 17B'; arrows indicate that 3-4 chromosomes were displaced). In the presence of 0.5 mg/L Cr, most of the oocytes had asymmetrical spindles with one

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small and one broad spindle pole (Figure 16G and 17C) with unaligned undefined irregular chromosomal masses (Figure 16 G') or with scattered chromosomes around the equator (Figure 17C'; arrows point to 4 displaced chromosomes). Moreover, the centrosomal microtubular organizing center at the spindle poles appeared positioned in a zig-zag pattern rather than being spatially organized and forming a flat plate as in the control group (Figure 17C, 17 A).

Interestingly, at 2.5 mg/L Cr, most of the MI arrested oocytes showed complete destruction and disappearance of spindles with the presence of round undefined chromosomal masses (Figure 17D, D'). To exclude the possibility of being GV oocytes (without assembled microtubules), images of GV oocytes were taken and compared with 2.5 mg/L Cr images. Although in both of them no spindles were recognized, the appearance of centralized round small faintly blue nuclear material surrounded by a nuclear membrane was characterized for GV oocytes (Figure 17E, E').

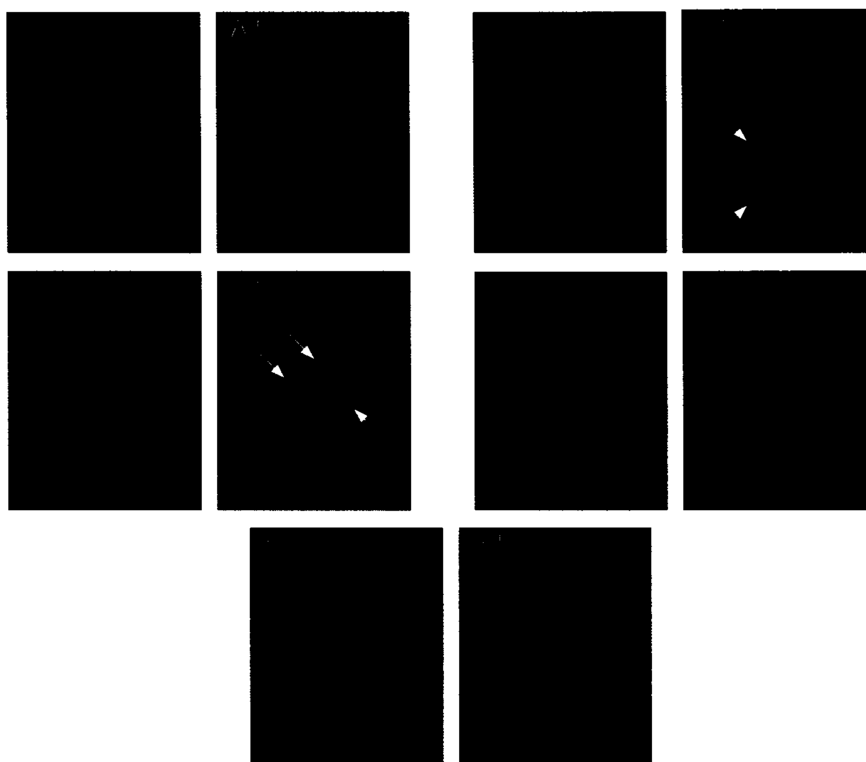

Figure 17. Spindle and chromosome behavior in Cr-exposed meiosis I oocytes. **(A, A')** Control. **(B, B')** 0.1 mg/L Cr. **(C, C')** 0.5 mg/L Cr. **(D, D')** 2.5 mg/L Cr. **(E, E')** GV oocyte. Green staining = spindle immunofluorescence; blue staining = DAPI-stained chromosomes. Arrows indicate chromosome misalignment.

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Spindle Morphology and Chromosome Alignment in Cu-exposed Oocytes

Copper presence in trace amount in oocyte maturation medium showed a deforming toxic effect on spindle and chromosome morphology. The percentage of abnormal spindles and unaligned chromosomes significantly appeared ($P < 0.0001$) at 0.05 mg/L copper in comparison with control, reaching its maximum value (100% aberrant spindle with 92.1% misaligned chromosome) at 5 mg/L copper (Table XXIV). Although there was slight reduction in the percentage of aberrant spindles and misaligned chromosomes between the 0.1 and 0.5 mg/L copper-treated groups, no statistically significant differences were found between them. A clear significant concentration-dependant response arose between the percentage of aberrant spindles and misaligned chromosomes with an increasing copper concentration in the culture medium.

Table XXIV. Analysis of spindle morphology and chromosome alignment in oocytes 16 hr post-copper treatment

Concentration [mg/L]	Oocyte number	Spindle		Chromosome	
		Normal (%)	Aberrant (%)	Aligned (%)	Unaligned (%)
Control	101	76 (75.2)	25 (24.8)	87 (86.1)	14 (13.9)**
0.05	44	14 (31.8)	30 (68.2)$^{**, a}$	22 (50.0)	22 (50.0)$^{**, e}$
0.10	54	11 (20.4)	43 (79.6)$^{**, b}$	23 (42.6)	31 (57.4)$^{**, f}$
0.50	61	14 (23.0)	47 (77.0)$^{**, c}$	30 (49.2)	31 (50.8)$^{**, g}$
2.50	27	4 (14.8)	23 (85.2)$^{**, d}$	8 (29.6)	19 (70.4)**
5.00	38	0 (0)	38 (100)$^{**, a, b, c, d}$	3 (7.9)	35 (92.1)$^{**, e, f, g}$

** Highly significantly different from control, $P < 0.0001$.
Pairs of values compared together indicated by same superscript.
a – g pairwise comparison are highly significantly different, $P < 0.0001$.
c pairwise comparison is significantly different, $P < 0.05$.

Most of the MI arrested Cu-exposed oocytes possessed abnormal spindles and unaligned chromosomes even at low, 0.05 mg/L, copper concentration. Spindle and chromosome abnormalities progressively increased with elevating copper concentration in the culture medium (Table XXV). At 0.05 mg/L Cu, the number of oocytes with aberrant spindles and misaligned chromosomes increased significantly ($P < 0.0001$), ~ 2 fold compared with control. All 2.5 mg/L and 5 mg/L copper MI arrested oocytes

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exhibited highly significant misaligned chromosomes compared with the control and all other treated groups (Table XXV).

Table XXV. Analysis of spindle morphology and chromosome alignment in MI oocyte 16 hr post-copper treatment

Concentration [mg/L]	Oocyte number	Spindle		Chromosome	
		Normal (%)	Aberrant (%)	Aligned (%)	Unaligned (%)
Control	32	19 (59.4)	13 (40.6)	21 (65.6)	11 (34.4)
0.05	20	6 (30.0)	14 (70.0)$^{*, a, b}$	7 (35.0)	13 (65.0)$^{*, f, g}$
0.10	34	5 (14.7)	29 (85.3)$^{**, c}$	14 (41.2)	20 (58.8)$^{*, h, i}$
0.50	34	8 (23.5)	26 (76.5)$^{**, d}$	14 (41.2)	20 (58.8)$^{**, j, k}$
2.50	15	2 (13.3)	13 (86.7)$^{*, a, e}$	0 (0)	15 (100)$^{**, f, h, j}$
5.00	29	0 (0)	29 (100)$^{**, b, c, d, e}$	0 (0)	27 (100)$^{**, g, i, k}$

* Significantly different from control, $P < 0.05$.
** Highly significantly different from control, $P < 0.0001$
Pairs of values compared together indicated by same superscript.
c, d, f and g pairwise comparisons are significantly different, $P < 0.05$.
a, b, e, h, I, j and k pairwise comparisons are highly significantly different, $P < 0.0001$.

Interestingly, regardless of spindle morphology, chromosome misalignment was observed in 100% of 2.5 mg/L of the MI arrested Cu- exposed oocytes compared with 86.7% with aberrant spindle (Table XXV). In the presence of 5 mg/L copper in the cultured medium, all MI oocytes exhibited abnormal spindles with misaligned chromosomes compared with the control and other treated groups ($P < 0.0001$). This explained why only 1.6% of 5 mg/L copper exposed oocytes were able to develop to MII (Table XVII).

Metaphase II Cu-exposed oocytes exhibited abnormalities in spindle morphology and chromosome behavior. Abnormalities progressively increased in a dose-dependant manner to copper's presence in the culture medium. Disturbance in spindle formation showed a higher tendency with increasing copper concentration compared with chromosome misalignment. All MII Cu-exposed oocytes had a higher percentage of aberrant spindles in compared with misaligned chromosomes. Apparently at 5 mg/L Cu, all MII Cu-exposed oocytes possessed aberrant spindles with only 66.7% of them having misaligned chromosomes (Table XXVI).

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Table XXVI. Analysis of spindle morphology and chromosome alignment in MII oocyte 16 hr post-copper treatment

Concentration [mg/L]	Oocyte number	Spindle		Chromosome	
		Normal (%)	Aberrant (%)	Aligned (%)	Unaligned (%)
Control	69	57 (82.6)	12 (17.4)	66 (95.7)	3 (4.3)
0.05	24	8 (33.3)	16 (66.7)**, a	15 (62.5)	9 (37.5)**
0.10	20	6 (30.0)	14 (70.0)**	9 (45.0)	11 (55.0)**
0.50	27	6 (22.2)	21 (77.8)**	16 (59.3)	11 (40.7)**
2.50	12	2 (16.7)	10 (83.3)*	8 (66.7)	4 (33.3)**
5.00	9	0 (0)	9 (100)**, a	3 (33.3)	6 (66.7)**

* Significantly different from control, $P < 0.05$.
** Highly significantly different from control, $P < 0.0001$.
Pairs of values compared together indicated by same superscript.
a pairwise comparison is significantly different, $P < 0.05$.

All MII copper-treated oocytes showed significantly higher percentage of misaligned chromosomes compared with control ($P < 0.0001$). Although the percentage of misaligned chromosomes at 0.5 mg/L and 2.5 mg/L showed a slight drop compared with 0.1 mg/L, no significant differences were found between them ($P = 0.333$ for 0.5 mg/L Cu; $P = 0.234$ for 2.5 mg/L Cu; Table XXVI).

The severity of the abnormality in spindle morphology and chromosome behavior in both MI arrested Cu-exposed oocytes and MII treated oocytes, was related to copper concentration. Most of the MI-arrested copper-treated oocytes exhibited abnormal spindle morphology with misaligned chromosomes compared with typical anastral, barrel-shaped spindles with aligned chromosomes at the equator of the control (Figure 18A, A'). At 0.05 mg/L copper, some oocytes showed slim, elongated spindles with or without aligned chromosomes. Others showed round oval-shaped spindles with chromosomes spread all over the spindle (Figure 18B, B'). With increasing copper concentration in the culture medium to 0.1 mg/L copper, some of the oocytes still had the oval-shape spindles but the remaining had completely disrupted irregular-shaped spindles with chromosomes scattered all over the spindles (Figure 18C, C'). Some of the 0.5 mg/L, most of the 2.5 mg/L and all of the 5 mg/L MI arrested Cu-exposed oocytes had no microtubule or spindle structure (Figure 18D; E; F) with chromosomes spread throughout the cytoplasm (Figure 18D'; E'; F'; arrows point to some of the scattered chromosomes).

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Most of the Cu-exposed oocytes reached to MII exhibited aberrant spindles with misaligned chromosomes. Some of them possessed slim shaped elongated spindles. In some of these, condensed chromosomes still aligned at the spindle equator (Figure 16H, H'). In others, individual chromosomes were displaced or sometimes they were even positioned outside the central array of microtubules between opposite spindle poles (Figure 16I, I'; arrow points to two displaced chromosomes). Increasing copper concentration in the culture medium was associated with complete disruption of spindles with chromosomes spread throughout the disrupted spindles (Figure 16J, J' and K, K'; arrows point to some of scattered chromosomes).

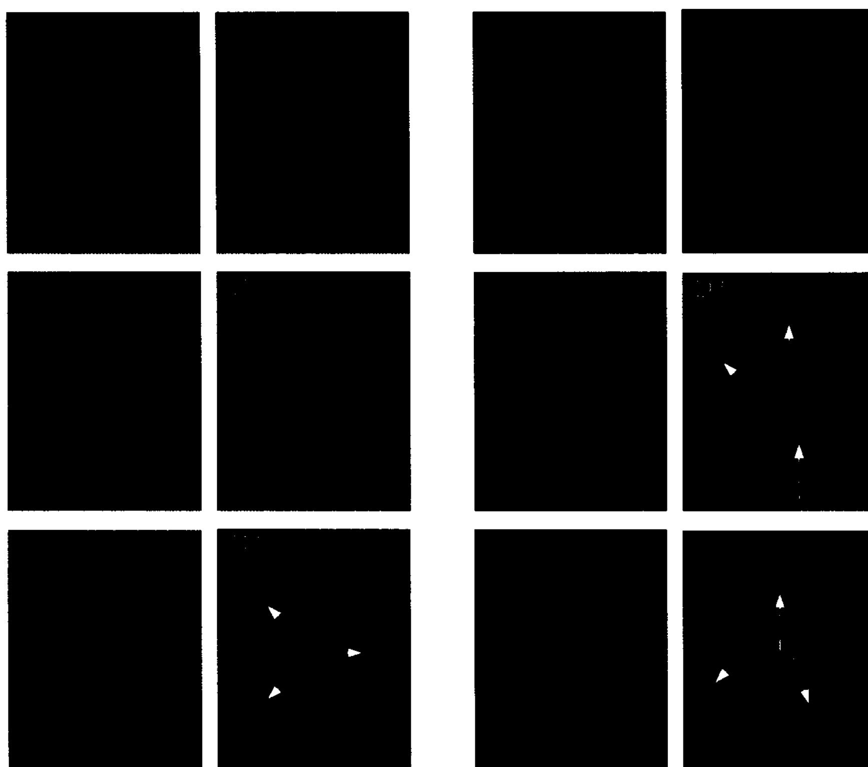

Figure 18. Spindle and chromosome behavior in Cu-exposed meiosis I oocytes. **(A, A')** Control. **(B, B')** 0.05 mg/L Cu. **(C, C')** 0.1 mg/L Cu. **(D, D')** 0.5 mg/L Cu. **(E, E')** 2.5 mg/L Cu. **(F, F')** 5 mg/L Cu. Oocytes observed under a fluorescent microscope. **A-F** Anti-tubulin stained meiotic I spindles; **A'-F'** DAPI-stained chromosomes. Arrows indicate displaced chromosomes.

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3.3 Evaluation of Sperm Function and Viability

3.3.1 Evaluation of Sperm Function in the Presence of Individual CCA Components

When sperm isolated form the vas deference of sexually mature CD1 male mice were diluted and incubated in Earle's balanced salt solution (EBSS), they exerted vigorous forward progressive movement (fpm). After about 30 min, they slowed down and began to associate head to head clusters with sluggish circular movements. By 4 hr incubation, most of the clusters had broken up and most of the control untreated sperm exhibited category 4 motility with rapid purposeful fpm without hyperactivation. At this time, sperm motility index (SMI) of the control group reached its peak. A significant reduction in the SMI at 4 hr indicated the presence of toxic contaminants in the medium.

To determine if the SMI reduction was due to percentage of motile sperm reduction or forward progressive quality (FPQ) score reduction, these two components of the SMI were analyzed for each treated group and compared with the control and the other treated groups.

Sperm Function in the Presence of Arsenic

With arsenic presence in the sperm medium, SMI exhibited a significant reduction ($P < 0.0001$) compared with the control group. At the same time, sperm motility percentage showed a significantly lower value in the As-treated groups compared with the control group. However, the FPQ score was significantly higher with a 4.5 score in the As-treated groups compared with 4 for the control group. A score of 4.5 indicted that 50% of sperm showed grade 4 motility and the remaining were in the hyperactivated stage.

Sperm motility and SMI exhibited a significant reduction in the presence of 0.5 mg/L As, this drop became more obvious and significant ($P < 0.0001$) with increasing arsenic concentration in incubation medium, reaching the maximum value at 1.5 mg/L As concentration (Table XXVII). Therefore, a concentration-dependant response for SMI and sperm motility percentage was demonstrated with arsenic present in the sperm medium. On the other hand, the FPQ showed a constant significantly higher score in all As-exposed sperm compared with the control group.

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Table XXVII. Analysis of sperm function 4 hr post-arsenic treatment

Concentration [mg/L]	Number	Mobility percentage mean ± SEM	FPQ mean ± SEM	SMI mean ± SEM
Control	12	57.8 ± 1.1	4.0 ± 0	920.0 ± 16.3
0.5	12	41.8 ± 1.8[**, a]	4.5 ± 0[**]	845.5 ± 35.4[c, d]
1.0	12	35.0 ± 4.0[**, b]	4.5 ± 0[**]	573.8 ± 3.9[**, c, e]
1.5	12	18.8 ± 0.5[**, a, b]	4.5 ± 0[**]	379.7 ± 9.7[**, d, e]

** Highly significantly different from control, $P < 0.0001$.
Pairs of values compared together indicated by same superscript.
a – e pairwise comparisons are significantly different, $P < 0.0001$.

Arsenic TC_{50} for sperm function seemed to be located between 1-1.5 mg/L. In the presence of 1-1.5 mg/L arsenic, the percentage of motile sperm was highly reduced and sperm exhibited half the value of the control group (57.8 ± 1.1% of the control were motile compared with 35.0 ± 4.0 - 18.8 ± 0.5% for 1-1.5 mg/L As concentration). In addition, the SMI value at 1-1.5 mg/L showed half the value of the control (920.0 ± 16.3 for control compared with 573.8 ± 3.9 for 1 mg/L As; 379.7 ± 9.7 for 1.5 mg/L As; Table XXVII).

Sperm Function in the Presence of Chromium

After 4 hr incubation of sperm in chromium containing medium, there was a dose-dependant reduction in SMI. All the Cr-treated groups showed significantly lower SMI compared with the control group. These lower SMI values resulted from the reduction in motility percentage and FPQ score (Table XXVIII). No significant differences were found between the control group and the other treated groups or between the treated groups with each other concerning FPQ score.

At 1.5 mg/L chromium concentration, only 23.0 ± 1.2% of sperm were motile compared with 57.8 ± 1.1% for control. Moreover, at 1.5 mg/L chromium concentration there was more than 50% reduction in SMI value compared with the control group (368.0 ± 19.6 versus 920.0 ± 16.3 respectively; Table XXVIII). Therefore, 1.5 mg/L Cr was considered as the TC_{50} for sperm.

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Table XXVIII. Analysis of sperm function 4 hr post-chromium treatment

Concentration [mg/L]	Number	Mobility percentage mean ± SEM	FPQ mean ± SEM	SMI mean ± SEM
Control	12	57.8 ± 1.1	4.0 ± 0	920.0 ± 16.3
0.5	12	43.0 ± 2.3[*, a]	3.5 ± 0.6	523.5 ± 58.4[**]
1.0	12	35.8 ± 3.8[**, b]	3.5 ± 0.6	426.3 ± 47.5[**]
1.5	12	23.0 ± 1.2[**, a, b]	4.0 ± 0	368.0 ± 19.6[**]

* Significantly different from control, $P < 0.05$.
** Highly significantly different from control, $P < 0.0001$.
Pairs of values compared together indicated by same superscript.
a pairwise comparison is highly significantly different, $P < 0.0001$.
b pairwise comparison is significantly different, $P < 0.05$.

Sperm Function in the Presence of Copper

Exposure of sperm to copper containing medium appeared to have a dramatic toxic effect on sperm function. The percentage of motile and FPQ score of the Cu-treated groups showed significantly lower values compared with the control group (Table XXIX).

Correspondingly, the SMI of Cu-treated groups was significantly reduced. Percentage of motile sperm, FPQ and SMI exhibited a concentration-dependant response to the presence of copper in the medium. A highly significant drop in all of the previously mentioned values was observed when copper concentration increased from 0.5 to 1.5 mg/L in the sperm medium, reaching the maximum reduction at 2.5 mg/L Cu, where only 0.6 ± 0.5% of the treated sperm were motile and expressing twitching without any forward progressive movement.

At 0.5 mg/L copper, SMI was about half of the control value (500.0 ± 43.4 versus 920.0 ± 16.3; Table XXIX). In addition, the FPQ score and sperm motility percentage exhibited significant reduction in their mean values compared with the control group. Therefore, 0.5 mg/L Cu was considered as the TC_{50} for sperm function.

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Table XXIX. Analysis of sperm function 4 hr post-copper treatment

Concentration [mg/L]	Number	Mobility percentage mean ± SEM	FPQ mean ± SEM	SMI mean ± SEM
Control	12	57.8 ± 1.1	4.0 ± 0	920.0 ± 16.3
0.5	12	47.3 ± 1.5**, a, b	3.3 ± 0.6c, d	500.0 ± 43.4**, e, f
1.5	12	3.8 ± 0.5**, a	1.9 ± 0.1**, c	10.8 ± 1.0**, e
2.5	12	0.6 ± 0.5**, b	0.9 ± 0.5**, d	1.3 ± 1.1**, f

** Highly significantly different from control, $P < 0.0001$.
Pairs of values compared together indicated by same superscript.
a – f pairwise comparisons are highly significantly different, $P < 0.0001$.
c pairwise comparison is significantly different, $P < 0.05$.

3.3.2 Evaluation of Sperm Viability

When mouse sperm were incubated for 5 min with propidium iodide, percentage of the cells (dead cells) incorporated the stain and were identified by fluorescent microscopy (Figure 19). Immotile dead sperm showed a bright red orange fluorescent head while the immotile viable one did not stain. The average sums of the percentage of immotile unstained viable sperm of control and treated groups were determined. The lower the value the more toxic the tested compound on sperm viability.

From the previous experiment's results, 0.5 mg/L appeared to be the lowest toxic-subtoxic concentration for all tested compounds on sperm function. In the presence of this concentration, sperm motility percentage, FPQ and SMI showed a slight reduction compared with the control group. Therefore, sperm viability was used as a second indicator to detect sperm toxicity at 0.5 mg/L concentration.

Sperm Viability in the Presence of Arsenic

Even though the presence of 0.5 mg/L arsenic in sperm medium trended to a lower but insignificant effect on SMI (Table XXVII) compared with control, viability of 0.5 mg/L As-treated sperm was greatly reduced. When sperm incubated with 0.5 mg/L As for 4 hr were stained with propidium iodide, the number of immotile unstained viable cells was significantly lower when compared with the control group ($P < 0.0001$) (6.4 ± 3.9% versus 22.5 ± 0.4% respectively; Table XXX). Correspondingly, the number of stained immotile non-viable cells was greater than the number of control stained cells (Figure 19A', A). This indicates that 0.5 mg/L arsenic has a spermicidal effect.

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Using Nomarski differential interference contrast optics, images of control and treated sperm were taken (Figure 19B, B'), and superimposed on the corresponding fluorescent images (Figure 19C, C'). Control superimposed images showed 3 out of 5 sperm were fluorescent in that field (Figure 19C) while sperm in the 0.5 As-treated groups were all fluorescent in that field (Figure 19C').

Table XXX. Sperm viability in the presence of arsenic

Concentration [mg/L]	Number	Mean ± SEM
Control	12	22.5 ± 0.4
0.5	12	6.4 ± 3.9**

** Highly significantly different from control, $P < 0.0001$.

Sperm Viability in the Presence of Chromium

Sperm viability was greatly affected by the presence of chromium in the medium. At 0.5 mg/L chromium, only 7.1 ± 2.0% of immotile sperm were viable compared with 22.5 ± 0.4% for the control (Table XXXI). Thus most (92.9%) of the immotile chromium-treated sperm were non-viable.

Table XXXI. Sperm viability in the presence of chromium

Concentration [mg/L]	Number	Mean ± SEM
Control	12	22.5 ± 0.4
0.5	12	7.1 ± 2.0**

** Highly significantly different from control, $P < 0.0001$.

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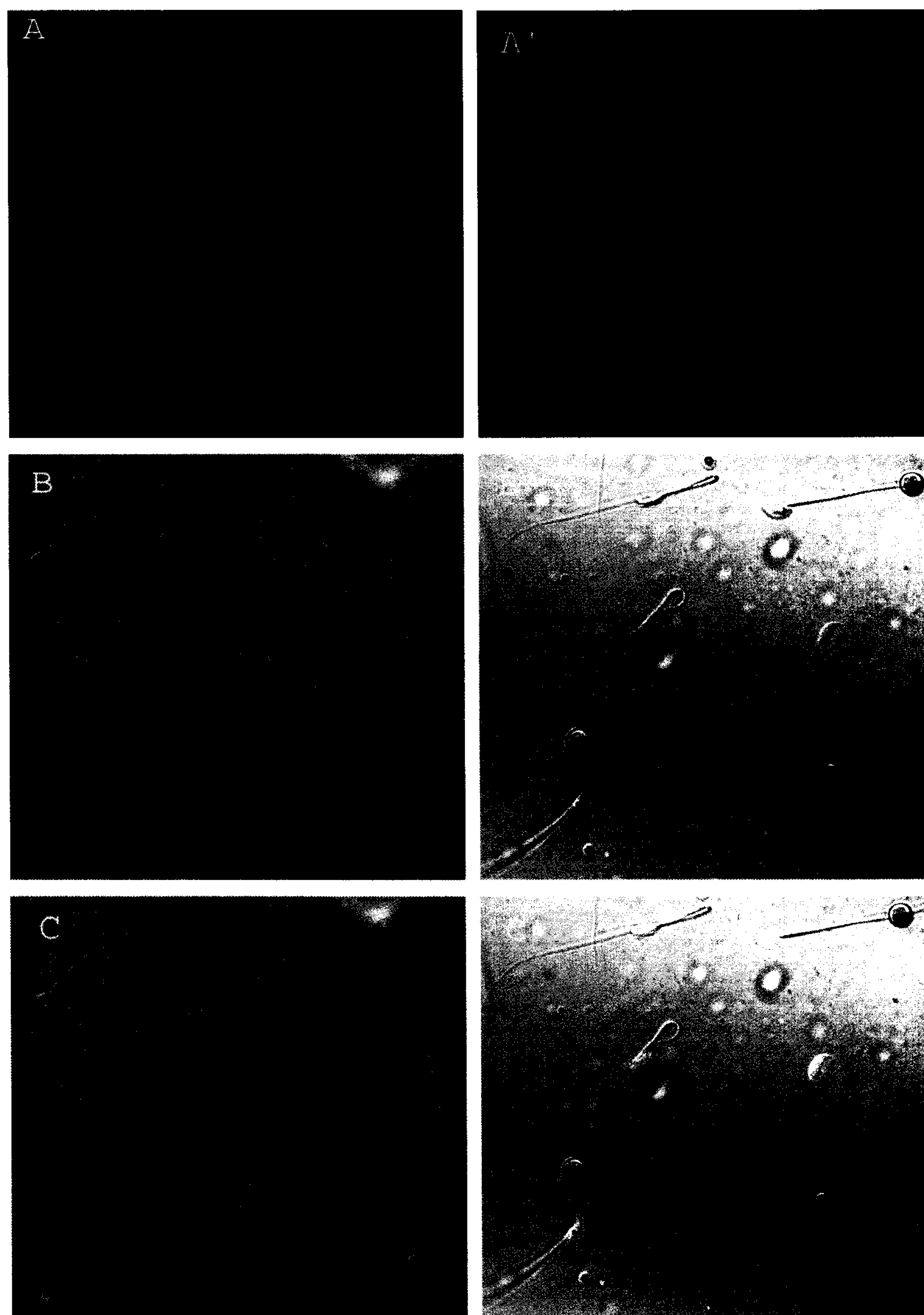

Figure 19. Viability of mouse sperm in the presence of arsenic. Left panel **(A, B, C)** represents control. Right Panel **(A', B', C')** represents 0.5 As-treated sperm. **(A, A')** Under fluorescent microscope. **(B, B')** Under Nomarski optics. **(C, C')** Superimposed images.

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Sperm Viability in the Presence of Copper

Sperm viability was significantly reduced when 0.5 mg/L copper was added to the sperm medium. Only 4.9 ± 2.2% of the immotile sperm were viable compared with 22.5 ± 0.4% for the control (Table XXXII). Therefore, 0.5 mg/L copper is a potent toxin for sperm viability.

Table XXXII. Sperm viability in the presence of copper

Concentration [mg/L]	Number	Mean ± SEM
Control	12	22.5 ± 0.4
0.5	12	4.9 ± 2.2**

**Highly significantly different from control, $P < 0.0001$.

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CHAPTER IV

DISCUSSION

A considerable degree of controversy has surrounded the safety of CCA-treated wood. This controversy has centered primarily on the toxicity of arsenic and chromium and the potential for these metals to be released from the wood. Several studies have reported the possibility of these toxic metals to leach from the wood and be bioavailable under certain circumstances.

CCA toxicity on the reproductive system may be demonstrated as abnormality in the gametes in the form of decrease in number or increase in abnormal morphology and/or abnormality in embryonic and fetal development. CCA compounds are found in many places around us. The resulting long-term exposure may cause chronic toxicity within sensitive reproductive developing organs. Both male and female gametes are very sensitive cells. Chronic toxicity could cause such severe changes that the damage to the germ cells could result in permanent decrease in number or increase in abnormal morphology. In addition, maternal exposure during the prenatal or natal period to the CCA compound and its individual components may have an irreversible toxic effect on embryo development and quality. The manifestation of CCA toxicity and its individual components may be noted as alternations in fertility (gamete effect) or pregnancy outcome (embryo effect).

To illustrate the validity of our hypothesis, explanations for the obtained results of each topic, their significance (in this particular study or other studies) and the potential postulated mechanisms will be the theme for this chapter.

4.1 Impacts on Embryo Development and Quality

The hypothesis to be tested was whether CCA components and their mixtures have deleterious effects on embryo development (growth and differentiation).

4.1.1 The Obtained Results

In vitro culture of two-cell mouse embryos has been used routinely as a quality control standard for media and supplements for human IVF. One of the driving forces for

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its usage until now is its sensitivity to detect toxins in the culture medium. The data presented in this thesis demonstrate a sensitive model for exploring metal embryotoxicity in early mammalian embryos and provide a system for direct comparison of the toxicities of various metals.

Arsenic

Arsenic often gets the most attention in any discussion of the hazards of CCA and pressure treated wood. Several studies have reported that prenatal arsenic-exposed laboratory animals produced birth defects and malformations in their offspring (Aschengrau et al., 1989; Shalat et al., 1996; Shephard, 1998). However, these results depend on route of administration (intravenous, intraperitonial or oral), timing of administration and the general conditions of the animal. All these factors were eliminated in this study with the usage of a whole embryo culture system.

In the present study, murine embryonic development in the presence of 0.5 mg/L or less arsenic concentration in the culture medium did not produce any significant difference compared with the control group. After 72 hr of culture, all 0.5 mg/L As-treated two-cell embryos reached the morula-blastocyst stage (Table III). Increasing arsenic concentration above 0.5 mg/L was accompanied by a dose-dependant decrease in embryonic development in the form of a drop in the number of morula-blastocyst embryos and elevation in fragmented-degenerated embryos. Arsenic concentration of 1.5 mg/L or more caused severe toxic lethal effect on embryo development with 100% lethality (Table III and Table A. 2). Between the previous nontoxic (0.5 mg/L or less) and highly toxic (1.5 mg/L or more) concentrations, TC_{50} appeared to be around 1.1 mg/L As.

Total cell numbers, an index of cell proliferation, have been used as a second parameter of embryo development and a good indicator of embryo quality. The embryo's total cell number was found to be a more sensitive parameter than blastocyst formation for determining a toxicity threshold concentration. Even though embryonic development after 72 hr of culture in the presence of 0.5 mg/L did not show any significant difference compared with control regarding the blastocyst formation rate or blastocyst morphology, morphologically normal blastocyst cultured in the presence of 0.5 mg/L As exhibited highly significantly lower total cell numbers compared with the control group (Table XII;

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Figure 6). Lower total cell numbers characteristic of delayed blastocyst development may be attributed to a slower proliferation in both trophectoderm cells (TE) and inner cell mass (ICM). Blastocysts with lower TE suggested that events associated with placental development and implantation may be greatly affected (Van Soom et al., 2001). Minimal numbers of ICM are required to obtain a pregnancy, blastocysts with lower ICMs are less viable, at the same time they are responsible for biochemical pregnancies where there is an initial increase in the levels of hCG but no fetal development. Whether these blastocysts with low cell numbers resulted from TE or ICM reduction is a topic that requires further investigation.

Finally, two main parameters should be considered. The first one relates to how the obtained results can be applied to determine arsenic toxicity on human preimplantation embryos. The National Academy of Science noted that humans may be even more sensitive to the adverse arsenic reproductive effect than the standard laboratory animals because rodents are more efficient methylators than humans and completely detoxify inorganic arsenicals (Hughes et al., 1994). Moreover, it was shown that humans are found to be more sensitive to arsenic than most laboratory animals by a factor of as much as 300 (ATDSR, 2000a). The second parameter relates to the form of arsenic in the CCA compound. Although the form of arsenic in CCA is pentavalent, the same form has been used in this study, more in vivo embryotoxicity is expected with the tested arsenic concentration because this pentavalent form will be reduced in vivo to the more toxic trivalent form, prior to metabolism by methylation (Machado et al., 1999). From all of the above, we conclude that embryonic exposure to any trace dose of arsenic may have a predominant embryotoxic effect that may be presented by embryo-fetal lethality, fetal anomalies or birth defects.

Chromium

Although the form of chromium in CCA is hexavalent (classified as type A human carcinogen; ATSDR, 2000b), less attention is paid to the chromium in CCA because the treatment process converts it to the more benign trivalent chromium. However, if during the pressure treating process the hexavalent chromium is not fixed completely and converted to the more benign trivalent form, it can leach out of the wood in its original form even if virtually all of the chromium is fixed.

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Several studies have demonstrated that chromium embryotoxicity and teratogenicity depend on the time, the dose and the route of administration (Gale, 1978; Iijima et al., 1979; Trivedi et al., 1989). Common features of chromium toxicity were embryotoxicity (embryocidal), fetotoxicity (reduced fetal weight and increase fetal anomalies), higher incidence of resorption and postimplantation loss. The mode of action was not explained in any of these previous studies. Only one study, using in vitro mammalian cell culture showed that chromium exposure increases oxidative stress and DNA breaks, with no decrease in cell viability, when the chromium concentration exceeded 20 mg/L (De Flora and Wetterhahn, 1989).

The results presented in this thesis were able to mix and reshape the previous information to obtain a final clear conclusion regarding chromium toxicity. In a nutshell, 0.5 mg/L of hexavalent chromium appeared to have no effect on cell viability because embryonic development in its presence did not show any significant difference from control in embryo cultured media. However, their total cell numbers were much lower compared to control. Reduction in the total embryonic cell numbers especially in TE cells may explain the higher incidence of resorption and postimplantation loss with chromium exposure. Moreover, fetotoxicity in the form of reduced fetal weight and increase fetal anomalies can be explained if the reduction in the total cell numbers was localized to the ICM. One other outcome of the performed experiment is that, chromium embryocidal effect appeared if its concentration exceeded 1.9 mg/L and reached maximum at 3 mg/L Cr. The difference in the toxic chromium concentration in this study (1.1 mg/L) from the previous reported information (20 mg/L) indicates the sensitivity of the mouse embryo culturing system in detecting a toxin in the culture medium compared with other mammalian cell cultures.

Copper

Embryonic development and blastocyst formation rate did not exhibit any changes with exposure to 5 mg/L or less copper (Table A.6). However, both of them showed marked reduction if copper concentration exceeded 7 mg/L. Copper toxicity may be through inhibition of embryonic division at 7 mg/L Cu, then by increasing embryo lethality (embryocidal effect) at 8 mg/L or more. Surprisingly, at 0.5 mg/L Cu, exposed blastocyst had poor quality with low total cell numbers. Even though, 0.5 mg/L copper

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did not show any harmful effects on embryonic development, blastocyst quality was poor.

CCA Compound

The combination of known toxins and carcinogens into a single chemical formula may result in an even stronger toxin, carcinogen and teratogen. CCA may present a greater acute toxicity hazard than exposure to its constituent compounds separately, particularly if the dose of at least one of the compounds is at a level which would be toxic if administrated alone (Mason and Edwards, 1989). Although, our study confirmed the previous conclusion, we proved here that even mixing them in their subtoxic doses or even in nontoxic doses (the dose of each one at the level that would not be toxic if ~2-3 fold of the compound was administered alone) appeared to having very high embryotoxicity. The only CCA mixture that did not show toxic effects on embryo development and blastocyst formation rate was one-tenth the nontoxic-subtoxic concentration of each compound. However, most of these blastocysts were morphologically abnormal and were characterized by one or more of the following features: unincorporated blastomeres, multiple cavities (possibly intracellular vacuoles), no discrete ICM visible or low numbers of mural TE cells.

From the above discussion, we conclude that embryonic exposure to arsenic, chromium and copper concentrations of even 0.5 mg/L, appeared to have a bad toxic effect on embryo quality with no obvious reduction in embryonic developmental stages. Combining these individually-tested compounds, in a CCA mixture, produced a synergistic embryotoxic effect.

4.1.2 Expected Mechanisms.

Numerous mechanisms may help to explain the toxicity of each metal tested in this study on embryo development. For examples, oxidative damage to cells may be associated with each of the compounds tested here, with the damage occurring through either direct oxidative interactions (arsenic, chromium) or activation of reactive oxygen species through secondary mechanisms (copper). Additionally, metalic interactions with DNA, either through direct metal complexion (as in arsenic which bind to sulphhydryl groups of histones and nucleic acid) or metal-catalyzed oxidative damage (as in

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chromium and copper), may be another route for embryotoxicity. Moreover, arsenic impairs assembly and disassembly of microtubules, presumably by binding to protein sulphhydryl groups, and thus may interfere with the mitotic spindle formation and embryonal cell division (Leonard and Lauwerys, 1980). Such actions could interfere with embryonic cell proliferation and/or differentiation.

4.2 Impacts on Oocyte Maturation and Spindle-Chromosome Behavior

The hypothesis to be tested was whether CCA components have toxicological effects on oocyte maturation and integrity.

4.2.1 The Obtained Results

In mitosis, checkpoints exist which sense the integrity of the mitotic spindle at metaphase (Hartwell and Weinert, 1989). There appear to be similar checkpoints at the first meiotic metaphase of mouse oogenesis, one that monitors spindle integrity and one activated by drugs interfering with actin polymerization (Wassarman et al., 1976). Several studies concluded that the potential of a drug to induce abnormalities in mammalian oocytes is related to its mode of activity by either inducing damage, which triggers meiotic arrest, or affecting checkpoint controls. Cytochalasin D and chloral hydrate appear to belong to the former class (Eichenlaub-Ritter and Betzendahl, 1995). In contrast, cycloheximide and diazepam (Soewarto et al., 1995) as well as maternal age (Liu and Keefe, 2002) seem to have the opposite activity in promoting or delaying meiosis while checkpoints become leaky.

The present study shows unambiguously that a trace amount of arsenic, chromium or copper inhibits maturation of mouse oocytes. The metal compounds block some oocytes in GV stage but predominantly delay the cell-cycle progression and arrest oocytes irreversibly in meiosis. This meiotic arrest became more potent with the increase of metal compound concentration in culture medium reaching TC_{50} at 0.7 mg/L arsenic, 0.1-0.5 mg/L chromium and 0.1 mg/L copper (Table XV; XVI; XVII). Oocyte maturation appeared to be inhibited in the presence of low concentrations of arsenic, chromium or copper. The higher the metal concentration, the greater the number of arrested MI oocytes. At low tested concentrations, a higher percentage of oocytes were able to

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mature to MII compared with the higher concentrations. However, spindle aberration and chromosome misalignment of these MII oocytes did not show any significant difference compared with the other MII treated oocytes. Indicating that low concentrations of the tested compounds may affect the metaphase/anaphase checkpoint, allowing oocytes with aberrant spindles and misaligned chromosomes to progress to MII. Studies of oocytes from XO females indicate that chromosome misalignment at metaphase does not induce a delay in anaphase onset (Le Marie-Adkins et al., 1997). Despite an apparent delay in the congression of the X chromosome during prometaphase and a significant proportion of metaphase cells with a misaligned X chromosome, no evidence of accumulation of cells at metaphase I among oocytes from XO females was found.

Given the role of the metaphase/anaphase checkpoint in ensuring proper chromosome segregation, the lack of this important control mechanism during female meiosis is surprising. In fact, the most plausible explanation (for our results and the other study result) was that the cell cycle control machinery does indeed exist, but is operationally impaired because of the excessive volume of the mammalian oocyte (LeMarie-Adkins et al., 1997). Moreover, it becomes more leaky with maternal age or chemical exposure (Liu and Keefe, 2002; Soewarto et al., 1995).

The obtained results in this thesis, suggested that there is a meiosis checkpoint control. This checkpoint became more permissive with chemical exposure (especially at lower concentration) and thereby allowed the meiotically incompetent oocytes to resume maturation and to progress from MI to MII regardless chromosome misalignment. Most control MII oocytes had normal bipolar spindles with well aligned chromosomes in equator. Apparently, most of the abnormality in spindles and chromosomes of control groups localized to MI arrested oocytes. Aberrant spindles were found in 56.3, 40.6 and 40.6% of MI oocytes compared with 5.3, 14.7 and 14.7% in MII oocytes. In addition, unaligned chromosomes were represented in 18.8, 34.4 and 34.4% of MI oocytes compared with 4, 4.3 and 4.3%. However, this was not the case of metaphase II oocytes which escaped the As, Cr or Cu –induced meiosis I arrest. Most of these MII oocytes have aberrant spindles with displaced chromosomes. This was only seen at lower concentration of the tested compounds. However, at higher dose all/most of the oocytes failed to progress to MII.

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4.2.2 Expected Mechanisms

Numerous mechanisms may help to explain the disturbance in spindle organization of each metal tested in this study. Direct effect on the integrity, function and positioning of centrosomes deduced from the asymmetric alignment of microtubule organizing centers (MTOCs) in the first meiosis and the fusiform shapes of the poles of the treated oocytes is one mechanism. Oocytes possess multiple MTOCs rather than pairs of centrioles at their spindle poles. These MTOCs are essential for the formation of the spindle and proved to be very sensitive for the presence of many drugs. MTOCs acquire components such as γ- tubulin, centrin, cell-cycle regulating enzymes and other proteins (Eichenlaub-Ritter and Betzendahl, 1995) at or before the transition to metaphase.

Tested compounds- As_2O_5, CrO_3, and CuO, may interfere with these processes and therefore lead to spindle abnormalities which trigger meiosis I arrest. Furthermore, metal interactions with mitochondria either through direct metal complexion (as in arsenic which substitutes phosphorous in mitochondria) or metal-catalyzed oxidative stress (as in arsenic, chromium and copper) may be another mechanism for spindle aberrations. Oxidative stress damages proteins that may involve microtubules and small molecules important for spindle organization. Lastly, arsenic, chromium and copper compounds may have a triggering action on the metaphase I checkpoint judging from the potent and irreversible arrest at MI.

Chromosome displacements were a common feature in all MI-arrested treated oocytes even at lower concentration (Table XIX; XXII; XXV). Many factors may be linked to this event. Mitochondrial dysfunction and deficiencies in energy production (arsenic uncouples oxidative phosphorylation and diminishes ATP production; Shalat et al., 1995) could indirectly influence microtubule turnover and the activity of motor proteins. This could disrupt microtubule motor proteins involved in assembling of the chromosomes at the metaphase plate, tethering the kinetochore to spindle microtubules (Wood et al., 1997) and triggering the spindle checkpoint in the response to the status of chromosome alignment (Chan et al., 1998). This explains the frequent disturbed alignment of chromosomes at the equator. On the other hand, oxidative stress has been publicized to induce disturbances in chromosomal distribution in mouse oocytes (Liu and Keefe, 2002). Arsenic, chromium and copper compounds toxicity have been related to an

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increase in the oxidative stress in the exposed cells (Huang and Lee, 1996; De Flora and Wetterhahn, 1989; Bremner, 1998). Oxidative stress may directly involve DNA and chromosomal damage and/or indirectly damage the chromosome or kinetochore associated proteins ending with chromosomal displacement.

The fidelity of cell division is ensured by checkpoint mechanisms assuring that strategic events occurring during one phase of the cell cycle are completed before the next phase of the cycle is initiated. One such mechanism operates at metaphase/anaphase transition to delay the onset of anaphase in the cells with defective spindle formation or chromosome misalignment. The imposed delay provides an opportunity for the correction of defects that would predispose to errors in chromosome segregation at anaphase. Meiotic segregation errors have disastrous consequences, since they result in the production of abnormal gametes. Thus, the metaphase/anaphase checkpoint would be expected to be of particular importance during meiotic cell division.

4.3 Impacts on Sperm Function and Viability

The hypothesis to be tested was whether CCA components have deleterious effects on sperm function.

4.3.1 The Obtained Results

All the tested compounds had an inhibitory effect on sperm motility and SMI. In our experiments, all the treated sperm, except those arsenic-treated, exhibited a great reduction in forward progressive movement compared with the control group. This result was expected as the reduction in motility percentage is usually accompanied with reduction in FPQ. However, this was not the scenario in arsenic-treated sperm. Although, there was a reduction in sperm motility percentage, 50% of sperm exhibited grade 4 FPQ and the remaining showed hyperactivation after 3-4 hr incubation and even persist at 6 hr. The tested compound not only affected sperm motility but also affected sperm viability. Almost all immotile sperm were non-viable even at the lower tested concentration (0.5 mg/L) indicating that, arsenic, chromium and copper are very potent spermicidal.

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Copper is considered the least problematic metal in the CCA compound (Fields, 2001). In this study, copper appeared to have the least toxic effect on embryos. However, this was not the case for sperm function. Copper exhibited the most detrimental effect on sperm function and viability compared with arsenic and chromium. Copper intrauterine devices (IUD) prove to be a highly effective method of contraception through affecting sperm motility and survival compared with non-medicated IUD. The amount of the copper released from IUD in vivo was around 11.4 ± 4.7 μg/mL (11.4 ± 4.7 mg/L) and varied according to the age of the device and the uterine stage (Arancibia et al., 2003). Results showed that copper, at 100 μg/mL (100 mg/L) concentrations, caused significant decreased in sperm motility, viability and acrosomal reaction in vitro (Roblero et al., 1996). Our study found that the presence of even 0.5 mg/L of copper in sperm medium greatly affected sperm motility and viability. The difference in our values and the previous reported toxic copper values may be related to the absence of protein from our medium. On the other hand, most of the copper in the previous studies was in the form of complexes with protein.

4.3.2 Expected Mechanisms

Sperm toxicity may be related to many factors, however the most common one among them was reactive oxygen species (ROS). Like all cells living under aerobic conditions, spermatozoa produce ROS during normal metabolic activity. At the same time, spermatozoa and seminal plasma contain a battery of ROS scavengers, including many enzymes such as superoxide dismutase (SOD), catalase and the glutathione (GSH) peroxidase/reductase system (De Lamirande et al., 1997). The balance between ROS generation and scavenging is very important. Excessive ROS generation that overcomes the scavenging ability was found to be related to male infertility (Iwasaki and Gagnon, 1992). Although several studies demonstrated that high concentrations of ROS induce sperm pathology (ATP depletion leading to insufficient axonemal phosphorylation, lipid peroxidation and loss of motility and viability (Jones et al., 1979), low and controlled concentrations of ROS have an important role in sperm physiology (capacitation). This means that fine balance between ROS production and scavenging is important for sperm function and fertilization ability. Therefore, sperm motility reduction of the tested compound may be related to the increase of ROS concentration (oxidative stress) in the

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culture medium. This may be directly through increasing ROS production (as in arsenic, chromium and copper which produce oxyredicals) or indirectly through decreasing ROS destruction through inhibiting scavenging enzymes (arsenic reduces cytosolic glutathione level).

Mammalian spermatozoa are rich in polyunsaturated fatty acids and are thus very susceptible to ROS attack. Such an attack results in decreased sperm motility, presumably by a rapid loss of intracellular ATP leading to axonemal damage and loss of viability in addition to the decreased motility. Lipid peroxidation of sperm membranes is considered to be the key mechanism of this ROS-induced sperm damage (Alvarez et al., 1987). Lipid peroxidation (LPO) is the most extensively studied manifestation of oxygen activation in biology. The most common types of LPO are: (a) nonenzymatic membrane LPO, and (b) enzymatic (NADPH and ADP dependent) LPO. The enzymatic reaction involves NADPH-cytochrome P-450 reductase (Ernster, 1993).

In general, the most significant effect of LPO in all cells is the perturbation of membrane (cellular and organellar) structure and function (transport processes, maintenance of ion and metabolite gradients, receptor mediated signal transduction, etc.). Besides membrane effects, LPO can damage DNA and proteins, either through oxidation of DNA bases (primarily guanine via lipid peroxyl or alkoxyl radicals) or through covalent binding to malondialdehyde (MDA) resulting in strand breaks and cross-linking (Ernster, 1993). Sperm DNA is vulnerable to oxidative stress, in part because semen has a weak antioxidant system (Zini et al., 1993) and in part because spermatozoa lack DNA repair enzyme activity (Matsuda et al., 1989).

Hyperactivation in arsenic-treated sperm may be related to increased calcium concentration at their axoneme. A number of physiological factors, such as Ca^{2+}, cAMP, bicarbonate and metabolic substrate, are essential for initiation and maintenance of hyperactivated motility in vitro. It appeared that Ca^{2+} played a major role in regulating hyperactivation. The crucial site for the action of Ca^{2+} is the axoneme. The increase in the axoneme Ca^{2+} whether from extracellular or intracellular sources is required for hyperactivation (Ho and Suarez, 2001). . A rise in intracellular calcium was elicited at once after application of As_2O_3 to esophageal carcinoma cells (Shen et al., 2002). Although, the mechanism of how arsenic increases intracellular calcium levels was not

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clear, many explanations were given. One of them, is that arsenic has been shown to disrupt mitochondria and may elevate intracellular calcium via a signal transduction pathway. Arsenite has also been reported to activate protein kinase C and mitogen-activated protein kinase. These kinases are known to be involved in the calcium signal transduction pathway (Jun et al., 1999).

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CHAPTER V

CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

Deterioration in embryonic and gamete development and quality in the presence of CCA's individual components (arsenic pentaoxide, chromium trioxide and cupric oxide) and their mixtures was the hypothesis for this research.

The first aim was to determine the effect of CCA (single, mixtures and synergism-potentiation) on embryo development. We found that embryonic exposure to arsenic, chromium and copper concentrations of even 0.5 mg/L, have a detrimental toxic effect on embryo quality represented by significant reduction in total cell numbers although there was no obvious reduction in embryonic developmental stages. Increasing concentration above 0.5 mg/L was accompanied by a dose-dependant decrease in embryonic development in the form of drop in the number of morula-blastocyst embryos and elevation in fragmented-degenerated embryos. TC_{50} was determined to be at 0.7 mg/L arsenic, 0.1-0.5 mg/L chromium and 0.1 mg/L copper. To determine the synergistic effect of CCA compound on embryo development, our findings indicated that combining these individually-tested compounds, in m-CCA even in their nontoxic concentrations, produce a synergistic embryotoxic effect. The only CCA mixture that did not show toxic effects on embryo development and blastocyst formation rate was one-tenth the nontoxic concentration of each compound. However, most of these blastocysts were morphologically abnormal.

The second aim was to detect the developmental and genetic effect of CCA components on in vitro matured oocytes. Arsenic, chromium and copper were found to induce spindle-chromosomal damage to mammalian oocytes that triggered meiotic arrest at MI and affected checkpoint controls at low concentrations. These compounds become potent meiotic arresters and fertility reducing chemicals at high concentrations. TC_{50} is 0.7 mg/L arsenic, 0.1-0.5 mg/L chromium and 0.1 mg/L copper. The lack of chromosome-mediated checkpoint control imposed by the metaphase/anaphase

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checkpoint would be predicted to result in an elevated meiotic error rate in female meiosis. In our own species, this is indeed the case. An estimated 20% of human conceptions are chromosomally abnormal and virtually all errors are maternal in origin (Jacobs, 1992). This overstresses the toxicity of CCA and its metal compounds. Exposure to these metal compounds not only will affect oocyte maturation and reduce the fertility but also may increase the teratogenicity if these abnormal MII oocytes succeed to ovulate and be fertilized.

The third aim was to assess sperm function and viability in the presence of CCA components. The tested compounds not only have an inhibitory effect on sperm motility but also have deleterious effects on sperm viability. TC_{50} is 1-1.5 mg/L arsenic, 1.5 mg/L chromium and 0.5 mg/L copper. Almost all immotile sperm were non-viable even at the lower tested concentration (0.5 mg/L) indicating that arsenic, chromium and copper are very potent spermicidal.

We conclude that the presence of arsenic, chromium or copper compounds in embryo and gamete environmental medium have a severe detrimental effect on their development and quality. Even though small amounts of these metal compounds did not show an obvious toxic effect on embryo and gamete development, greater reduction in their quality and subcellular structure damage was observed. Consequently, it is recommended to avoid any unnecessary exposure to arsenic, chromium and copper compound.

The main contribution of this study is that it may have a significant impact on reproductive biology-toxicology of gametes and embryos.

5.2 Future Work

The above study is one step toward detecting the behavior of embryo and gametes development and function in the presence of individual components of the CCA compound. Like many research efforts, the doors are opened for further improvements and extensions. In this section, we briefly explain some possible extensions to each proposed experiments.

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5.2.1 Embryo Development

- Total cell count of blastocyst embryos was used in this study to detect the difference in embryo quality between the treated and untreated embryos. As there was obvious significant reduction in total cell numbers, differential staining of inner cell mass and trophectoderm cells can be used to allocate the reduction.
- In addition, assessment of blastomere viability using fluorescent markers of cell viability may help in ascertaining embryo viability.
- To detect whether CCA toxicity on embryo development is mediated through interfering on the mitotic spindle formation and embryonal cell division and / or through affecting on mitochondrial function two tests can be performed:
  - Immunocytochemical staining methods may be used for labeling of nuclei and cytoskeleton of preimplantation embryo.
  - Ratiometric confocal microscopy with the mitochondrion-specific membrane potential-sensitive fluorescence dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide) may be used to measure the membrane potential and hence the activity of mitochondria in preimplantation embryos.

5.2.2 Oocyte Integrity

- Although, chromosomal behavior of treated oocytes used indirect antitubulin immunofluorescence was accurate and efficient, chromosomal analysis using combined fluorescence in-situ hybridization (FISH) with an X chromosome-specific probe may localize the toxicity of the tested compound.
- Detecting the possibility of the tested compounds to cause aneuploidy in the exposed oocytes could be determined using C-banding.
- Redundancy in cell cycle checkpoint especially at low tested compound concentrations is a disastrous. Metaphase II treated oocytes that escaped the meiotic arrest may either fail to ovulate (causing infertility) or develop to embryo if they were fertilized (causing teratogenic effect). Correspondingly, detecting their ability to be fertilized (through IVF) will be another topic for further research.

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- By taking advantage of Pol-Scope's ability non-invasively to observe spindle and chromosome structures in the living oocytes using polarized light, Pol-Scope may be used for selection of healthy looking treated oocytes before doing IVF.

5.2.3 Sperm Function

- There is growing evidence that oxidative stress significantly impairs sperm function, and plays a major role in the etiology of defective sperm function. The measurement of the rate of ROS generation by luminol-induced chemiluminescence has been the most common method for quantifying ROS. Other methods that can be used for measuring ROS can be categorized as:
    - Methods involving nitroblue tetrazolium (NBT) or cytochrome c-Fe^{+++} complexes that measure ROS on the cell membrane surface.
    - Methods that measure ROS (generated inside or outside the cell) utilizing luminol-dependent chemiluminescence.
- The electron spin resonance methods are more sensitive and can identify the type of ROS generated inside the cell. These methods are considered a new research direction that needs further investigation.
- Spermatozoa, unlike other cells, are unique in structure, function, and susceptibility to damage by LPO. In spermatozoa, production of malondialdehyde (MDA), an end product of LPO induced by ferrous ion promoters, is a simple and useful diagnostic tool for the measurement of LPO for in vitro systems.
- Since any changes in mitochondrial function may be reflected in sperm motility, evaluation of mitochondrial function using JC-1 staining may be a useful tool to assess the mechanism of sperm toxicity.

5.2.4 Others

- One of the mechanisms of substance toxicity is through reducing the cytosolic glutathione level. GSH assay (Anderson, 1989), a colorimetric assay may be carried out to quantify intracellular level of GSH and determine the embryo and gamete toxicity in the presence of CCA.
- Mitochondria are intracellular organelles, existing in eukaryotic cells, responsible for production of ATP, intracellular calcium regulation, and reactive oxygen species

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generation and are involved in apoptosis and cell death. These organelles are extremely sensitive to minimal variations of cellular physiology. Small changes in pH, cellular oxygenation and the presence of several substances may interfere in mitochondrial respiratory metabolism. Arsenic, chromium and copper may be one of these substances. Based on that, mitochondrial respiration (measured polarographically with a Clarktype oxygen electrode, Rodrigues et al., 2003) can be used as a sensitive parameter to evaluate the biocompatibility of arsenic, chromium and copper exposed cells (embryos and gametes).

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APPENDIX A

RAW DATA FOR EMBYRONIC DEVELOPMENT

Table A.1. Analysis of embryonic stage of two-cell mouse embryos after 48 hr culture in the presence of arsenic

As_2O_5 [mg/L]	Embryo number	2 cell	3-4 cell	5-8 cell	Morula	Blastocyst	Fragmented-Degenerated
Control	30	0 (0)	0 (0)	0 (0)	6 (20)	24 (80)	0 (0)
0.1	30	0 (0)	0 (0)	1 (3.3)	4 (13.4)	24 (80)	1 (3.3)
0.3	30	0 (0)	0 (0)	0 (0)	4 (13.4)	25 (0)	1 (3.3)
0.5	30	0 (0)	0 (0)	0 (0)	7 (23.3)	23 (76.7)	0 (0)
0.7	30	0 (0)	3 (10)	7 (23.3)	9 (30)	10 (33.4)	1 (3.3)
0.9	30	2 (6.7)	4 (13.4)	5 (16.4)	15 (50)	4 (13.4)	0 (0)
1.1	30	3 (10)	12 (40)	5 (16.7)	7 (23.3)	2 (6.7)	1 (3.3)
1.3	30	12 (40)	15 (50)	1 (3.3)	0 (0)	1 (3.3)	1 (3.3)
1.5	30	20 (66.7)	10 (33.4)	0 (0)	0 (0)	0 (0)	0 (0)

Table A.2. Analysis of embryonic stage of two-cell mouse embryos after 72 hr culture in the presence of arsenic

As_2O_5 [mg/L]	Embryo number	2 cell	3-4 cell	5-8 cell	Morula	Blastocyst	Fragmented-Degenerated
Control	30	0 (0)	0 (0)	0 (0)	0 (0)	30 (100)	0 (0)
0.1	30	0 (0)	0 (0)	0 (0)	0 (0)	28 (93.3)	2 (6.7)
0.3	30	0 (0)	0 (0)	0 (0)	1 (3.3)	29 (96.7)	0 (0)
0.5	30	0 (0)	0 (0)	0 (0)	2 (6.7)	28 (93.3)	0 (0)
0.7	30	0 (0)	0 (0)	0 (0)	0 (0)	19 (63.3)	11 (36.7)
0.9	30	0 (0)	0 (0)	0 (0)	0 (0)	19 (63.3)	11 (36.7)
1.1	30	0 (0)	0 (0)	0 (0)	0 (0)	11 (36.7)	19 (63.3)
1.3	30	0 (0)	0 (0)	0 (0)	0 (0)	2 (6.7)	28 (93.3)
1.5	30	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	30 (100)

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Table A.3. Analysis of embryonic stage of two-cell mouse embryos after 48 hr culture in the presence of chromium

CrO_3 [mg/L]	Embryo number	2 cell	3-4 cell	5-8 cell	Morula	Blastocyst	Fragmented-Degenerated
Control	50	1 (2)	1(2)	0 (0)	37 (74)	5 (10)	6 (12)
0.1	10	0 (0)	0 (0)	0 (0)	7 (70)	3 (30)	0 (0)
0.2	18	0 (0)	1 (5.6)	2 (11.1)	15 (83.3)	0 (0)	0 (0)
0.3	21	1 (4.8)	2 (9.5)	2 (9.5)	13 (61.9)	2 (9.5)	1 (4.8)
0.5	18	0 (0)	2 (11.1)	0 (0)	14 (77.8)	0 (0)	2 (11.1)
0.7	22	1 (4.5)	0 (0)	0 (0)	19 (86.4)	2 (9.1)	0 (0)
0.9	21	0 (0)	0 (0)	1 (4.8)	18 (85.7)	2 (9.5)	0 (0)
1.1	25	0 (0)	1 (4)	1 (4)	21 (84)	2 (8)	0 (0)
1.2	17	1 (5.9)	2 (11.8)	0 (0)	13 (76.5)	1 (5.9)	0 (0)
1.5	34	0 (0)	6 (17.6)	1 (2.9)	24 (70.6)	2 (5.9)	1 (2.9)
1.7	17	0 (0)	5 (29.4)	1 (5.9)	11 (64.7)	0 (0)	0 (0)
1.9	6	0 (0)	2 (33.3)	0 (0)	4 (66.7)	0 (0)	0 (0)
2.0	10	2 (20)	2 (20)	2 (20)	4 (40)	0 (0)	0 (0)
2.5	7	0 (0)	4 (57.1)	0 (0)	1 (14.3)	0 (0)	2 (28.6)
3.0	9	1 (11.1)	1 (11.1)	6 (66.7)	0 (0)	0 (0)	1 (11.1)

Table A.4. Analysis of embryonic stage of two-cell mouse embryos after 72 hr culture in the presence of chromium

CrO_3 [mg/L]	Embryo number	2 cell	3-4 cell	5-8 cell	Morula	Blastocyst	Fragmented-Degenerated
Control	50	1 (2)	1 (2)	0 (0)	1 (2)	40 (80)	7 (14)
0.1	10	0 (0)	0 (0)	0 (0)	1 (10)	9 (90)	0 (0)
0.2	18	0 (0)	1 (5.6)	0 (0)	0 (0)	17 (94.4)	0 (0)
0.3	21	1 (4.8)	2 (9.5)	0 (0)	2 (9.5)	15 (71.4)	1 (4.8)
0.5	18	0 (0)	0 (0)	0 (0)	2 (11.1)	14 (77.8)	2 (11.1)
0.7	22	1 (4.5)	0 (0)	0 (0)	0 (0)	21 (95.5)	0 (0)
0.9	21	0 (0)	0 (0)	0 (0)	0 (0)	21 (100)	0 (0)
1.0	25	0 (0)	1 (4)	0 (0)	1 (4)	23 (92)	0 (0)
1.1	17	1 (5.9)	2 (11.8)	0 (0)	1 (5.9)	13 (76.5)	0 (0)
1.5	34	1 (2.9)	5 (14.7)	3 (8.8)	2 (5.9)	22 (64.7)	1 (2.9)
1.7	17	0 (0)	0 (0)	3 (17.6)	4 (23.5)	8 (47.1)	2 (11.8)
1.9	6	0 (0)	0 (0)	1 (16.7)	1 (16.7)	3 (50)	1 (16.7)
2.0	10	2 (20)	3 (30)	0 (0)	1 (10)	0 (0)	4 (40)
2.5	7	0 (0)	4 (57.1)	0 (0)	0 (0)	1 (14.3)	2 (28.6)
3.0	9	1 (11.1)	1 (11.1)	1 (11.1)	0 (0)	0 (0)	6 (66.7)

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Table A.5. Analysis of embryonic stage of two-cell mouse embryos after 48 hr culture in the presence of copper

CuO [mg/L]	Embryo number	2 cell	3-4 cell	5-8 cell	Morula	Blastocyst	Fragmented-Degenerated
Control	28	0 (0)	0 (0)	0 (0)	17 (60.7)	10 (35.7)	1 (3.6)
0.1	11	0 (0)	1 (9.1)	0 (0)	1 (9.1)	9 (81.8)	0 (0)
1.0	16	0 (0)	2 (12.5)	0 (0)	2 (12.5)	12 (75)	0 (0)
5.0	30	0 (0)	0 (0)	0 (0)	29 (96.7)	0 (0)	1(3.3)
6.0	20	0 (0)	0 (0)	0 (0)	18 (90)	0 (0)	2 (10)
7.0	22	2 (9.1)	16 (72.7)	1 (4.6)	3 (13.6)	0 (0)	0 (0)
8.0	19	6 (31.6)	13 (68.4)	0 (0)	0 (0)	0 (0)	0 (0)
9.0	21	5 (23.8)	9 (42.9)	0 (0)	0 (0)	0 (0)	7 (33.3)
10.0	10	2 (20)	1 (10)	0 (0)	0 (0)	0 (0)	7 (70)

Table A.6. Analysis of embryonic stage of two-cell mouse embryos after 72 hr culture in the presence of copper

CuO [mg/L]	Embryo number	2 cell	3-4 cell	5-8 cell	Morula	Blastocyst	Fragmented-Degenerated
Control	28	0 (0)	0 (0)	0 (0)	2 (7.1)	24 (85.7)	2 (7.1)
0.1	11	0 (0)	1 (9.1)	0 (0)	0 (0)	10 (90.1)	0 (0)
1.0	16	0 (0)	0 (0)	1 (6.2)	0 (0)	15 (93.8)	0 (0)
5.0	30	0 (0)	0 (0)	0 (0)	1 (3.3)	28 (93.3)	1 (3.3)
6.0	20	0 (0)	0 (0)	0 (0)	3 (15)	14 (70)	3 (15)
7.0	22	1 (4.6)	10 (45.5)	0 (0)	1 (4.6)	2 (9.1)	8 (36.4)
8.0	19	2 (10.5)	0 (0)	0 (0)	0 (0)	0 (0)	17 (89.5)
9.0	21	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	21 (100)
10.0	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	10 (100)

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Table A.7. Analysis of embryonic stage of two-cell mouse embryos after 48 hr culture in the presence of CCA mixture

CCA [mg/L]	Embryo number	2 cell	3-4 cell	5-8 cell	Morula	Blastocyst	Fragmented-Degenerated
Control	50	3 (6)	0 (0)	2 (4)	43 (86)	0 (0)	2 (4)
m-CCA1	82	4 (4.9)	44 (53.7)	18 (21.9)	16 (19.5)	0 (0)	0 (0)
m-CCA2	41	7 (17.1)	18 (43.9)	6 (14.6)	2 (4.9)	0 (0)	8 (19.5)
m-CCA3	43	2 (4.7)	16 (37.2)	6 (13.9)	19 (44.2)	0 (0)	0 (0)
m-CCA4	41	7 (17.1)	4 (9.8)	0 (0)	30 (73.1)	0 (0)	0 (0)
m-CCA5	41	0 (0)	0 (0)	0 (0)	1 (2.4)	39 (95.2)	1 (2.4)

The m-CCAi is a chromated copper arsenate mixture arranged from highest to lowest concentration.

Table A.8. Analysis of embryonic stage of two-cell mouse embryos after 72 hr culture in the presence of CCA mixture

CCA [mg/L]	Embryo number	2 cell	3-4 cell	5-8 cell	Morula	Blastocyst	Fragmented-Degenerated
Control	50	0 (0)	0 (0)	0 (0)	0 (0)	45 (90)	5 (10)
m-CCA1	82	0 (0)	7 (8.5)	3 (3.7)	4 (4.9)	0 (0)	68 (82.9)
m-CCA2	41	0 (0)	3 (7.3)	3 (7.3)	0 (0)	2 (4.9)	33 (80.5)
m-CCA3	43	2 (4.7)	2 (4.7)	0 (0)	2 (4.7)	4 (9.3)	33 (76.7)
m-CCA4	41	0 (0)	0 (0)	1 (2.4)	4 (9.8)	8 (19.5)	28 (68.3)
m-CCA5	41	0 (0)	0 (0)	0 (0)	0 (0)	40 (97.6)	1 (2.4)

The m-CCAi is a chromated copper arsenate mixture arranged from highest to lowest concentration.

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VITA

Nervana Talaat-Elsebaei Mahmoud was born in Zagazig, Sharkia, Egypt in 1973. She received here Medical Bachelor and Bachelor of Surgery from Zagazig University, Zagazig, Egypt, in December 1997. Then, she was appointed as an intern at Zagazig University Hospitals and Ministry of Public Health Hospitals for one year. After that, she spent six months of practicing in a maternal-fetal health center. In 2000, Nervana joined the Biomedical Science Program, a joint program between Old Dominion University and Eastern Virginia Medical School, as a Ph.D. student. She successfully passed her comprehensive and candidacy exams in 2002. Nervana Mahmoud has extensive expertise in various topics in her specialization. She is a member of the American Society of Reproductive Medicine.

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