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The Interactive Effects of Toxaphene, Toxaphene Congeners, and Hyperglycemia on Cultured Rat Embryos

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PREFACE

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ABSTRACT

The presence of persistent organic pollutants, including the pesticide toxaphene, has been reported even in remote regions such as the Arctic and is becoming a health concern. The technical mixture of toxaphene contains over 800 different congeners; however, the numbers decrease along the food chain. Only two major congeners, *2-exo,3-endo,5-exo,6-endo*,8,8,10,10-octachlorobornane (T_2) and *2-exo,3-endo,5-exo, 6-endo*,8,8,9,10,10-nonachlorobornane (T_{12}) have been found in humans. Information on the embryotoxicity of toxaphene is based only on studies using the toxaphene technical mixture and not its major congeners. Diabetes mellitus, one of the most common maternal illness resulting in congenital defects, is now on an upgrowth trend in many native communities. Xenobiotics, such as toxaphene, may induce interactive effects with hyperglycemia, a teratogenic metabolic agent.

In the present study, the relative dysmorphogenic effects of the toxaphene technical mixture or its congeners (T_2 and T_{12}) and the interactive effects of these compounds and high glucose concentrations were investigated using rat embryo culture. Early stage embryos (0-2 somite) were treated with three different doses of the toxaphene technical mixture, T_2, T_{12}, or 50:50 mixture of T_2 and T_{12} and incubated in normal or hyperglycemic culture media for 48 h. Both the technical mixture and the two congeners had significant adverse effects on the total morphological score, somite number, head diameter, crown rump length, and the central nervous system scores of embryos. Differences in the type of toxicity and the target sites were observed between the technical mixture and the congeners. The embryos cultured under hyperglycemic conditions exhibited a dose related interactive effect reflected in the total morphological score, head diameter, and the central nervous system scores of embryos. The results suggest environmentally predominant toxaphene congeners can have organ specific embryotoxic effects not predicted by the toxaphene technical mixture. Moreover, there is a site-specific and dose-related interactive dysmorphogenesis elicited by toxaphene or its congeners in combination with high levels of glucose in rat embryonic development.

RESUME

La présence constante de poluants tels que le toxaphène, un pesticide, a été observe jusque dans les régions reculées de l'Artique et cela est devenu un important problème. Un mélange technique de toxaphène contient plus de 800 congénères toutefois ce nombre diminue avec la chaine alimentaire. Seuls deux congénères, le 2-exo, 3-endo,5-exo,6-endo,8,8,10,10-octachloroborane (T2) et le 2-exo,3-endo,5-exo, 6-endo,8,8,9,10,10-nonachloroborane (T12), furent touvés chez les humains. Des informations sur les effets toxiques du toxaphène sur l'embryon ont juste utilisé le mélange technique et non ses principaux congénères. Le diabète mellitus, une des maladies les plus communes chez la mère occasionnant des malformations congénitales, est en nettte progression dans les communautés amerindiennes. Des xénobiotiques tels que le toxaphène pourrait avoir un effet interactif avec l'hyperglycémie et être un agent tératogène.

Dans cette etude, les effets morphogénétiques du mélange technique du toxaphène, de ses congénères (T2 et T12) en interaction ou non avec des concentrations en glucose élevées furent étudiées en utilisant la culture d'embryons de rat. Des embryons de rat au debut de leur dévelopment (0 à 2 somites) furent traites avec trois différentes doses de mélange technnique de toxaphène, de congénère T2, de congénère T12 et de mélange T2-T12 (50:50) puis furent cultivés pendant 48 h dans un milieu de culture normal ou hyperglycémique. Le mélange technique de toxaphène et ses deux congénères montrèrent un effet très significatif sur le score morphologique total, le nombre de somites, le diamêtre de la tête, la longueur du corps des embryons et le score total du système nerveux des embryons. Des différences dans le type de toxicité et le site affecté furent observées entre les différents congénères et le mélange technique. Les embryons cultivés en conditions hyperglycémiques montèrent une relation dose-effet sur le score morphologique total, le diamêtre de la tête et le score total du système nerveux des embryons. Les resultats montèrent que les congénères de toxaphène présents dans l'environement ont des effets cytotoxiques spécifiques sur des organes cibles qui ne

pouvaient être prédits à partir des effets obtenus avec le mélange technique. En plus du site d'effet spécifique, une relation dose-effet fût observée lorsque le mélange technique de toxaphène ou ses congénères étaient utilisés en présence de serum hyperglycémique lors de la culture des embryons.

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LITERATURE REVIEW

CHAPTER 1.

Because of the relatively high levels of toxaphene found in the northern ecosystem, toxicity of toxaphene and its potential health impact on Indigenous Peoples in Canada are of major concern. The congener mixture detected in the environment represents only a few of the congeners present originally in toxaphene. Preliminary results from monitoring programs showed that there were only two major congeners in human milk and serum. The toxicological significance of identifying these specific toxaphene congeners in human tissues is unknown and hence there is no regulatory guideline level for toxaphene in human blood.

Diabetes mellitus, one of the most common maternal illnesses resulting in congenital defects is now on an upgrowth trend in many native communities. Hyperglycemia is the major metabolic disturbance of diabetes and, since glucose crosses the placenta, maternal hyperglycemia might result in the early embryo exposure to elevated levels of glucose. A close relation has been found between fetal malformations and high maternal blood glucose levels in the first trimester. An interactive adverse effect of glucose on hepatic xenobiotic metabolism has also been shown. Thus, as both hyperglycemia and toxaphene are known to adversely affect embryonic development in rodents, these agents may interact additively or synergistically to exacerbate dysmorphogenic effects.

TOXAPHENE

1.1 Historical Background

Toxaphene is a complex mixture of polychlorinated bornanes, bornenes, and bornadienes containing chlorine atoms varying in number from four to ten. Toxaphene has an average formula of $C_{10}H_{10}Cl_8$. Several thousands of congeners of toxaphene are theoretically possible, but the technical mixture comprises of at least 800 congeners. Toxaphene has a broad spectrum of pesticidal activity, once the most heavily used. Hercules Co. introduced it in the United States in 1945 as a new insecticide to control a variety of insect pests. Its primary use has been on cotton, but it has also been sprayed on soybeans and peanuts. Annual world consumption reached maximum in 1976, 24 mil. kg, and decreased to 1.7 mil. kg in 1983. In 1984, U.S. cancelled most uses of toxaphene. Despite its global threat similar to DDT and PCB, toxaphene is still used in some countries of South America, Africa and Europe (Saleh, 1991).

Toxaphene is carried through the atmosphere, due to its high vapor pressure, from the sites of application and is a widespread contaminant in fresh water and marine environment (Ribick et al., 1982). Atmospheric transport has been suggested as a cause for the contamination found in remote areas. Bidleman and Onley (1975) reported an average of 0.63 ng/m^3 of toxaphene in air samples from the northwestern Atlantic, 1100 km from the North American continent. In a study of atmospheric removal processes for

several organochlorines in South Carolina, the rain water mean was as high as 159±133 ng/l toxaphene (Bidleman et al., 1989). Despite the fact that products containing toxaphene have not been registered for use in Sweden since 1956, Sündstrom (1981) found 3-6 ng/l toxaphene in rainwater collected 100 km southwest of Stockholm. Wania and Mackey (1993) called "cold condensation/global fractionation" the process by which pesticides are volatilized from temperate and tropical zones and redeposit in colder regions. Volatile toxaphene congeners were preferentially transported to the Arctic. Heavier congeners were enriched in the snow and less water-soluble congeners were accumulated in lipids by aquatic organisms (Mackay, 1992). The high lipid levels (>20% of dry weight) typical of Arctic Ocean fauna that store energy for long periods of starvation, could lead to the bioaccumulation of toxaphene (Bidleman et al., 1989) by biota inhabiting regions hundreds and thousands of kilometers away from its usage.

The presence of chlorinated contaminants in the Arctic and Subarctic has been documented as a global phenomenon. A spectrum of chlorinated compounds including toxaphene has been shown in terrestrial, freshwater, and marine systems (Andersson et al., 1988). Surveys of organochlorine compounds in fish across the Canadian Northwest Territories have shown that polychlorinated compounds are the most abundant pesticide residue (Norstrom, 1988).

Indigenous Peoples, who live in rural areas and are more dependent on wildlife for food, are at greater risk as a result of higher exposure to chlorinated contaminants

from the wildlife they consume. Traditional food system includes many marine mammals, which are high on the food chain. Muir et al. (1992) noted that in some locations consumption of fish and their livers by native people could lead to an intake of polychlorinated compounds that exceeds the tolerable daily intake of 0.2 μg/kg body weight/day (Food Directorate, Health Protection Branch, 1992). Results of a dietary study (Kuhnlein et al., 1995) indicated that over 50% of the intake records collected from the Buffin Inuit exceeded the tolerable daily intake for toxaphene. Effects of toxaphene are therefore of public health concern; however, the impact on an individual level is not known. Primary contributing foods to toxaphene for the Buffin Inuit were narwhal (*Monodon monoceros*) blubber (45% of the total toxaphene intake), walrus (*Odobenus rosmarus*) blubber (23% of the total toxaphene intake) and narwhal mattak (15% of the total toxaphene intake). It is worth noting that foods containing the highest levels of toxaphene were not usually those items consumed in greatest quantities. At present, consumption advisories based on total toxaphene contamination have been issued for lake trout (*Salvenilus namaycush*) muscle and burbot (*Lota lota*) liver from Lake Laberge and Atlin in Yukon Territory and for burbot liver from Slave River, Northwest Territories (Lockhart et al., 1988). It is worth noting that organochlorines were previously documented not only in human dietary items but also in breast milk (Müller et al., 1988). Stern et al. (1992) reported that concentrations of T_2 and T_{12} in the breast milk of Inuit women were 70 and 150 μg/kg lipid, respectively. Preliminary results from cord blood study conducted in Northwest Territories show that levels in maternal blood are around 1-40 μg/L (Walker et al., 1994).

Due to environmental degradation, selected metabolism and bioaccumulation toxaphene residue patterns in the environment, especially in the biological species such as fish, marine mammal, terrestrial mammals, and humans, are highly altered from the patterns in the technical mixture. Toxaphene technical mixture contains over 800 different congeners. The number of prevalent congeners, however, decreases along the food chain. About twenty major congeners are found in fish, eight in marine mammals, and only two major ones in humans, *2-exo,3-endo,5-exo,6-endo*,8,8,10,10-octachlorobornane (T_2) and *2-exo,3-endo,5-exo,6-endo*,8,8,10,10-nonachlorobornane (T_{12}).

1.2 Toxicity

Because of the complex mixture of toxaphene congeners, coupled with the difficulty in analyzing it and isolating its individual components, little is known about its mode of action. However, several reports have appeared in the literature describing the effect of toxaphene on different enzyme systems and biochemical processes. Lawrence and Casida (1984) showed that mammalian toxicity of toxaphene and its purified components was closely related to the potency for inhibition of TBPS (t-butylbicyclophosphorothionate) binding to brain specific sites, an action at the GABA (γ-aminobutyric acid) regulated chloride channel. Trottman et al. (1980) showed that

exposure of rats to toxaphene increased cytochrome P-450 and NADPH-cytochrome c-reductase and dehydrogenase activity in hepatic microsomal fractions.

Studies carried out to look for target organs for toxaphene recorded histological changes in the thyroid, liver, kidney, and brain (Chu et al., 1988). Dosing mice with a mixture of pesticides, including toxaphene, Chaturvedi (1992) conducted a study on mice. These workers showed an increase in liver weight and increases in cytoplasmic density, homogeneity, vacuolation, and basophilic aggregations. These mixtures have the potential to induce the xenobiotic-metabolizing enzymes in liver.

Most toxicity studies were conducted using technical mixture of toxaphene, which reflects the current limitation of the lack of individual congener preparations. Toxaphene is of intermediate acute toxicity to most mammals when compared to other organochlorine insecticides. Dogs are estimated to be 2-5 times more sensitive to toxaphene than other mammals (Parker and Beacher, 1947). Estimated acute toxicity of toxaphene to an average 70 kg man was predicted to be 2-3 g (Guyer et al., 1971) and based on records of poisoning the lethal dose has been estimated to be from 2-7 g (Stormont and Conley, 1952). Moreover, toxaphene is absorbed more rapidly and more completely from the alimentary tract than from skin (U.S. EPA, 1976).

Toxaphene was found to be also chronically toxic to most mammals (Gaines, 1960). It is highly carcinogenic (Reuber, 1979) in rodents (mice and rats), inducing

malignant neoplasm of the liver. Toxaphene is classified as a suspected human carcinogen. Hooper et al. (1979) and Saleh (1980) showed that toxaphene is mutagenic in the Ames Salmonella test without requiring liver homogenate for activity. They also showed that the most easily isolated major toxic component, heptachlorobornane-1, did not have mutagenic activity in any of the standard tester strains. Epstein et al. (1972) showed that toxaphene does not produce chromosomal abnormalities that inhibit zygote development.

The actual toxicity of toxaphene residues in biological samples may be different from that of the technical toxaphene mixture. This is supported by the work of Olson et al. (1980). Two toxaphene congeners (Toxicant A and Toxicant B) were isolated from lake trout collected from the Great Lakes. There was a differential toxicity between toxaphene congeners and technical mixture as indicated by behavioral changes in perinatal rats, such as inferior swimming ability, delayed righting reflex ability, which demonstrated retarded maturation. The toxicity in ascending order was: toxicant A, toxicant B, and with toxaphene showed the highest toxicity. Gooch and Matsumura (1987), however, reported that toxaphene residues isolated from lake trout from the Great Lakes were as toxic as the technical mixture. They concluded that composition estimates of toxicant A and B were not sufficient to predict toxicological potency and the toxicity was attributed to the other components of the toxaphene residues. These results further demonstrate the need to determine the relative toxicity of the prevalent toxaphene congeners in the ecosystem.

1.3 Teratogenicity

The teratogenicity of toxaphene has been documented. Khera (1984) hypothesized that maternal toxicity caused by diverse chemical and physical agents could per se induce a consistent pattern of major malformations in fetal mice. This hypothesis is now fully supported by the teratology data on mice (Kavlock et al., 1985) and rats (Chernoff et al., 1990). A strong relationship was also found between embryo-fetal mortality and maternal toxicity and maternal toxicity may be regarded as an etiological factor for fetal malformations and embryo-fetal mortality. It was demonstrated that the effects on the fetus depend largely on the dose, route, and duration of exposure.

Following implantation, organogenesis takes place. This period is characterized by division, migration and association of cells into primitive organ rudiments. The basic structural templates for organization of tissues and organs are established on the molecular, cellular, and morphologic levels. The most characteristic susceptibility of the embryo to xenobiotics during the organogenesis period is the induction of structural birth defects. Within this period, individual organ systems possess highly specific periods of vulnerability to teratologic insult. Administration of a teratogen on day 10 of rat gestation would result in a high level of brain and eye defects. If the same agent was administered on day 11, a different spectrum of malformations would be anticipated, with brain and palate malformations predominating.

1.3.1 In vivo animal studies

Early studies conducted to demonstrate the teratogenicity of toxaphene showed little evidence that toxaphene may have teratogenic effects in rats (Kennedy et al., 1973), in mice (Keplinger et al., 1970), or in guinea pigs (DiPasquale, 1977). In Kennedy's study, five generations of mice were fed diets supplemented with toxaphene. The experiment was designed to determine effects in the offspring caused by the absorption of the pesticide via the placenta, ingestion of mother's milk, and ingestion of contaminated food. No adverse effects were noted through five generations fed 25 ppm toxaphene.

Kavlock et al. (1982) conducted a study in which five compounds, including toxaphene technical mixture, were administered during the organogenesis period and the effects on organ differentiation were determined in day 21 fetuses. Growth retardation and compromised liver and kidney functions of rat fetuses were observed. Brain and lungs appeared to be less sensitive to the insult of toxaphene.

Kavlock et al. (1985) demonstrated the effects of toxaphene-induced acute maternal toxicity on fetal development in CD-1 mice. Doses of toxaphene technical mixture were calculated to exert either a low or a moderate degree of maternal lethality. Day 8 of gestation was the day of treatment and fetuses were examined for gross

malformations on day 18 of gestation. Supernumerary ribs were the significant malformation in the fetuses. The treatment, however, had no influence on the litter size and birth rate.

Chu et al. (1988) investigated the reproductive effects of toxaphene in Sprague-Dawley rats. In a first generation two litter reproduction study, the toxaphene technical mixture was administered orally up to 500 ppm. Toxaphene treatment had no effects on the litter size, pup weight, fertility, or gestation and survival indices. Toxic effects, however, were noted in the parent rats which included depressed weight gain and increased hepatic microsomal enzyme activities.

Chernoff et al. (1990) studied the effects of toxaphene-induced maternal toxicity on prenatal development in Sprague-Dawley rat. Female rats were dosed by oral gavage with toxaphene in five doses which were chosen using reported acute LD_{50} (80 mg/kg). The treatment-related malformations were supernumerary ribs.

The interpretation of adverse developmental effects on the fetus or new born at dose levels which are inducing maternal toxicity is a difficult task since it is often impossible to separate developmental effects resulting from the specific agent studied from those induced by alterations in maternal homeostasis. Rodent embryo culture has some unique capabilities in terms of risk assessment with respect to xenobiotics and mammalian metabolism. This experimental model allows an assessment of the direct

effect of the toxicant on the embryo, free of maternal interaction, and precise control of the variables of interest.

The teratogenicity of the individual congeners is not known, as previous studies have focused on the toxaphene technical mixture, which contains over 800 congeners. The actual toxicity and teratogenicity of toxaphene residues in biological or food samples may differ from that of the technical mixture as only two major congeners (T_2 and T_{12}) were found in the Canadian Arctic food system.

1.3.2 In vitro studies

Rodent embryo culture has some unique capabilities in terms of hazardous risk assessment with respect to mammalian metabolism. The post-implantation embryo culture system described by New (1978) has been applied mainly in the study of embryonic development during early organogenesis. Cultured embryos have been shown to have an impressively high predictive capacity with respect to the effects observed in vivo in a wide range of different chemical classes such as drugs, agrochemicals, industrial chemicals (Cicurel and Schmid, 1988). This method offers major advantages over conventional in vivo teratogenicity testing such as:

- accurate control of exposure conditions (i.e., concentrations of teratogen, duration of exposure, developmental stage of the embryo);
- shorter investigation time;
- fewer animals used

Furthermore, the effect of the chemical compound on the embryo can be assessed directly free of maternal interaction and its embryotoxic or teratogenic activities can be distinguished from one another.

The teratogenicity of toxaphene was demonstrated by Crawford et al. (1985) in a teleost (*Fundulus heteroclitus*) embryo culture model using the toxaphene technical mixture at doses ranging from 0.1 ppm to 10 ppm. The observed effects included inhibition of gastrulation, abnormal axis formation, diminished pigmentation, slowed rate of development, reduced frequency of hatching, loss of neuromuscular control and reduction or inhibition of heart beat.

CHAPTER 2

HYPERGLYCEMIA

2.1 Historical Background of Congenital Malformations and Diabetes

Diabetes mellitus has a major impact upon the reproductive events and the developing fetus. It is one of the most common maternal illnesses resulting in congenital defects and is now on an upgrowth trend in many native communities. The association between congenital malformations and diabetes in pregnancy was first reported by LeCorche (1885). Congenital malformations have remained the leading cause of perinatal mortality in the offspring of diabetic mothers (Mills, 1982). Pederson et al. (1974) have reported that anomalies are responsible for 40% of perinatal deaths. Furthermore, several studies have demonstrated that the incidence of malformations was three times higher in the offspring of women with diabetes than in the general population (Pedersen, 1977) and fatal congenital abnormalities occur six times more frequently (Breidahl, 1966). Retrospective data of 7101 infants of diabetic mothers showed a 4.8% incidence of congenital malformations affecting all major organs systems, including heart, kidneys and skeleton (Kucera, 1971). Abnormalities of the central nervous system, such as anencephaly and spina bifida, are also commonly found in infants of diabetic

mothers (Gabber, 1977). In addition, caudal regression syndrome, a condition in which agenesis of both femora and the lower vertebrae, showed a strong correlation to diabetes (Eriksson, 1991).

Congenital malformations in diabetic pregnancy have also been reported experimentally in animal models using mice (Sadler, 1980 and Baker et al., 1990), rats (Baker et al., 1981 and Giavini et al., 1986) and rabbits (Brismade et al., 1956). Malformations seen in these studies primarily affected the skeleton and central nervous system. Increased incidence of cataract and eye defects, such as microphthalmia and anophthalmia has been reported in the offspring of severely diabetic rats (Giavini and Prati, 1990).

2.2 Potential Mechanisms of Diabetic Teratogenicity

The overall risk for malformations for diabetic patient population was found to be approximately 8-12% (Beccera et al., 1990). Diabetes-induced dysmorphogenesis occurs very early in pregnancy, between the third and the sixth week of gestation (Mills et al., 1979). This is the period of organogenesis which has the greatest teratogenic susceptibility. There has been ongoing research in understanding the etiology of malformations in infants of diabetic mothers. One proposed concept of "fuel-mediated teratogenesis" by Feinkel (1980) suggests that the alterations in the maternal glucose,

amino acid, and lipid levels are instrumental in the induction of dysmorphogenesis in the offspring. Disturbances in the maternal metabolism alter the fuel mixture offered to the embryo and these effects on the offspring can manifest as malformations in early gestation.

The etiology of the congenital malformations has not been elucidated yet. The diabetic state itself results in a variety of metabolic disturbances including the presence of hyperglycemia (Sadler et al., 1980), hyperketonemia (Lewis et al., 1983), arachidonic acid or myo-inositol deficiency (Baker et al., 1990), increased glycation (Kubow et al., 1993), and the increased generation of free oxygen radicals (Eriksson et al., 1991). In vitro studies have demonstrated a synergistic effect between glucose and another teratogenic metabolic agent, ß-hydroxybutyrate. Minimally teratogenic amounts of glucose combined with minimally teratogenic amounts of ß-hydroxybutyrate, during culture of rat embryos, resulted in a significantly greater reduction in the crown rump length and mean embryonic protein content than either of these metabolites alone (Lewis et al., 1983). Somatomedin inhibitors are also found in high concentrations in streptozocin-induced diabetic rats and are being considered as a possible teratogen. Sadler et al. (1986), using a whole-embryo rodent culture model, were able to demonstrate that the presence of a low-molecular-weight fraction of somatomedin inhibitors was associated with an increased incidence of malformations and impaired growth.

There may also be some type of "genotoxic effects" as a result of these aberrant fuels either occurring singly, or in combination (Reece et al., 1996). Alterations in the maternal glucose, amino acid, and lipid levels appear to play a significant interactive role in dysmorphogenesis elicited by diabetes in embryonic development. Studies performed in animal models have reported that diabetic teratogenesis appears to be enhanced in the presence of genetically predisposed embryos (Otani et al., 1991). These data, however, have not been consistent with human clinical trials. Chung et al. (1975) have found no significant difference in the incidence of congenital malformations in the offspring of diabetic and non-diabetic fathers, but the incidence was significantly higher among infants whose mothers were diabetic as compared with the offspring of non-diabetic parents.

An interactive effect of glucose on hepatic xenobiotic metabolism has also been shown. Lamson et al. (1952) observed that injection of a solution of glucose or its intermediary products of metabolism influenced the barbiturate anesthesia in dogs, hamsters, rabbit, and guinea pig. Moreover, Yau et al. (1987) showed glucose intake increased the drug sensitivity. Such studies indicate the possibility that high glucose concentrations could interact negatively with teratogenic xenobiotics.

2.3 Role of Hyperglycemia in Diabetes Teratogenicity

Hyperglycemia is the marker of uncontrolled diabetes, which is the most easily assessed clinically and most commonly associated with major diabetes-induced congenital abnormalities (Fuhrmann et al., 1983). Human clinical trials demonstrate that diabetic women who are hyperglycemic during organogenesis and who have elevated glycosylated hemoglobin levels (an indicator of abnormal glycemic control) in the first trimester of pregnancy have an increased occurrence of birth defects. Measurement of hemoglobin A1c provides a retrospective index of glycemic control over the previous four to eight weeks and therefore first trimester levels are believed to reflect the degree of metabolic control during organogenesis (Greene et al., 1984).

Studies have examined the relationship of hyperglycemia, duration of diabetes, and vascular complications with the occurrence of anomalies. A 10-year study, performed by Karlsson and Kjellmer (1972), reported a higher rate of malformations among women with hyperglycemia, long-standing diabetes, and diabetic vasculopathy than those without these complications. Wittaker et al. (1983), in a prospective study, have also reported a 30% frequency of spontaneous abortion among insulin-dependent diabetic women compared to 15% reported in the general population of pregnant women.

In vitro animal studies have demonstrated that hyperglycemia has an atherogenic effect during organogenesis. Cockroft and Coppola (1977) demonstrated that rat embryos explanted at 9.5 days of gestation and cultured with 12-15 mg/ml of D-glucose

for 48 hours have severe abnormalities and retarded development. Reece et al. (1985) showed, in a post-implantation rat embryo culture model, that malformations were induced in a dose-related fashion. Glucose levels twice high as normal induced a 20% malformation rate and an approximately 100% rate at glucose levels six times the normal level. Sadler et al. (1986) compared the effects of different concentrations of glucose on younger (0-2 somites) and older (4-6 somites) embryos using the whole embryo culture technique. The frequency of malformations was higher in younger embryos and in the embryos exposed to the higher glucose concentration, showing a dose- and age-related effect of glucose on early stages of embryogenesis.

The possibility that the embryopathies induced by hyperglycemia are yolk sac-dependent has been raised. Freeman et al. (1981) showed that the visceral yolk sac endoderm, which is an epithelium with transport function, mediated the selective transfer of macromolecules to the developing embryo. Moreover, Pinter et al. (1986) showed that embryos exposed to a hyperglycemic culture (eight times the normal level) have abnormal yolk sac formation and embryonic development. Reece et al. (1989) demonstrated that during hyperglycemia-induced embryopathy there is concomitant yolk sac failure evidenced by morphologic and functional alterations.

A more recent hypothesis for the mechanism of diabetic embryopathy has been proposed by Eriksson et al. (1991). Glucose is oxidized to CO_2, H_2O, and free oxygen

radicals. The latter are eliminated, under normal conditions, by scavenging enzymes in the mitochondria. In the situation of hyperglycemia the immature scavenging enzymes are overwhelmed resulting in excess free radicals which cause teratogenesis. The increased free oxygen radical activity can enhance lipid peroxidation which in turn leads to an imbalance in prostaglandin synthesis. In addition, Kubow (1993) demonstrated the protective role of acetylsalicylic acid against hyperglycemia-induced glycation and neural tube defects in cultured early somite mouse embryos, which supports the role of glycation in diabetic teratogenesis.

RATIONALE

Because of the relatively high levels of toxaphene found in the northern ecosystem, toxicity of toxaphene and its potential health impact on Indigenous Peoples in Canada are of major concern. The congener mixture detected in the environment represents only a few of the congeners present originally in toxaphene. Moreover, the composition of toxaphene congeners differs among geographical areas and among animal groups. Therefore, existing information obtained largely from toxicological studies using the commercial technical toxaphene is unlikely to be applicable to an assessment of risk associated with consumption of tissues of fish and animals.

Preliminary results from monitoring programs showed that there were only two major congeners in human milk and serum. The toxicological significance of identifying these specific toxaphene congeners in human tissues is unknown and hence there is no regulatory guideline level for toxaphene in human blood.

It is difficult to separate sufficient quantities of individual congeners from the technical mixture for in vivo testing. Therefore, an in vitro model will be the most cost-effective way in determining the relative toxicity of the congeners. It also provides an advantage of the relative simplicity for studying the interactive effects.

Because the potential for human exposure in utero to teratogens, xenobiotics or metabolic agents, remains very high it is important to study the interactive effects of

different compounds on embryonic dysmorphogenesis. Teratogens with very different mechanisms of action can interact and potentiate the deleterious developmental effects. Diabetes mellitus, one of the most common maternal illness resulting in congenital defects is now on an upgrowth trend in many native communities. No information is available on whether there is any relationship between body contaminant burden and hyperglycemia. Hyperglycemia is the major metabolic disturbance of diabetes and, since glucose crosses the placenta, maternal hyperglycemia might result in the early embryo exposure to elevated levels of glucose. A close relation has been found between fetal malformations and high maternal blood glucose levels in the first trimester and rodent embryo culture studies have confirmed the teratogenic effects of hyperglycemia. In vitro studies have demonstrated a synergistic effect between glucose and other teratogenic metabolic agents, such as ß-hydroxybutyrate. An interactive adverse effect of glucose on hepatic xenobiotic metabolism has also been shown. Thus, as both hyperglycemia and toxaphene are known to adversely affect embryonic development in rodents, these agents may interact additively or synergistically negatively to exacerbate dysmorphogenic effects.

HYPOTHESIS

The studies were designed to test three hypotheses:

1. There is a difference in the degree of teratogenicity between the toxaphene technical mixture and individual congeners.

2. There is an interactive teratogenic effect between the two congeners.

3. There is an interactive effect between the toxaphene technical mixture, its congeners and hyperglycemia.

OBJECTIVES

This project will assess the relative teratogenicity of toxaphene residues which are prevalent in wildlife and human serum. The objectives of this study are as follows:

1. To assess the dysmorphogenesis of toxaphene and of the individual congeners using rat embryo culture at the 0-2 somite stage.

2. To identify possible interactive teratogenic effects using combination of the two major toxaphene congeners (T_2 and T_{12}).

3. To identify possible interactive effects of toxaphene or its congeners with elevated levels of glucose on embryo development.

MANUSCRIPT 1

Thesis Chapter 3

Toxaphene congeners differ from toxaphene mixtures in their teratogenic effects on cultured rat embryos

SUMMARY

The presence of persistent organic pollutants, including the pesticide toxaphene has been reported even in remote regions such as the Arctic and is becoming a health concern. The technical mixture of toxaphene contains over 800 different congeners. The numbers of prevalent congeners, however, decrease along the food chain. About twenty major congeners are found in fish, eight in marine mammals and only two major ones in humans, *2-exo,3-endo,5-exo,6-endo*, 8,8,10,10-octachlorobornane (T_2) and *2-exo,3-endo,5-exo,6-endo*,8,8,9,10,10-nonachlorobornane (T_{12}). Embryotoxicity of these individual congeners is not known, as previous toxicity studies have focused on the toxaphene technical mixture. We studied the relative dysmorphogenic activity of toxaphene technical mixture and individual congeners (T_2 and T_{12}) using rat embryo culture. Extracted embryos (0-2 somite) were treated for 48 h with doses of 0 ng/ml (DMSO 0.01%), 100 ng/ml, 1000 ng/ml and 5000 ng/ml of either (a) toxaphene technical mixture; (b) T_2 ; (c) T_{12} ; and (d) a 50:50 mixture of T_2 and T_{12}. The treatment period corresponds to gestational days (GD) 10-12, a period within the critical time of morphogenesis and organogenesis. Both the technical mixture and the two individual congeners had significant adverse effects on the total morphological score, somite number, head and crown rump length, and the central nervous system scores of embryos. All treatments caused a high incidence of central nervous system defects. The T_2 and T_{12} congeners differed in their spectrum of abnormalities as exposure to T_2 caused limb and flexion defects which were not observed with the T_{12} congener. Differences were

observed, however, in the type of toxicity and the target sites between the technical mixture and the congeners. T_2 showed a more potent, adverse effect on the morphological score, as compared to the technical mixture. Both T_2 and T_{12} were less inhibitory on growth than the technical mixture as indicated by crown-rump length but they showed a stronger inhibitory effect on otic system development. The mixture of T_2+T_{12} showed a synergistic effect on decreasing crown rump and head length. Conversely, the combination of T_2 and T_{12} inhibited the strong adverse effect of the individual congeners on otic development. The results suggest environmentally predominant toxaphene congeners can have organ specific embryotoxic effects not predicted by the toxaphene technical mixture.

Key words: Toxaphene congeners, teratogenicity, embryo culture

INTRODUCTION

Toxaphene is a complex mixture of polychlorinated bornanes with a broad spectrum of pesticidal activity. It was introduced in 1945 and reached maximum world consumption in 1976, when it started to replace DDT. Before its ban in 1982, it was the most heavily used insecticide in the United States and many parts of the world (Saleh et al., 1991). Despite its global threat similar to DDT and PCB, toxaphene is still used in South America, Africa and Mexico (Saleh et al., 1991). Toxaphene can be transported through the atmosphere from the sites of application due to its high vapour pressure and spread in the environment (Ribick et al., 1982). It is persistent in soils and lakes sediments (Bidleman et al., 1988) and has been found in fish (Lach and Parlar, 1990), marine mammals (Bidleman et al., 1993), terrestrial mammals (Zhu and Norstrom, 1993) and human milk (Bidleman et al., 1988).

Recently, the toxicology of toxaphene has received renewed attention because it was found to be the most prevalent organochlorine compound in Great Lakes fish (Glassmeyer, 1997). Moreover, in the Arctic where consumption of fish and marine mammals is common, a high percentage of the people had dietary exposure levels exceeding the tolerable daily intake level (TDI) of 0.2 μg/kg body weight/day established by the Health Protection Branch of Health Canada (Chan et al., 1997).

Relatively little is known about the mode of action of toxaphene, partly because of its complicated mixture nature. The toxaphene technical mixture was, however, shown to be acutely (Gaines et al., 1960) and chronically (Ohsawa et al., 1975) toxic to aquatic life and pose a carcinogenic risk to humans. Effects of toxaphene technical mixture on different enzyme systems and biochemical processes have also been described (Chu et. al, 1990).

A strong relationship has been found between embryo-fetal mortality and maternal toxicity in rodents upon toxaphene exposure (Kavlock et al., 1982). Chernoff et al. (1990) found that rat litters of dams treated with toxaphene had elevated incidence of supernumerary ribs. However, in a one generation two litter reproduction study in rats, toxaphene technical mixture treatment of up to 290 to 380 μg/kg/ day given orally had no effects on litter size, pup weight, fertility, or gestation and survival indices (Chu et al., 1988). Teratogenicity of toxaphene was also demonstrated in a teleost (*Fundulus heteroclitus*) embryo culture model (Crawford et. al, 1985). Results of epidemiological studies have shown that the developing embryos are the most susceptible target organ for organochlorine toxicity. Information on the toxicity and teratogenicity of toxaphene, however, is based on studies using the toxaphene technical mixture of over 800 congeners. The toxicity of toxaphene residues in biological or food samples differs from that of the technical mixture; only two major congeners, *2-exo,3-endo,5-exo,6-endo*, 8,8,10,10-octachlorobornane (T_2) and *2-exo,3-endo,5-exo,6-endo*,8,8,9,10,10-nonachlorobornane (T_{12}), were found in the Canadian Arctic traditional food system and

in the breast milk of indigenous women (Stern et al., 1992). Therefore, it is important to verify the relative toxicity of these two environmentally prevalent congeners.

In this study, the dysmorphogenic effects of the toxaphene technical mixture and T_2 and T_{12} were studied using the rodent embryo culture model to test the hypothesis that there are differences in the dysmorphogenic potency between the toxaphene technical mixture and the environmentally prevalent toxaphene congeners. The embryo culture model was used because it offered the advantage that the exposure concentrations on the embryo could be more accurately controlled and the effects of mixtures of the chemical could be easily studied. A mammalian system was chosen to provide a more physiologically relevant system with respect to implications on human health.

MATERIALS AND METHODS

Animal maintenance and mating procedures

Virgin female Sprague-Dawley rats (180-200 g) (Charles River Canada, St. Constant, PQ, Canada) were used in all experiments. Upon arrival, the females were housed in a temperature-controlled room (20°C) with a 14L-10D reversed light cycle (lights on at 1600). The animals were kept in plastic shoe box cages with Beta-Chip bedding (Beta-Chip, Northeastern Products Corp., Warrenburg, NY, USA). Purina Rat Chow (Ren's Feed and Supply, Oakville, Ontario, Canada) and tap water were fed *ad libitum*. Females were housed three per cage and males caged individually. After three weeks acclimation period, for mating purposes, three or four females were housed one to one with a male from 0900 to 1500. The presence of sperm in the vaginal smear indicated insemination and this day was considered as gestational day (GD) 0.5.

Toxaphene preparation

Technical toxaphene mixture was purchased from Ultra Scientific (North Kingstown, RI.). Toxaphene congeners, T_2, and T_{12} (1 mg) were obtained from Promochem (Wesel, Germany). Stock solutions were prepared by dissolving the toxaphene technical mixture, T_2, T_{12} and a mixture of T_2: T_{12} mixture (50:50) in 0.01% DMSO (dimethylsulfoxide) and added to 10 ml of male rat serum. Culture media were mixed with these stock solutions to final concentrations of 100, 1000 and 5000 ng/ml. The choice of exposure concentrations was based on results of a preliminary experiment

that showed dysmorphogenic effects at these exposure levels. Control embryos were treated with 0.01% solution of DMSO.

Animal treatments

A 4 x 4 factorial experimental design was used. On GD 10, 40 pregnant rats were killed and a total of 120 embryos containing 0-2 somites were randomly allocated to four culture groups. Only embryos of 0-2 somites were used for two reasons: 1) to ensure that there was minimal variability among responses of embryos to the treatments as developmental stages of embryos obtained on gestational day 10 can vary greatly from litter to litter; and 2) stronger dysmorphogenic effects appear at younger stages of embryonic development (New, 1978). Each group of embryos was dosed with one of the following compounds: toxaphene technical mixture, T_2, T_{12}, and a 50:50 mixture of T_2 and T_{12}. The T_2 + T_{12} mixture was used to study possible synergistic effect of the two congeners. Four concentrations were administered for each treatment: control (0.01% DMSO), 100 ng/ml, 1000 ng/ml, and 5000 ng/ml. The embryos were incubated for 48 h.

Whole embryo culture

The techniques for explanting and culturing embryos followed the methods described by New (1978). To provide serum for embryo culture, male Sprague-Dawley rats (Charles River Canada) were exsanguinated under halothane anaesthetic. The collected blood was centrifuged immediately and then allowed to stand until the plasma clot had formed. The plasma clot was removed and the serum was collected; after

recentrifugation, the serum was pooled. Pooled serum was heat-inactivated at 56°C for 30 min and filter-sterilised using 0.45 μm mesh filter (Millipore Corp., Bedford, MA). The heat-inactivated, pooled serum was stored at –80°C and thawed immediately before use in embryo culture. At 0900 h on day 10 of gestation, rats were killed under halothane anaesthetic and their uteri excised immediately. Under aseptic conditions, the decidua was dissected from the uterus in Hanks' Balanced Salt Solution (HBSS; Gibco, Burlington, ON, Canada) with a dissecting stereomicroscope. Decidual tissue and Reichert's membrane were removed and the ectoplacental cone, amnion, and visceral yolk sac were left intact. Embryos containing 0-2 somites were chosen for culture. Randomly selected embryos were placed into 60-ml glass culture bottles containing 1.99 ml of warm sterile male rat serum per embryo. Streptomycin sulfate and penicillin G potassium were added to final concentration of 10000 IU/ml. The bottles were gassed for 2 min with 20% O_2:5% CO_2:75% N_2 immediately before and after addition of the embryos. The same gas mixture was also used to pass over the serum 4-6 h after embryo transfer. The culture flasks were rolled on a 30-rpm rotator wheel in a 37°C incubator. Embryos were re-gassed at a 12-h intervals on the second day of culture with 95%O_2.

Growth and morphological assessment of cultured embryos

After 48 h in culture, embryos were transferred in 0.9% saline solution and examined under a dissecting microscope. They were checked for survival as indicated by the presence of a heartbeat and yolk sac circulation. Dead embryos were not further analyzed. The yolk sac diameter, crown-rump length, and head length of live explanted

embryos were measured with the aid of an eyepiece micrometer. Embryos were given a differentiation score according to the method of Brown and Fabro (1981), under which 13 morphological criteria are each given a numerical score ranking from 0 to 5, corresponding to a given stage of development for each organ and tissue. The sum of the scores from each individual morphological feature gives a total morphological score that is indicative of the stage of development of each embryo. Somite counts were performed only on embryos that had turned completely. The following features were considered abnormal for midgestational (GD 10 to 12) embryos: unfused neural tube, hindbrain, midbrain or forebrain, malrotated, kinked or ventrally convex flexion of the tail, and underdeveloped forelimb or hindlimb.

Statistical analysis

Data are presented as means ± SEM. The dose response of the different toxaphene treatments was analysed by two-way ANOVA followed by the LSMEANS comparisons. The incidence of neural tube, limb, and flexion abnormalities was compared using a chi-square test with continuity correction. Statistical tests were performed with the Statistical Analysis System for personal computers (SAS Institute, Cary, NC, version 6.10). $P < 0.05$ was accepted as the minimal level of significance.

RESULTS

After the 48 h culture period, control embryos exhibited normal growth and development. The embryos underwent rotation to assume fetal position, the neural tube closure was completed, and brain vesicle formation occurred. Cardiac looping and a rapid uninterrupted heartbeat were established. Optic and otic vesicles were formed, visceral arches 1, 2, and 3 became apparent, and yolk-sac circulation was initiated. The forelimb and hindlimb buds showed development characteristic of GD 12.

The effects of toxaphene technical mixture and congeners (T_2 and T_{12}) on growth and development of specific morphological features are detailed in Tables 1 to 4. The total morphological score, crown-rump length, and head length were significantly decreased by treatments, indicating that toxaphene technical mixture and congeners (individually or in combination) caused retarded growth and morphological development of the embryos. The major malformations were abnormalities in the development of the nervous system (small midbrains and forebrains, unfused neural folds, open neural tube). Significant increases in frequencies of neural tube malformations were registered at 1000 ng/ml and at 5000 ng/ml when compared with the control. The 5000 ng/ml treatment also caused forelimb and hindlimb malformations. There also was a significant reduction in olfactory, branchial bars, maxillary, and somite parameters.

The yolk sac diameter, which is considered an embryo growth parameter, was significantly decreased in the 1000 ng/ml and 5000 ng/ml groups, except for toxaphene

technical mixture. The head length was significantly decreased by all treatments; the lowest head length was registered in the T_2 + T_{12} groups. All embryos cultured in the 5000 ng/ml T_2 and T_2 + T_{12} groups had significantly lower somite numbers than those in the toxaphene and T_{12} groups. Embryos that were cultured with toxaphene technical mixture exhibited a significantly lower score for branchial bars than those with T_2 and T_{12} at 100 ng/ml and 5000 ng/ml groups. T_{12} and T_2 + T_{12} treatments at 5000 ng/ml had significantly lower scores for mandibular process than toxaphene technical mixture and T_2. Moreover, on otic system development, a mean score as low as 0.85 was attributed to the T_{12} treatment at 5000 ng/ml (compared with the control which had a mean score of 3.83).

To compare the effects of the four toxaphene treatment groups on embryo development, the total morphological score, the crown rump length, and the scores for the otic and optic systems are presented in Figures 1 to 3. A significant dose-dependent adverse effect on the total morphological score was observed as decreases observed in the score were at the lowest 100 ng/ml dose for all treatment groups (Fig. 1). Significant differences among the different toxaphene treatment groups were also observed. Compared to the toxaphene technical mixture, T_2 had a stronger effect on morphological score at 1000ng/ml while T_{12} was less toxic at 5000 ng/ml. T_2+T_{12} was less toxic than T_2 at 1000 ng/ml and more toxic than T_2 and T_{12} at 5000 ng/ml.

All four treatments had a significant adverse concentration-response effect on crown-rump length (Fig. 2). T_2 and T_{12} had a significantly lower effect on crown-rump length than toxaphene at all concentrations. In contrast, the combination of T_2+T_{12} had a similar effect as the toxaphene mixture and was stronger than T_2 and T_{12} alone.

A significant adverse concentration-response effect on the otic system development was observed (Fig. 3). T_2 and T_{12} had a significantly greater effect than the toxaphene technical mixture at all concentrations. The effects of T_2+T_{12} on the otic system, however, were less than the effects of T_2 and T_{12} alone. In contrast, there was no difference between the treatment groups on the optic system (Tables 1 to 4).

Occurrence of neural tube malformations, as measured by open caudal neural tubes, hindbrains, midbrains and forebrains, were tested by Chi-square test against the control (Table 5). All treatments, except toxaphene technical mixture and T_{12} at 100 ng/ml induced neural tube malformations. T_2 at each concentration and T_2 + T_{12} at 1000 ng/ml and 5000 ng/ml significantly increased limb malformations. An increased frequency of limb abnormalities was observed at 5000 ng/ml toxaphene technical mixture treatment that was comparable with the effect of T_2 + T_{12} at the same concentration (Table 5). The percentage of tail abnormalities was relatively low among all the treatments groups. The T_2 + T_{12} treatment at 5000 ng/ml, however, had a tail malformation rate significantly higher when compared to the control (Table 5).

DISCUSSION

The most remarkable finding of this study was the differential toxicity of the toxaphene congeners versus the technical mixture. Although all treatments had a significant adverse concentration-response effect on total morphological score, the toxicity of toxaphene technical mixture or the T_2+ T_{12} mixture was different than that of the congeners. Exposure of embryos to treatment with either T_2 or T_{12} alone had a lesser effect on growth than did the toxaphene technical mixture or the $T_2 + T_{12}$ mixture at the same exposure levels. This suggests that there may be a synergistic effect on growth retardation among the toxaphene congeners. On the other hand, T_2 and T_{12} alone had a significantly stronger effect than both the toxaphene technical mixture and the $T_2 + T_{12}$ mixture on the otic system. Also, the two congeners differed with respect to their toxicity as T_2 caused limb and flexion abnormalities which were not observed with the T_{12} congener. This outcome suggests that both the target site and the type of toxicity are highly congener specific. These results agree with the findings of Matsumura et al. (1980) who demonstrated that there was differential toxicity between two toxaphene congeners isolated from lake trout versus the toxaphene technical mixture as indicated by behavioural parameters such as inferior swimming ability and delayed righting reflex ability. The mechanism involved in the toxicity of toxaphene on embryo development, however, is not clear at present.

The results show that both the toxaphene congeners and the technical mixture had a significant concentration-dependent adverse effect on the developing embryo. The

toxicological end points observed included retardation of growth as indicated by decreased crown rump length, malformation of limbs and neural tubes, and defects in the otic system. These effects, however, were only observed in the relative high dose range (100 - 5000 ng/ml) used in this study. Results of preliminary experiments showed no effects at lower dosages.

There are no published data available on toxaphene levels in human blood. Stein et al. (1992) reported that concentrations of T_2 and T_{12} in the breast milk of Inuit women were 70 and 150 μg/kg lipid, respectively. Assuming that the lipid content in cord blood is 2.5 μg/l, the levels of T_2 and T_{12} in cord blood can be estimated to be 175 and 375 ng/l, respectively. These levels are 1/1000th of the lowest dose used in this study. Bryce et al. (1997) showed that the blood level of toxaphene in the cynomolgus monkeys dosed with 1 mg/kg/day toxaphene for one year was 40 ppb. The cord blood level can, therefore, be estimated as 10 ppb or 10 μg/l assuming that the lipid level in cord blood is about one-quarter of maternal blood concentrations. This is approximately 1/10 of the lowest dose used in the present study. Therefore, the growth retardation and malformations observed in this study may not be observed in *in vivo* feeding experiments; the high concentrations of toxaphene mixture needed to exert embryotoxic action in the present study could explain the findings that toxaphene technical mixture treatment of up to 290 to 380 μg/kg/day given orally had no effects on litter size, pup weight, fertility, or gestation and survival indices in a one generation two litter reproduction study in rats (Chu et al., 1988). More subtle effects on embryo development, however, may be

observed at lower doses. For instance, Olso et al. (1980) observed retarded maturation as judged by the swimming test during the early development of juvenile rats from perinatal exposure to low levels of toxaphene (50 μg/kg) and its toxic components that they named as toxicant A and toxicant B (2 μg/kg).

The present use of embryo culture has pinpointed that environmentally prevalent congeners can have a specific toxicity different from the technical toxaphene mixture that has been typically used in toxaphene toxicity studies. The otic system of the developing fetus appears to be a particularly susceptible target organ to the action of congeners. Further study of health implications of toxaphene congener exposure particularly among the highly exposed Inuit populations should be of high priority.

Table 1. Effects of toxaphene technical mixture on growth parameters and morphological features in cultured rat embryos

	Dose			
Feature[1]	Control	100ng/ml	1000ng/ml	5000ng/ml
Number of cultured embryos	6	7	7	7
Yolk sac diameter (mm)	[2]4.5 ± 0.1	4.2 ± 0.1	4.2 ± 0.1	4.1 ± 0.1
Head length (mm)	2.3 ± 0.1	1.8 ± 0.1*	1.7 ± 0.1*	1.5 ± 0.1*
Yolk sac circulation	3.7 ± 0.1	2.9 ± 0.1*[b]	2.5 ± 0.0*[ab]	2.1 ± 0.1*[ab]
Allantois	3.0 ±0.0	2.5 ± 0.0*[a]	2.4 ± 0.1*	2.0 ± 0.0*[ab]
Flexion	3.0 ±0.0	2.6 ± 0.1	2.6 ± 0.1[ab]	2.4 ± 0.1*
Heart	3.0 ±0.1	2.6 ± 0.1*[ab]	2.5 ± 0.0*[b]	2.0 ± 0.0*[ab]
Caudal neural tube	3.8 ±0.1	3.6 ± 0.1[a]	3.4 ± 0.1[a]	2.8 ± 0.1*[a]
Hindbrain	3.0 ± 0.0	2.6 ± 0.1[a]	2.3 ± 0.1*[a]	2.0 ± 0.0*[a]
Midbrain	3.0 ± 0.0	2.7 ± 0.1	2.5 ± 0.0	2.1 ± 0.1
Forebrain	3.2 ± 0.1	2.6 ± 0.1*	2.6 ± 0.1*[ab]	2.1 ± 0.1*
Optic system	3.2 ± 0.1	2.3 ± 0.1	2.3 ± 0.0	2.0 ± 0.0
Olfactory system	3.2 ± 0.1	2.2 ± 0.1*[ab]	1.6 ± 0.1*[b]	1.3 ± 0.1*
Branchial bars	3.8 ± 0.1	2.9 ± 0.1*[ab]	2.1 ± 0.1*[b]	1.5 ± 0.0*[ab]
Maxillary process	2.0 ± 0.0	1.5 ± 0.1*[a]	1.4 ± 0.1*[a]	1.0 ± 0.0*
Mandibular process	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0[ab]	1.0 ± 0.0[b]
Forelimb	3.0 ± 0.0	2.7 ± 0.1[a]	2.5 ±0.1*[b]	1.6 ± 0.1*[ab]
Hindlimb	2.0 ± 0.0	2.0 ± 0.0[a]	1.6 ± 0.1*[ab]	1.1 ± 0.1*[b]
Somites	4.0 ± 0.0	3.0 ± 0.0*	3.0 ± 0.0*	3.0 ± 0.0*[a]

[1]Morphological features were scored according to Brown and Fabro (1981)
[2]Values are means ± SEM
*Indicate the comparisons to Control are significantly different ($P < 0.05$)
[a]Indicate the comparisons T_2 to Toxaphene are significantly different ($P < 0.05$)
[b]Indicate the comparisons T_{12} to Toxaphene are significantly different ($P < 0.05$)

Table 2. Effects of T_2 on growth parameters and morphological features in cultured rat embryos

	Dose			
Feature[1]	Control	100ng/ml	1000ng/ml	5000ng/ml
Number of cultured embryos	6	8	8	8
Yolk sac diameter (mm)	[2]5.0 ± 0.1	4.7 ± 0.1*	4.6 ± 0.1*	4.2 ± 0.1*
Head length (mm)	2.3 ± 0.1	2.0 ± 0.1*	1.9 ± 0.2*[c]	1.6 ± 0.1*[c]
Yolk sac circulation	3.7 ± 0.1	3.0 ± 0.0*	3.0 ± 0.0*	3.0 ± 0.0*[c]
Allantois	3.0 ± 0.0	3.0 ± 0.0[c]	2.5 ± 0.0*	2.7 ± 0.1*[c]
Flexion	3.0 ± 0.0	3.0 ± 0.0[c]	2.1 ± 0.3*	2.6 ± 0.3[c]
Heart	3.7 ± 0.1	3.5 ± 0.0[c]	2.4 ± 0.1*[c]	2.7 ± 0.2*
Caudal neural tube	4.0 ± 0.1	3.0 ± 0.0*	2.6 ± 0.4	1.5 ± 0.4*
Hindbrain	3.0 ± 0.0	2.2 ± 0.1*	1.8 ± 0.2*[c]	0.8 ± 0.3*[c]
Midbrain	3.0 ± 0.0	2.7 ± 0.1	2.2 ± 0.2	1.6 ± 0.3
Forebrain	3.3 ± 0.1	2.6 ± 0.1*[c]	1.9 ± 0.1*[c]	1.9 ± 0.1*
Optic system	3.6 ± 0.1	2.4 ± 0.1*	2.1 ± 0.1*	1.9 ± 0.2*
Olfactory system	3.2 ± 0.1	1.7 ± 0.1*[c]	1.2 ± 0.1*	1.3 ± 0.1*
Branchial bars	3.7 ± 0.1	2.6 ± 0.1*	2.1 ± 0.1*	2.1 ± 0.1*[c]
Maxillary process	2.0 ± 0.0	1.3 ± 0.1*	1.0 ± 0.0*[c]	1.0 ± 0.0*
Mandibular process	1.0 ± 0.0	1.0 ± 0.0	0.8 ± 0.1*	0.9 ± 0.1[c]
Forelimb	3.0 ± 0.0	2.3 ± 0.1*	2.4 ±0.1*	2.4 ± 0.1*[c]
Hindlimb	2.0 ± 0.0	1.5 ± 0.0*	1.1 ± 0.2*[c]	1.0 ± 0.1*
Somites	4.0 ± 0.0	3.0 ± 0.0*	2.9 ± 0.1*	1.9 ± 0.3*

[1]Morphological features were scored according to Brown and Fabro (1981)
[2]Values are means ± SEM
*Indicate the comparisons to Control are significantly different ($P < 0.05$)
[c]Indicate the comparisons T_2 to $T_2 + T_{12}$ are significantly different ($P < 0.05$)

Table 3. Effects of T_{12} on growth parameters and morphological features in cultured rat embryos

	Dose			
Feature[1]	Control	100ng/ml	1000ng/ml	5000ng/ml
Number of cultured embryos	6	9	9	9
Yolk sac diameter (mm)	[2]4.7 ± 0.1	4.5 ± 0.1	4.4 ± 0.1*	3.8 ± 0.1*
Head length (mm)	2.2 ± 0.1	1.8 ± 0.1*	1.6 ± 0.1*	1.6 ± 0.1*
Yolk sac circulation	3.7 ± 0.1	3.3 ± 0.1*[d]	3.5 ± 0.0[d]	3.2 ± 0.1*[d]
Allantois	2.9 ± 0.1	2.5 ± 0.0*	2.5 ± 0.0*	2.4 ± 0.1*[d]
Flexion	3.0 ± 0.0	2.5 ± 0.0*	2.3 ± 0.1*	2.3 ± 0.1*[d]
Heart	3.7 ± 0.1	3.3 ± 0.1*[d]	3.1 ± 0.1*	3.0 ± 0.0*[d]
Caudal neural tube	4.0 ± 0.0	3.4 ± 0.1*[d]	2.9 ± 0.1*	2.9 ± 0.1*[d]
Hindbrain	3.0 ± 0.0	2.7 ± 0.1[d]	2.3 ± 0.1*	2.2 ± 0.1*[d]
Midbrain	3.0 ± 0.0	2.7 ± 0.1	2.5 ± 0.0	2.0 ± 0.0
Forebrain	3.3 ± 0.1	2.8 ± 0.1*	2.9 ± 0.1*	2.0 ± 0.0*
Optic system	3.3 ± 0.1	2.2 ± 0.1	1.9 ± 0.1	1.8 ± 0.1
Olfactory system	3.1 ± 0.1	1.3 ± 0.1*	1.0 ± 0.2*	1.3 ± 0.1*
Branchial bars	3.7 ± 0.1	2.4 ± 0.1*	2.6 ± 0.1*[d]	2.2 ± 0.1*[d]
Maxillary process	2.0 ± 0.0	1.7 ± 0.1*[d]	1.4 ± 0.1*	1.0 ± 0.0*
Mandibular process	1.0 ± 0.0	1.0 ± 0.0	0.7 ± 0.1*[d]	0.5 ± 0.0*
Forelimb	3.0 ± 0.0	2.8 ± 0.1[d]	2.8 ±0.1[d]	2.8 ± 0.1[d]
Hindlimb	2.0 ± 0.0	1.8 ± 0.1[d]	1.8 ± 0.1[d]	1.8 ± 0.1[d]
Somites	4.0 ± 0.0	3.0 ± 0.1*	3.0 ± 0.0*	3.0 ± 0.0*[d]

[1]Morphological features were scored according to Brown and Fabro (1981)
[2]Values are means ± SEM
*Indicate the comparisons to Control are significantly different ($P < 0.05$)
[d]Indicate the comparisons T_{12} to ~ - T_{12} are significantly different ($P < 0.05$)

Table 4. Effects of $T_2 + T_{12}$ on growth parameters and morphological features in cultured rat embryos

	Dose			
Feature[1]	Control	100ng/ml	1000ng/ml	5000ng/ml
Number of cultured embryos	6	8	8	8
Yolk sac diameter (mm)	[2]4.8 ± 0.1	4.6 ± 0.1	4.5 ± 0.1*	4.1 ± 0.1*
Head length (mm)	2.1 ± 0.1	1.7 ± 0.1	1.5 ± 0.1*	1.3 ± 0.1*
Yolk sac circulation	3.7 ± 0.1	2.9 ± 0.1	2.9 ± 0.0*	2.7 ± 0.1*
Allantois	2.9 ± 0.1	2.4 ± 0.1*	2.4 ± 0.1*	2.4 ± 0.1
Flexion	3.0 ± 0.0	2.3 ± 0.1*	2.2 ± 0.1*	1.6 ± 0.2*
Heart	3.4 ± 0.1	2.9 ± 0.1*	2.9 ± 0.1*	2.5 ± 0.2*
Caudal neural tube	3.6 ± 0.1	2.7 ± 0.1*	2.7 ± 0.1*	1.7 ± 0.2*
Hindbrain	3.0 ± 0.0	2.2 ± 0.1*	2.2 ± 0.1*	1.6 ± 0.2*
Midbrain	3.0 ± 0.0	2.5 ± 0.0	2.5 ± 0.0	1.7 ± 0.1
Forebrain	3.3 ± 0.1	2.9 ± 0.1*	2.9 ± 0.1*	1.8 ± 0.1*
Optic system	3.2 ± 0.1	2.1 ± 0.1	2.1 ± 0.1	1.8 ± 0.1
Olfactory system	3.0 ± 0.1	1.3 ± 0.1*	1.2 ± 0.1*	1.1 ± 0.1*
Branchial bars	3.4 ± 0.1	2.3 ± 0.1*	2.3 ± 0.1*	1.9 ± 0.2*
Maxillary process	1.9 ± 0.1	1.4 ± 0.1*	1.4 ± 0.1*	1.0 ± 0.0*
Mandibular process	1.0 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	0.5 ± 0.0*
Forelimb	3.0 ± 0.0	2.4 ± 0.1*	2.4 ±0.1*	1.7 ± 0.1*
Hindlimb	2.2 ± 0.1	1.4 ± 0.1*	1.4 ± 0.1*	0.9 ± 0.1*
Somites	4.0 ± 0.0	3.0 ± 0.0*	3.0 ± 0.0*	1.9 ± 0.2*

[1]Morphological features were scored according to Brown and Fabro (1981)
[2]Values are means ± SEM
*Indicate the comparisons to Control are significantly different ($P < 0.05$)

Table 5. Chi-square test with Continuity Correction

	Specific Malformations		
	Flexion (%)	Neural Tube (%)	...mbs (%)
Toxaphene[1]	0	42.9	0
Toxaphene[2]	0	46.2*	0
Toxaphene[3]	0	53.8*	53.8*
T_2[1]	0	47.1*	17.6
T_2[2]	6.7	46.7*	26.7
T_2[3]	15.5*	50.0*	37.5*
T_{12}[1]	0	13.3	0
T_{12}[2]	0	56.3	0
T_{12}[3]	0	62.5	0
$T_2 + T_{12}$[1]	0	0	0
$T_2 + T_{12}$[2]	7.1	57.1*	21.4
$T_2 + T_{12}$[3]	21.4*	57.1*	57.1*

[1]100 ng/ml
[2]1000 ng/ml
[3]5000 ng/ml
* $P < 0.05$ as compared to control

Figure 1 **Total Morphological Score**

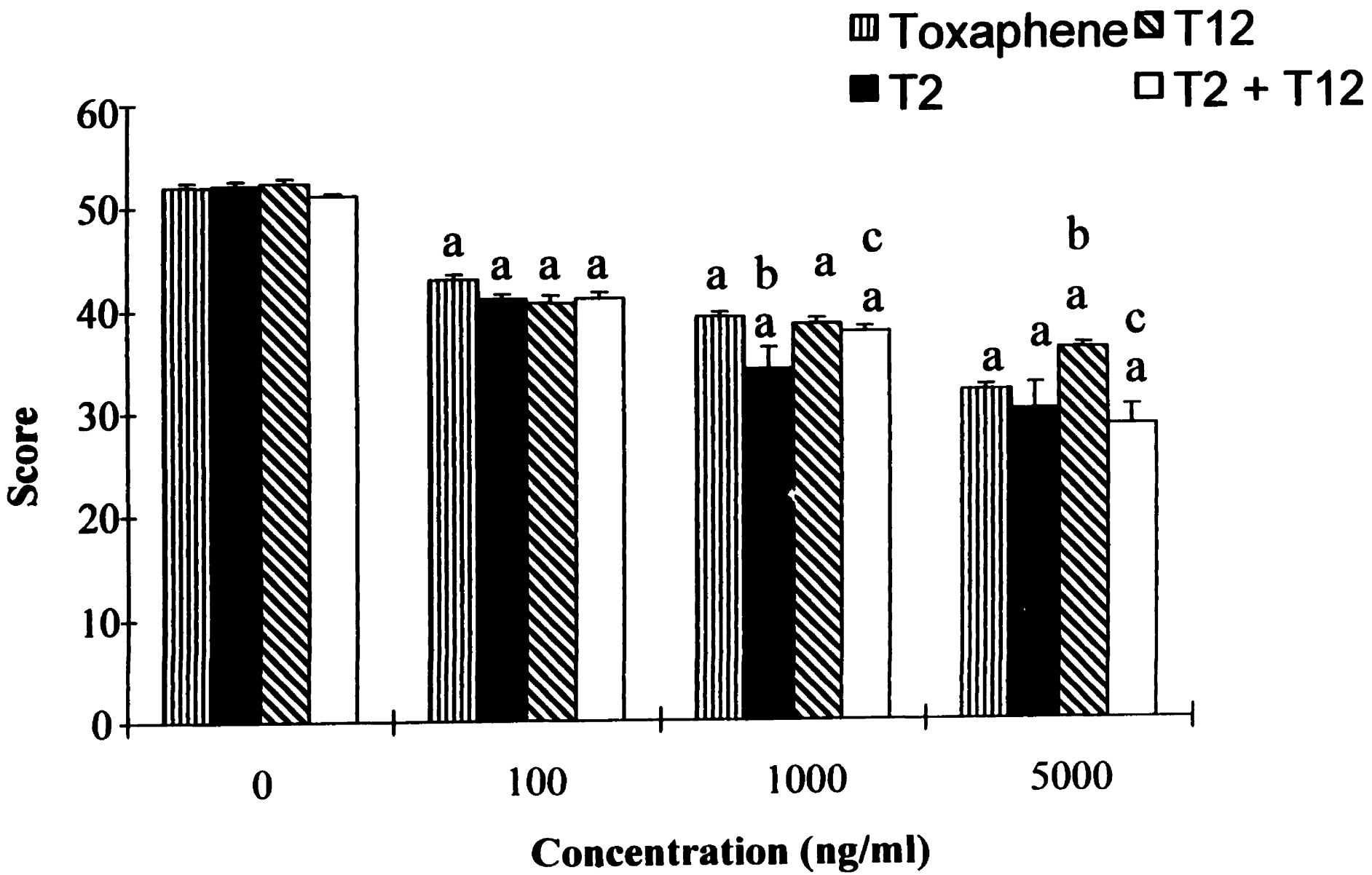


Figure 2 **Crown Rump Length**

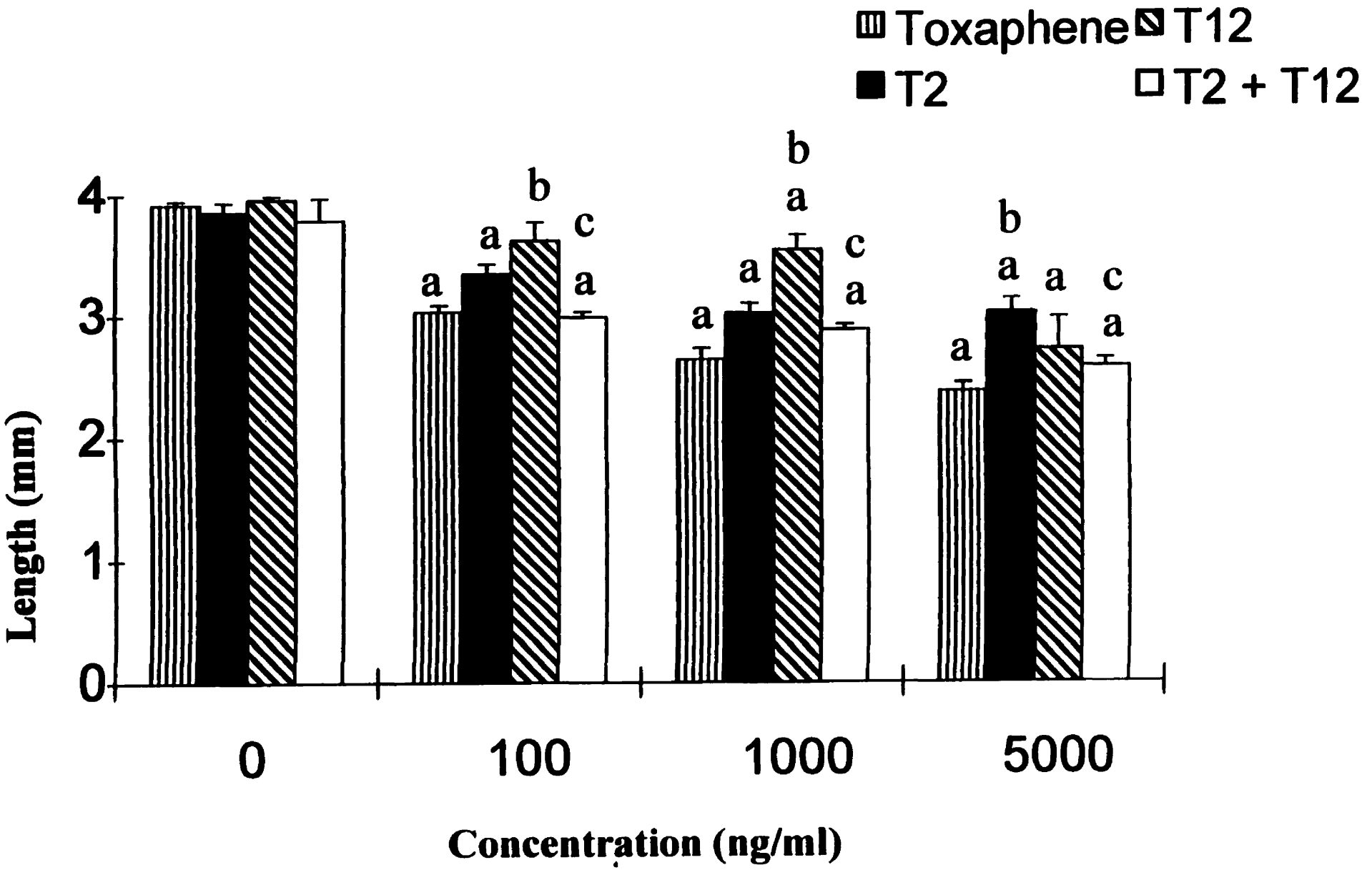
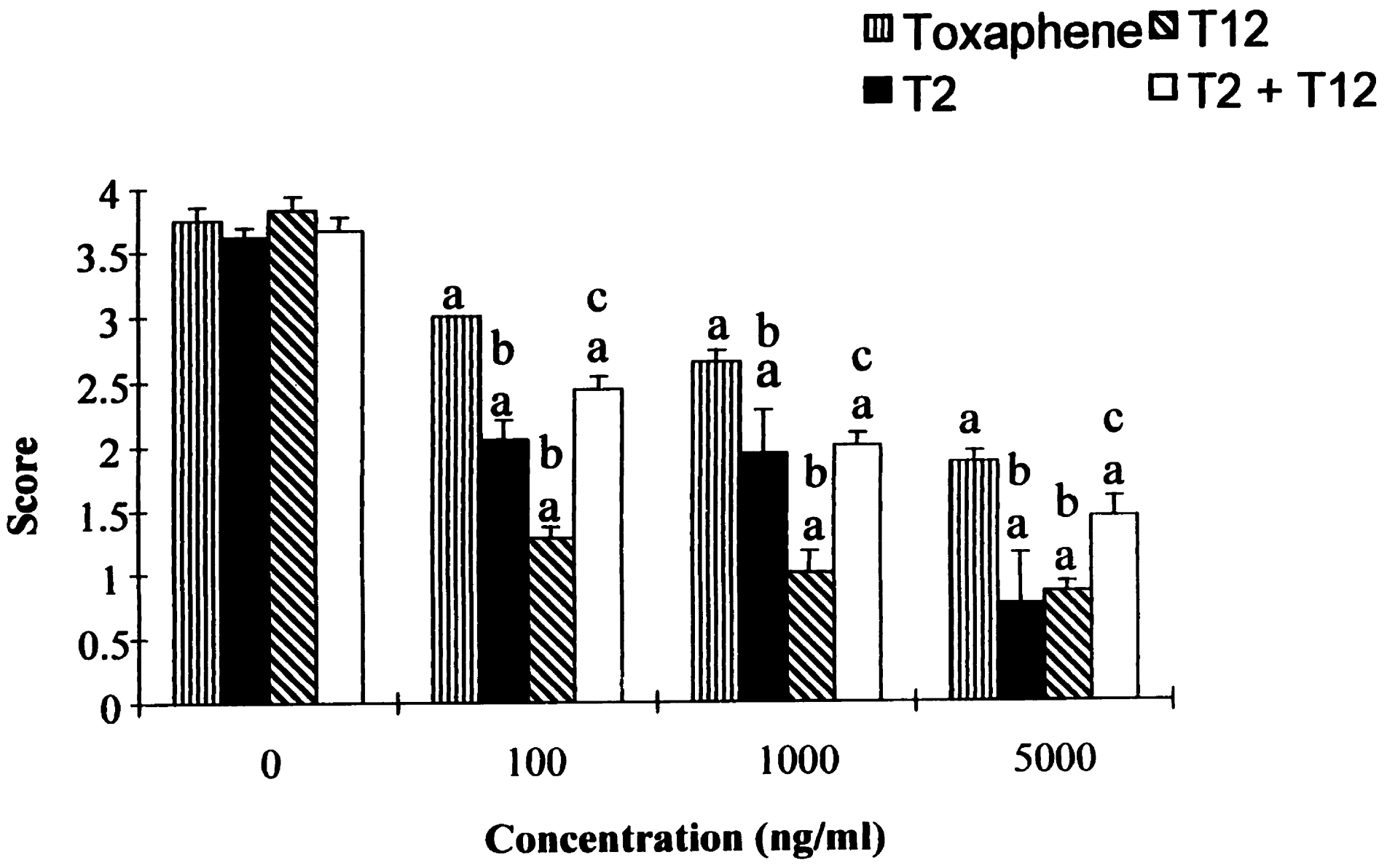


Figure 3

Otic System



LEGENDS TO FIGURES

Figure 1.

Total morphological score of embryos cultured for 48 h and dosed with toxaphene mixture, T_2, T_{12}, and $T_2 + T_{12}$

[a] significantly different than control ($P < 0.05$)

[b]significantly different from toxaphene mixture at the same dose ($P < 0.05$)

[c]significantly different from $T_2 + T_{12}$ at the same dose ($P < 0.05$)

Figure 2.

Crown rump length of embryos cultured for 48 h and dosed with toxaphene mixture, T_2, T_{12}, and $T_2 + T_{12}$

[a] significantly different than control ($P < 0.05$)

[b]significantly different from toxaphene mixture at the same dose ($P < 0.05$)

[c]significantly different from $T_2 + T_{12}$ at the same dose ($P < 0.05$)

Figure 3.

Otic system score of embryos cultured for 48 h and dosed with toxaphene mixture, T_2, T_{12}, and $T_2 + T_{12}$

[a] significantly different than control ($P < 0.05$)

[b]significantly different from toxaphene mixture at the same dose ($P < 0.05$)

[c]significantly different from $T_2 + T_{12}$ at the same dose ($P < 0.05$)

MANUSCRIPT 2

Thesis Chapter 4

Interactive Dysmorphogenic Effects of Toxaphene or Toxaphene Congeners and Hyperglycemia on Cultured Whole Rat Embryos During Organogenesis

SUMMARY

Diabetes mellitus, one of the most common maternal illnesses resulting in congenital defects is now on an upgrowth trend in many native communities. In addition, the presence of persistent organic pollutants, such as the pesticide toxaphene, which has been reported even in remote regions, such as in the Canadian Arctic, is becoming a health concern. In earlier work, we established the embryotoxicity of the toxaphene technical mixture (TOX) and its two major congeners, T_2 (*2-exo,3-endo,5-exo,6-endo*,8,8,10,10-octachlorobornane) and T_{12} (*2-exo,3-endo,5-exo,6-endo*,8,8,9,10,10-nonachlorobornane). The interactive effects of toxaphene or its two congeners and high glucose concentration were studied using rat embryo culture. Whole rat embryos (0-2 somite) were explanted and cultured into a normal (8 mM) or hyperglycemic 12.5 mM (12.5 G) or 18.75 mM (18.75 G) culture medium containing TOX, T_2, or T_{12} at various concentrations (0 ng/ml, 100 ng/ml, 1000 ng/ml, 5000 ng/ml) for 48 h at 37°C. The treatment period corresponds to gestational days (GD) 10-12, the critical time of morphogenesis and organogenesis. All treatments, except mild hyperglycemic exposure (12.5 G), had significant adverse effects on the total morphological score, somite number, head and crown rump length, and the central nervous system scores. The embryos cultured with TOX under severe hyperglycemic conditions exhibited a concentration related interactive effect. Interactive effect with hyperglycemia (18.75 G) was shown in two, five, and eight parameters at 100, 1000, and 5000 ng/ml TOX exposure respectively. Similar concentration related additive effects were present between T_2 or T_{12} and hyperglycemia (18.75 G). T_{12} was less toxic compared with TOX

and T_2. The major malformations were abnormalities in the development of the central nervous system. Embryos exposed to 18.75 G did not show central nervous system, limb, or flexion abnormalities. Hyperglycemia, however, when combined with higher doses of TOX or T_2 showed synergistic effects on CNS malformations. The results suggest that there is a site-specific and dose-related interactive dysmorphogenesis elicited by TOX or its congeners and high levels of glucose in rat embryonic development.

INTRODUCTION

Diabetes mellitus is one of the most common maternal illness resulting in congenital defects. Major congenital anomalies occur in 8 to 12 % of pregnancies complicated by pregestational diabetes mellitus, which represents a three- to fivefold increase over the rate seen in the general population (Becerra et al., 1990). The malformations occur early in the course of development, during the embryonic period, but there are no diabetes specific birth defects. Diabetic-related malformations, however, are major anomalies, which can involve multiple organs. These include heart, kidneys, skeleton, and genitalia. Abnormalities of the central nervous system such as anencephalia and spina bifida are commonly found in infants of diabetic mothers (Gabbe, 1977).

Studies of pregnant diabetic women, who are hyperglycemic during organogenesis, have suggested that elevated glucose levels adversely affect the developing embryo. In rodent embryo culture studies have demonstrated that hyperglycemia has a teratogenic effect during organogenesis (Cochroft et al., 1977; Sadler et al., 1980). The mechanism whereby hyperglycemia may induce malformations has not been completely elucidated. One proposed concept of "fuel-mediated teratogenesis" by Freinkel (1980) looks at the alterations in the maternal glucose, amino acid, and lipid levels as instrumental in the induction of dysmorphogenesis in the offspring. Damage of the developing yolk sac (Freeman et al., 1981), nonenzymatic glycosylation of proteins (Kubow et al., 1993), and the liberation of free oxygen radicals

(Eriksson et al., 1993) are a few theories which have been postulated as the etiology of diabetes-related malformations. The diabetic state itself results in a variety of metabolic disturbances including the presence of hyperglycemia, hyperketonemia, somatomedin inhibitors, zinc deficiency, arachidonic acid or myo-inositol deficiency, and the generation of free radicals (Reece et al., 1997). As a result of these aberrant fuels the teratogenic process in the diabetic pregnancy is likely multifactorial.

Toxaphene is a complex mixture of polychlorinated bornanes, which has a broad spectrum of pesticidal activity. It is persistent in soils and lake sediments (Bidleman et al., 1988) and has been found in fish (Stern et al., 1992), marine mammals (Bidleman et al., 1993), terrestrial mammals (Zhu and Norstrom, 1993) and in human milk (Bidleman et al., 1988). It has been shown to be acutely and chronically toxic and to posses a carcinogenic risk to humans (Saleh, 1991). Toxaphene was found to be the most prevalent organochlorine compound in Great Lakes fish (Glassmayer, 1997). In the Arctic a high percentage of the indigenous people have dietary exposure levels which exceed the tolerable daily intake level (TDI) of 0.2 μg/kg body weight/day (Chan et al, 1997). Therefore the toxicology of toxaphene is receiving renewed attention.

A strong relationship has been found between embryo-fetal mortality and maternal toxicity in rodents upon toxaphene exposure (Kavlock et al., 1982). Moreover, Chernoff et al. (1990) showed that rat litters of dams treated with toxaphene had an elevated incidence of supernumerary ribs. Teratogenicity of toxaphene was demonstrated in the teleost embryo culture model (Crowford et al., 1985) and more recently in the

rodent embryo culture model (Calciu et al., 1997). The two congeners (T_2 and T_{12}) had significant adverse effects on total morphological score, somite number, crown rump length, head length, and the central nervous system scores of embryos. Moreover, the mixture of $T_2 + T_{12}$ showed a synergistic effect on decreasing crown rump length and head diameter.

Because the potential for human exposure in utero to teratogens, xenobiotics or metabolic agents remains very high, it is important to study the interactive effects of different compounds on embryonic development. Teratogens with very different mechanisms of action can interact and potentiate developmental defects. In vitro culture technique for postimplantation rodent embryo has been extensively used in the search for critical factors and conditions with possible relevance to diabetic pregnancy. Reece et al. (1996) using a post-implantation rat embryo culture demonstrated that there is a critical age (GD 10-12) and duration of exposure of greater than 24 h to hyperglycemia was necessary to induce embryopathy. In vitro studies have also demonstrated a synergistic effect between glucose and an other teratogenic metabolic agent, ß-hydroxybutyrate. Minimally teratogenic amounts of glucose combined with minimally teratogenic amounts of ß-hydroxybutyrate, during culture of rat embryos caused a significantly greater reduction of crown rump length and mean embryonic protein content than either of these metabolites alone (Sadler et al., 1988).

Diabetes mellitus is now on an upgrowth trend in many native communities (Young et al., 1993). The same populations may have higher exposure to toxaphene as it

is present at elevated levels in their food systems. Technical mixture of toxaphene has over 800 congeners (Saleh, 1991). Since they are metabolized along the food chain, there are only two to ten major congeners in most fish and wildlife. The two major congeners are T_2 (*2-exo,3-endo,5-exo,6-endo*,8,8,10,10-octachlorobornane) and T_{12} (*2-exo,3-endo,5-exo,6-endo*,8,8,9,10,10-nonachlorobornane). Because the potential for human exposure in utero to teratogens, xenobiotics or metabolic agents, like glucose, remains very high it is important to study the interactive effects of different compounds on embryonic dysmorphogenesis. Xenobiotics, such as toxaphene or its two major congeners, may thus induce interactive dysmorphogenic effects with hyperglycemia since both hyperglycemia and toxaphene are known to adversely affect embryonic development in rodents. Thus, embryotoxic effects of toxaphene and its two major congeners (T_2 and T_{12}) may be exacerbated by metabolic factors of diabetes such as hyperglycemia. Because the potential for poor preconception control of hyperglycemia remains very high (Kitzmiller, 1991), particularly in areas with relatively poor access to medical care such as native communities, it is important to study the possible interactive effects between hyperglycemia and toxaphene on embryonic growth and development.

In this study, the direct interactive effects of toxaphene technical mixture (TOX), T_2 or T_{12} and hyperglycemic conditions were investigated. Whole rat conceptuses, at gestational day (GD) 10 were incubated with TOX, T_2 or T_{12} in normoglycemic or hyperglycemic serum for 48 h and the effects on growth and morphogenesis were examined. A mammalian system (rodent embryo culture) was chosen to provide a more physiologically relevant system with respect to implications on human health.

MATERIALS AND METHODS

Animal maintenance and mating procedures

Virgin female Sprague-Dawley rats (180-200 g) (Charles River Canada, St. Constant, PQ, Canada) were used in all experiments. Upon arrival, the females were housed in a temperature-controlled room (20°C) with a 14L-10D reversed light cycle (lights on at 1600). The animals were kept in plastic shoe box cages with Beta-Chip bedding (Beta-Chip, Northeastern Products Corp., Warrenburg, NY, USA). Purina Rat Chow (Ren's Feed and Supply, Oakville, Ontario, Canada) and tap water were fed *ad libitum*. Females were housed three per cage and males caged individually. After three weeks acclimation period, for mating purposes, three or four females were housed individually with a male, at random, from 0900 to 1500. The presence of sperm in the vaginal smear indicated insemination and this day was considered as gestational day 0.5.

Chemicals

Technical toxaphene mixture was purchased from Ultra Scientific (North Kingstown, RI.). Toxaphene congeners, T_2, and T_{12} (1 mg) were obtained from Promochem (Wesel, Germany). Toxaphene was dissolved in 0.01% DMSO (dimethylsulfoxide) and added to 10 ml of male rat serum. Culture media were dosed with this stock solution to final concentrations of 100, 1000 and 5000 ng/ml. Control embryos were treated with 0.01% solution of DMSO. D-Glucose (Sigma Chemical Co., St. Louise, MO) was dissolved in distilled water and added to 1.0 ml of male rat serum. Control embryos received equivalent volumes of distilled water.

Experimental design

The experimental design was set up as a 3 x 4 factorial for the toxaphene technical mixture groups and as a 2 x 4 factorial for the T_2 and T_{12} treatment groups. On gestational day (GD) 10, 46 to 50 pregnant rats were sacrificed and a total of 232 embryos containing 0-2 somites were randomly assigned to the treatment groups:

1) normoglycemic medium (control) in which the glucose concentration was 8 mM
2) mild hyperglycemic medium (12.5G) prepared by adding D-glucose (final culture concentration 12.5 mM) to the same rat serum used in the control group
3) severe hyperglycemic medium (18.75G) prepared by adding D-glucose (final culture concentration 18.75 mM) to the same rat serum used in the control group
4) toxaphene technical mixture in three dosages (100, 1000, and 5000 ng/ml) + 12.5G
5) toxaphene technical mixture in three dosages (100, 1000, and 5000 ng/ml) + 18.75G
6) T_2 in three dosages (100, 1000, and 5000 ng/ml) + 18.75G
7) T_{12} in three dosages (100, 1000, and 5000 ng/ml) + 18.75G

The embryos were incubated for 48 h.

Whole embryo culture

The techniques for explanting and culturing embryos followed the methods described by New (1978). To provide serum for embryo culture, male Sprague-Dawley rats (Charles River Canada) were exsanguinated under halothane anaesthetic. The collected blood was centrifuged immediately and then allowed to stand until the plasma clot had formed. The plasma clot was removed and the serum was collected. After recentrifugation, the serum was pooled. Pooled serum was heat-inactivated at 56°C for 30 min and filter-sterilised using 0.45 μm mesh filter (Millipore Corp., Bedford, MA). The heat-inactivated, pooled serum was stored at –80°C and thawed immediately before use in embryo culture. At 0900 h on day 10 of gestation, rats were killed under halothane anaesthetic and their uteri excised immediately. Under aseptic conditions, the decidua were dissected from the uterus in Hanks' Balanced Salt Solution (HBSS; Gibco, Burlington, ON, Canada) with a dissecting stereomicroscope. Decidual tissue and Reichert's membrane were removed and the ectoplacental cone, amnion, and visceral yolk sac were left intact. Embryos containing 0-2 somites were chosen for culture. Randomly selected embryos were placed into 60-ml glass culture bottles containing 1.99 ml of warm sterile male rat serum per embryo. Streptomycin sulfate and penicillin G potassium were added to final concentration of 10000 IU/ml. The bottles were gassed for 2 min with 20% O_2:5% CO_2:75% N_2 immediately before and after addition of the embryos. The same gas mixture was also used to pass over the serum 4-6 h after embryo transfer. The culture flasks were rolled on a 30-rpm rotator wheel in a 37°C incubator. Embryos were re-gassed at a 12 h intervals on the second day of culture with 95% O_2.

Growth and morphological assessment of cultured embryos

After 48 h in culture, embryos were transferred in 0.9% saline solution and examined under a dissecting microscope. They were checked for survival as indicated by the presence of a heartbeat and yolk sac circulation. Dead embryos were not further analyzed. The yolk sac diameter, crown-rump length, and head length of live explanted embryos were measured with the aid of an eyepiece micrometer. Embryos were given a differentiation score according to the method of Brown and Fabro (1981), under which 13 morphological criteria are each given a numerical score ranking from 0 to 5, corresponding to a given stage of development for each organ and tissue. The sum of the scores from each individual morphological feature gives a total morphological score that is indicative of the stage of development of each embryo. Somite counts were performed only on embryos that had turned completely. The following features were considered abnormal for midgestational (GD 10 to 12) embryos: unfused neural tube, hind brain, mid brain or fore brain, malrotated, kinked or ventrally convex flexion of the tail, and underdeveloped forelimb or hindlimb. The frequency of having malformations was defined as having scored all the four criteria for neural and brain development below the predetermined cutoff points. The cutoff points were determined by using control embryos as the standard reference. All control embryos had closed posterior and anterior neuropores, mesencephalic and prosencephalic folds were completely fused. Therefore, embryos having scores lower than these points for all of the four criteria were considered as CNS malformations. Similarly, the presence of limb malformations, as measured by fore limb and hind limb was tested against the control.

Statistical analysis

Data are presented as means ± SEM. The dose response of the different toxaphene and glucose groups was analysed by two-way ANOVA followed by Tukey's comparison test. The incidence of neural tube and limb abnormalities was compared using Fisher's Exact Test. Statistical tests were performed with the Statistical Analysis System for personal computers (SAS Institute, Cary, NC, version 6.10). $P < 0.05$ was accepted as the minimal level of significance.

RESULTS

Normoglycemic (control) 8.0 G embryos explanted at gestation day 10 and cultured for 48 h in vitro progressed normally from the 0-2 somites stage to the 30-34 somite stage (scored 4). During this period, embryos underwent rotation, neural folds had fused, major brain vesicles had formed and a yolk sac circulation had been initiated.

The effects of treatment on specific embryological growth parameters were compared, and the data are presented in Tables 1 to 4. The total morphological score, crown rump length, head length were significantly decreased by all treatments, except for the mild hyperglycemic (12.5 G) treatment. Embryos cultured under mild hyperglycemic conditions (12.5 G) progressed normally and were not adversely affected by this treatment (Table 1).

Embryos cultured under severe hyperglycemic conditions (18.75 G) consistently received lower morphological scores for all parameters, except for flexion, midbrain, mandibular process, and somite number. However, they did not show CNS, limb, or flexion abnormalities or any other malformations. The mean scores for all other features were significantly lowered when compared with the control group (8.0 G). The severe hyperglycemic media also adversely affected the yolk sac circulation (Table 2).

The total morphological score, crown rump length and head length were significantly decreased by TOX treatment at all concentrations, indicating that toxaphene

technical mixture caused retarded growth of the embryos. The morphological development of the embryos was also affected by TOX treatment. A significant score reduction in all features, except the mandibular process, was observed (Table 2). TOX treatments caused a high incidence of concentration-related central nervous system defects.

An interactive effect between mild hyperglycemic (12.5 G) conditions and TOX was observed for all three concentrations in the otic system (Table 1). At 1000 ng/ml and 5000 ng/ml TOX an interactive effect with 12.5 G exposure was observed in yolk sac circulation. The 5000 ng/ml dose TOX + 12.5 G group had a lower score for hindbrain and forelimb, when compared with both 12.5 G and TOX groups. Embryos cultured with TOX under mild hyperglycemic conditions did not show CNS, limb, or flexion abnormalities.

In general, embryos in TOX + 18.75 G groups consistently received lower morphological scores for a number of parameters when compared with their respective normoglycemic TOX groups (Table 2). Embryos cultured in the 1000 ng/ml and 5000 ng/ml TOX + 18.75 G groups received a significantly lower score for yolk sac diameter, head length, and olfactory system, when compared with embryos exposed to severe hyperglycemia or TOX alone. Embryos cultured with 100 ng/ml and 1000 ng/ml TOX and 18.75 G exhibited a lower score for branchial bars than all other normoglycemic groups. Somite number was also lower in the TOX + 18.75 G group than either of the treatment alone. Both mild and severe hyperglycemic groups exhibited normal tail

(rotated to the right side) development. Addition of TOX, however, produced various types of tail abnormalities. Embryos cultured in the 100 ng/ml + 18.75 G and 1000 ng/ml + 18.75 G TOX groups had kinked tails. Moreover, ventrally convex tail (unturned tails) was found in the 5000 ng/ml TOX + 18.75 G group (Table 5).

The embryos cultured with TOX under hyperglycemic conditions (18.75 G) exhibited a concentration related interactive effect. At 5000 ng/ml an interactive effect was present in eight parameters. There were five features at 1000 ng/ml and there were two features (branchial bars and somite number) at 100 ng/ml that showed an additive effect.

While the TOX + 18.75 G group showed no additive effect on yolk sac circulation, the yolk sac circulation was severely affected in the T_2 + 18.75 G group (Table 3). The embryos cultured with T_2 at all concentrations + 18.75 G exhibited a total morphological score significant lower when compared with 18.75 G group. However, there was no interactive effect between hyperglycemic conditions and T_2 for total morphological score. T_2 + 18.75 G decreased the growth of embryos as indicated by the crown rump length at 5000 ng/ml. They also showed an additive effect on morphological development of most features, except for mid- and forebrain, optic and olfactory system, maxillary process, and hind limb. At lower doses of 100 ng/ml and 1000 ng/ml, six parameters showed a significant difference when compared with T_2 or the 18.75 G group alone.

The embryos cultured with T_{12} at 1000 ng/ml and 5000 ng/ml + 18.75 G exhibited a total morphological score significantly lower when compared with T_{12} or 18.75 G group alone (Table 4). An interactive effect between T_{12} and 18.75 G was also observed at 5000 ng/ml in the head length. The embryos in the T_{12} + 18.75 G group had a significantly lower score for midbrain when compared with T_{12} and 18.75 G treatments. When the same comparison was made, the branchial bars score was lower at 1000 ng/ml and 5000 ng/ml doses. Yolk sac circulation was significantly affected by the presence of severe hyperglycemia and the 5000 ng/ml dose. The hyperglycemic T_{12} treatment showed additive effects in six parameters at both 1000 ng/ml and 5000 ng/ml dose and only in three parameters at 100 ng/ml dose.

Analyses of interactive effects of TOX and its congeners and hyperglycemia were performed with contingency tables using the chi-square criteria and results are summarized in Table 5. Exposure to 18.75 G did not cause abnormalities in the development of the central nervous system (i.e., unfused neural folds, open neural tube), limbs, or flexion. However, TOX + 18.75 G and T_2 + 18.75 G treatments caused CNS malformations at 1000 ng/ml and 5000 ng/ml (Table 5). Incidences of abnormal CNS development were higher than those expected on the basis of results obtained with embryos incubated with TOX or T_2 alone at the same concentrations. Limb malformations were observed in hyperglycemic treatment (18.75 G) + TOX (5000 ng/ml), + T_2 (1000 and 5000 ng/ml) and T_{12} (1000 ng/ml and 5000 ng/ml). However, there was no significant additive effect (Table 5). Thus, synergistic effects were observed in CNS defects between severe hyperglycemia and exposure to toxaphene compounds.

The results of total morphological score, yolk sac circulation score, and head length are presented in Figures 1-3 in order to illustrate the effects of the treatments on morphological development and growth of the embryos. All toxaphene treatments show concentration-dependent adverse effects on morphological score (Fig 1a, b, and c). Mild hyperglycemic conditions (12.5 G) did not affect the toxicity. Severe hyperglycemic serum (18.75 G) decreased the morphological score in 0 and 5000 ng/ml toxaphene treatment and 1000 and 5000 ng/ml T_{12} exposure.

All toxaphene treatments show concentration-dependent adverse effects on head length (Fig 2a, b, and c). Severe hyperglycemic serum (18.75 G) decreased the head length in 1000 and 5000 ng/ml toxaphene technical mixture treatment and 5000 ng/ml T_{12} treatment.

Yolk sac circulation was affected adversely by hyperglycemia in T_2 (1000 and 5000 ng/ml) and T_{12} (5000 ng/ml) treatments (Fig 3a,b, and c).

DISCUSSION

The morphological growth and development of the embryos in the present study clearly demonstrate that dysmorphogenic effects of toxaphene technical mixture and its two congeners (T_2 and T_{12}) were increased when coupled with the hyperglycemic media on early stage (0-2 somite) embryos. The most significant finding of this study was the synergistic negative effect of TOX, T_2, or T_{12} and severe hyperglycemia on CNS development. As previously observed, in vitro studies performed with cultured whole rat embryos showed that addition of either D-glucose, toxaphene, or its two major congeners to the culture media resulted in severe CNS abnormalities. Toxaphene technical mixture or its congeners caused CNS defects, but this effect was worsened by the addition of high glucose concentrations. For example, addition of D-glucose (18.75 mM) did not affect the normal development of neural tube and encephalon while addition of TOX + 18.75 G induced severe CNS abnormalities. The dysmorphogenic effects on CNS produced by toxaphene and hyperglycemia were observed at glucose concentrations (18.75 mM) approximately three times lower than the concentrations required of glucose alone (50 mM) to produce similar CNS abnormalities (Reece et al., 1996).

The combination of toxaphene technical mixture or its major congeners with hyperglycemia was clearly shown to be more deleterious for CNS than either of them separately. Similar synergistic dysmorphogenic effects have been previously observed between two teratogens in vivo. For example, when two drugs (cocaine and diazepam)

were administered together significantly greater reductions in fetal weight and length were observed than when these agents were given individually (Mehanny et al., 1991).

The present results are in agreement with previous studies which looked at the dysmorphogenic effects of hyperglycemia in rodent embryos in vitro (Sadler, 1980; Cockroft, 1984; Pinter et al., 1986; Styrud et al., 1992). The toxicity of the two glucose concentrations (12.5 G and 18.75 G) added to the culture media, however, was different. Exposure of embryos to 12.5 G treatment had a slight but not significant adverse effect on growth. In comparison, severe hyperglycemic media (18.75 G) caused a reduction in the rate of growth and differentiation, as evidenced by reduced somite number, yolk sac size, crown rump length, head length and the overall morphological score.

Moreover, these observations are consistent with the findings of Reece et al. (1996) who demonstrated that hyperglycemic culture media 1.5 times control levels caused no malformations or decrease in total morphological score as opposed to three times control levels which produced a decrease in the total morphological score. Therefore, there appears to be a concentration threshold of glucose above which growth retardation and malformations occur. On the other hand, the present results showed that both 12.5 G and 18.75 G concentrations worsened the dysmorphogenic effects of toxaphene technical mixture or its congeners. These results indicate that lower glucose concentrations can exacerbate dysmorphogenic effects of another teratogen such as toxaphene. Mild hyperglycemic exposure (12.5 G) had an adverse additive effect with toxaphene technical mixture only on the otic system. Severe hyperglycemic conditions,

however, had an adverse effect on morphological scores of all parameters, except flexion, midbrain, mandibular process, and somite number. Addition of 18.75 G to 5000 ng/ml dose of TOX significantly also affected the normal development of CNS, while more than a 2.5 fold higher concentration of glucose alone was required to produce a similar incidence of malformations on the cultured embryos (Reece et al., 1996). Such results are consistent with in vitro cell culture studies which have shown that it is not feasible to predict the toxicity of pesticide mixtures on the basis of the results of the toxicity of single components (Marinovich et al., 1996).

The effects of hyperglycemia on the toxicity of the congeners were also different. There was a significant interactive effect for hyperglycemia and T_{12} exposure at 1000 ng/ml and 5000 ng/ml but no such effect was observed for T_2 exposure. In vitro animal studies have demonstrated that addition of D-glucose up to 50 mM to the culture media resulted in severe abnormalities and retarded development in a high proportion of embryos. In this study glucose levels were chosen closer to the physiological values, which normally are not associated with major defects.

In addition, the dysmorphogenic effects of TOX, T_2, or T_{12} and 18.75 G were target site-specific (i.e., CNS, flexion, and limb abnormalities). TOX + 18.75 G specifically affected the yolk sac diameter, head length, and olfactory system. T_2 treatment + 18.75 G had an adverse effect on yolk sac circulation, allantois, branchial bars, and fore limb scores. On the other hand T_{12} + 18.75 G adversely affected the midbrain and branchial bars.

In summary, the interactive developmental toxicity of individual environmentally prevalent toxaphene congeners and hyperglycemia are demonstrated for the first time in the mammalian system using an embryo culture model. Morphological defects were observed at glucose levels only twice the normal values. These levels are typically seen in uncontrolled diabetic patients (Sherwin, 1996). Even though toxaphene levels in cord blood are likely about 1/10 of the concentration that we used in this study, the findings of interactive effect is significant in the public health context. Therefore, a close monitor of the situation of toxaphene exposure and blood glucose levels is needed in the concerned native communities.

Table 1. Effects of toxaphene technical mixture and glucose (12.5 G) on growth parameters and morphological features in cultured rat embryo

	Dose							
Feature[†]	Control	12.5 G	TOX[1]	TOX[1]+12.5G	TOX[2]	TOX[2]+12.5G	TOX[3]	TOX[3]+12.5G
Number of embryos cultured	6	7	7	7	7	9	7	9
Yolk sac diameter (mm)	[‡]5.1 ± 0.1	4.9 ± 0.1	4.2 ± 0.1[#]	4.2 ± 0.1[*]	4.2 ± 0.1[#]	4.2 ± 0.1[*]	4.1 ± 0.1[#]	4.2 ± 0.1[*]
Crown rump length (mm)	3.9 ± 0.1	3.6 ± 0.1	3.0 ± 0.1[#]	3.1 ± 0.1	2.7 ± 0.1[#]	2.7 ± 0.1	2.3 ± 0.1[#]	2.4 ± 0.1
Head length (mm)	2.4 ± 0.1	2.2 ± 0.1	1.8 ± 0.1[#]	1.8 ± 0.1[*]	1.7 ± 0.1[#]	1.6 ± 0.1[*]	1.5 ± 0.1[#]	1.5 ± 0.1[*]
Total morphological score	52.0 ± 0.5	51.6 ± 0.3	42.8 ± 0.5[#]	43.4 ± 0.6	39.1 ± 0.5[#]	38.4 ± 0.4	31.9 ± 0.5[#]	32.3 ± 0.9
Yolk sac circulation	3.7 ± 0.1	3.6 ± 0.1	2.9 ± 0.1[#]	3.0 ± 0.0[*]	2.5 ± 0.0[#]	3.0 ± 0.0[*,a]	2.1 ± 0.1[#]	2.6 ± 0.1[*,a]
Allantois	3.0 ± 0.0	3.0 ± 0.0	2.5 ± 0.0[#]	2.9 ± 0.1	2.4 ± 0.1[#]	2.5 ± 0.0[*]	2.0 ± 0.0[#]	2.1 ± 0.1[*]
Flexion	3.0 ± 0.0	3.0 ± 0.0	2.6 ± 0.1[#]	2.8 ± 0.1	2.6 ± 0.1[#]	2.6 ± 0.1[*]	2.4 ± 0.1[#]	2.4 ± 0.1[*]
Heart	3.3 ±0.1	3.5 ± 0.1	2.6 ± 0.1[#]	2.9 ± 0.1[*]	2.5 ± 0.0[#]	2.4 ± 0.1[*]	2.0 ± 0.0[#]	2.5 ± 0.1[*]
Caudal neural tube	3.8 ± 0.1	3.8 ± 0.1	3.6 ± 0.1	3.1 ± 0.1	3.4 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.5 ± 0.1
Hindbrain	3.0 ± 0.0	3.1 ± 0.1	2.6 ± 0.1[#]	2.7 ± 0.1	2.3 ± 0.1[#]	2.6 ± 0.1[*]	2.0 ± 0.0[#]	1.6 ± 0.2[*,a]
Midbrain	3.0 ± 0.0	3.0 ± 0.0	2.7 ± 0.1[#]	2.9 ± 0.1[*]	2.5 ± 0.0[#]	2.9 ± 0.1	2.1 ± 0.1[#]	2.1 ± 0.1[*]
Forebrain	3.2 ± 0.1	3.3 ± 0.1	2.6 ± 0.1[#]	2.7 ± 0.1[*]	2.6 ± 0.1[#]	2.6 ± 0.1[*]	2.1 ± 0.1[#]	2.1 ± 0.1[*]
Otic system	3.8 ± 0.1	3.5 ± 0.0	3.0 ± 0.0[#]	2.6 ± 0.1[*,a]	2.6 ± 0.1[#]	2.2 ± 0.1[*,a]	1.9 ± 0.1[#]	1.4 ± 0.3[*,a]
Optic system	3.2 ± 0.1	3.3 ± 0.1	2.3 ± 0.1[#]	2.5 ± 0.2[*]	2.3 ± 0.0[#]	2.2 ± 0.1[*,a]	2.0 ± 0.0[#]	1.8 ± 0.1[*,a]
Olfactory system	3.3 ± 0.1	3.0 ± 0.0	2.2 ± 0.1[#]	2.5 ± 0.2[*]	1.6 ± 0.1[#]	1.6 ± 0.1[*]	1.3 ± 0.1[#]	1.1 ± 0.1[*]
Branchial bars	3.8 ± 0.1	3.6 ± 0.1	2.9 ± 0.1[#]	2.9 ± 0.1[*]	2.1 ± 0.1[#]	2.3 ± 0.1[*]	1.5 ± 0.0[#]	1.8 ± 0.1[*]
Maxillary process	2.0 ± 0.1	1.9 ± 0.1	1.5 ± 0.1[#]	1.5 ± 0.0[*]	1.4 ± 0.1[#]	1.2 ± 0.1[*]	1.0 ± 0.0[#]	1.1 ± 0.0[*]
Mandibular process	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	0.9 ± 0.1	1.0 ± 0.0	0.9 ± 0.1	1.0 ± 0.0	0.9 ± 0.1
Fore limb	3.0 ± 0.0	3.0 ± 0.0	2.7 ± 0.1[#]	2.6 ± 0.1[*]	2.5 ±0.1[#]	2.4 ± 0.1[*]	1.6 ± 0.1[#]	2.4 ± 0.1[*,a]
Hind limb	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0[#]	1.5 ± 0.0[*]	1.6 ± 0.1[#]	1.5 ± 0.1[*]	1.1 ± 0.1[#]	1.3 ± 0.1[*]
Somites	4.0 ± 0.0	4.0 ± 0.0	3.0 ± 0.0[#]	3.7 ± 0.2[*]	3.0 ± 0.0[#]	3.0 ± 0.0[*]	3.0 ± 0.0[#]	3.0 ± 0.0[*]

[†]Morphological features were scored according to Brown and Fabro (1981)
[‡]Values are means ± SEM
[#]Indicate that comparisons to Control are significantly different ($P < 0.05$)
[*]Indicate that comparisons to 12.5G are significantly different ($P < 0.05$)
[a]Indicate that comparisons TOX + 12.5G to TOX are significantly different ($P < 0.05$)

Table 2. Effects of toxaphene technical mixture and glucose (18.75 G) on growth parameters and morphological features in cultured rat embryos

	Dose							
Feature[†]	Control	18.75 G	TOX[1]	TOX[1]+18.75G	TOX[2]	TOX[2]+18.75G	TOX[3]	TOX[3]+18.75G
Number of embryos	9	9	7	9	7	9	7	9
Yolk sac diameter (mm)	[‡]5.1 ± 0.1	4.8 ± 0.1[#]	4.2 ± 0.1[#]	4.2 ± 0.1[*]	4.2 ± 0.1[#]	4.0 ± 0.1[*a]	4.1 ± 0.1[#]	3.8 ± 0.1[*a]
Crown rump length (mm)	3.8 ± 0.1	3.3 ± 0.1[#]	3.0 ± 0.1[#]	3.0 ± 0.1[*]	2.7 ± 0.1[#]	2.5 ± 0.1	2.3 ± 0.1[#]	2.2 ± 0.1
Head length (mm)	2.4 ± 0.1	1.9 ± 0.1[#]	1.8 ± 0.1[#]	1.8 ± 0.1	1.7 ± 0.1[#]	1.4 ± 0.1[*a]	1.5 ± 0.1[#]	1.3 ± 0.1[*a]
Total morphological score	52.5 ± 0.4	43.6 ±0.4[#]	42.8 ± 0.5[#]	42.2 ± 0.3	39.1 ± 0.5[#]	37.2 ± 0.9[*]	31.9 ± 0.5[#]	28.9 ± 1.2[*a]
Yolk sac circulation	3.7 ± 0.1	3.0 ± 0.0	2.9 ± 0.1[#]	2.9 ± 0.1	2.5 ± 0.0[#]	2.6 ± 0.1[*]	2.1 ± 0.1[#]	2.2 ± 0.1[*]
Allantois	3.0 ± 0.0	2.8 ± 0.1[#]	2.5 ± 0.0[#]	2.7 ± 0.1[a]	2.4 ± 0.1[#]	2.3 ± 0.1[*]	2.0 ± 0.0[#]	2.1 ± 0.1[*]
Flexion	3.0 ± 0.0	3.0 ± 0.0	2.6 ± 0.1[#]	3.0 ± 0.0[a]	2.6 ± 0.1[#]	2.7 ± 0.1[*]	2.4 ± 0.1[#]	1.8 ± 0.1[*a]
Heart	3.6 ± 0.1	2.8 ± 0.1[#]	2.6 ± 0.1[#]	2.8 ± 0.1	2.5 ± 0.0[#]	2.6 ± 0.1	2.0 ± 0.0[#]	2.3 ± 0.1[*a]
Caudal neural tube	3.6 ± 0.1	3.0 ± 0.0[#]	3.6 ± 0.1	3.0 ± 0.0	3.4 ± 0.1	2.6 ± 0.3	2.8 ± 0.1	2.1 ± 0.2
Hindbrain	3.3 ± 0.1	2.8 ± 0.1[#]	2.6 ± 0.1[#]	2.6 ± 0.1	2.3 ± 0.1[#]	2.4 ± 0.1[*]	2.0 ± 0.0[#]	2.0 ± 0.2[*]
Midbrain	3.0 ± 0.0	3.0 ± 0.0	2.7 ± 0.1[#]	3.0 ± 0.0	2.5 ± 0.0[#]	2.4 ± 0.1[*]	2.1 ± 0.1[#]	1.7 ± 0.2[*a]
Forebrain	3.4 ± 0.1	2.8 ± 0.1[#]	2.6 ± 0.1[#]	2.9 ± 0.1	2.6 ± 0.1[#]	2.4 ± 0.1[*]	2.1 ± 0.1[#]	1.6 ± 0.1[*a]
Otic system	3.5 ± 0.0	2.6 ± 0.1[#]	3.0 ± 0.0[#]	2.3 ± 0.1[a]	2.6 ± 0.1[#]	2.1 ± 0.1	1.9 ± 0.1[#]	1.4 ± 0.1
Optic system	3.4 ± 0.1	2.3 ± 0.1[#]	2.3 ± 0.1[#]	2.3 ± 0.1	2.3 ± 0.0[#]	2.1 ± 0.1[a]	2.0 ± 0.0[#]	1.8 ± 0.1[*]
Olfactory system	3.0 ± 0.0	2.6 ± 0.1[#]	2.2 ± 0.1[#]	2.1 ± 0.1[*]	1.6 ± 0.1[#]	1.8 ± 0.1[*a]	1.3 ± 0.1[#]	0.9 ± 0.1[*a]
Brachial bars	3.6 ± 0.1	2.4 ± 0.1[#]	2.9 ± 0.1[#]	2.1 ± 0.1[*a]	2.1 ± 0.1[#]	1.8 ± 0.1[*a]	1.5 ± 0.0[#]	1.6 ± 0.1[*]
Maxillary process	1.9 ± 0.1	1.4 ± 0.1[#]	1.5 ± 0.1[#]	1.3 ± 0.1	1.4 ± 0.1[#]	1.3 ± 0.1	1.0 ± 0.0[#]	1.0 ± 0.0[*]
Mandibular process	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	0.9 ± 0.1	1.0 ± 0.0	0.8 ± 0.1
Fore limb	3.0 ± 0.0	2.3 ± 0.1[#]	2.7 ± 0.1[#]	2.5 ± 0.0	2.5 ±0.1[#]	2.8 ± 0.1[a]	1.6 ± 0.1[#]	1.7 ± 0.1[*]
Hind limb	2.4 ± 0.1	1.8 ± 0.1[#]	2.0 ± 0.0[#]	2.0 ± 0.0	1.6 ± 0.1[#]	1.8 ± 0.1	1.1 ± 0.1[#]	1.3 ± 0.1[*]
Somites	4.0 ± 0.0	4.0 ± 0.0	3.0 ± 0.0[#]	2.7 ± 0.2[*a]	3.0 ± 0.0[#]	2.7 ± 0.0[*]	3.0 ± 0.0[#]	2.6 ± 0.2[*a]

[†]Morphological features were scored according to Brown and Fabro (1981)
[‡]Values are means ± SEM
[#] Indicate that comparisons to Control are significantly different ($P < 0.05$)
[*]Indicate that comparisons to 18.75 G are significantly different ($P < 0.05$)
[a]Indicate that comparisons TOX + 18.75G to TOX are significantly different ($P < 0.05$)
[1]100 ng/ml
[2]1000 ng/ml
[3]5000 ng/ml

Table 3. Effects of T_2 and glucose (18.75 G) on growth parameters and morphological features in cultured rat embryos

	Dose							
Feature[†]	Control	18.75 G	T_2[1]	T_2[1]+18.75G	T_2[2]	T_2[2]+18.75G	T_2[3]	T_2[3]+18.75G
Number of embryos cultured	9	9	8	8	8	8	8	8
Yolk sac diameter (mm)	[‡]5.1 ± 0.1	4.8 ± 0.1[#]	4.7 ± 0.1[#]	4.3 ± 0.1	4.6 ± 0.1[#]	4.2 ± 0.1	4.2 ± 0.1[#]	4.0 ± 0.1
Crown rump length (mm)	3.8 ± 0.1	3.3 ± 0.1[#]	3.3 ± 0.1	3.1 ± 0.1	3.0 ± 0.1	2.9 ± 0.1	3.0 ± 0.1	2.6 ± 0.1
Head length (mm)	2.4 ± 0.1	1.9 ± 0.1[#]	2.0 ± 0.1	1.7 ± 0.1	1.9 ± 0.2	1.6 ± 0.1	1.6 ± 0.1	1.4 ± 0.1
Total morphological score	52.5 ± 0.4	43.6 ± 0.3[#]	40.9 ± 0.6[#]	38.1 ± 0.7[*]	34.0 ± 2.2[#]	31.6 ± 0.7[*]	30.1 ± 2.6[#]	29.0 ± 0.8[*]
Yolk sac circulation	3.7 ± 0.1	3.0 ± 0.0[#]	3.0 ± 0.0[#]	2.9 ± 0.1	3.0 ± 0.0[#]	2.4 ± 0.1[*a]	3.0 ± 0.0[#]	2.0 ± 0.0[*a]
Allantois	3.0 ± 0.0	2.8 ± 0.1[#]	3.0 ± 0.0	2.4 ± 0.1[*a]	2.5 ± 0.0[#]	2.2 ± 0.1[*a]	2.7 ± 0.1[#]	2.0 ± 0.0[*a]
Flexion	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0 0	2.6 ± 0.1[*a]	2.1 ± 0.3[#]	2.4 ± 0.1[*]	2.6 ± 0.3	1.9 ± 0.1[*a]
Heart	3.6 ± 0.1	2.8 ± 0.1[#]	3.5 ± 0.0	3.2 ± 0.1[*a]	2.4 ± 0.1[#]	2.4 ± 0.1[*]	2.7 ± 0.2[#]	2.1 ± 0.1[*a]
Caudal neural tube	3.6 ± 0.1	3.0 ± 0.0[#]	3.0 ± 0.0[#]	2.9 ± 0.1	2.6 ± 0.4[#]	2.4 ± 0.1[*]	1.5 ± 0.4[#]	2.2 ± 0.1[*a]
Hindbrain	3.3 ± 0.1	2.8 ± 0.1[#]	2.2 ± 0.1[#]	2.9 ± 0.1[a]	1.8 ± 0.2[#]	2.4 ± 0.1[a]	0.8 ± 0.3[#]	1.5 ± 0.1[*]
Midbrain	3.0 ± 0.0	3.0 ± 0.0	2.7 ± 0.1	2.6 ± 0.1	2.2 ± 0.2	2.3 ± 0.1	1.6 ± 0.3	1.7 ± 0.2
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Otic system	3.5 ± 0.0	2.6 ± 0.1[#]	2.1 ± 0.1[#]	1.8 ± 0.1[*]	1.9 ± 0.3[#]	1.4 ± 0.1[*]	0.8 ± 0.4[#]	1.3 ± 0.1[*a]
Optic system	3.4 ± 0.1	2.3 ± 0.1[#]	2.4 ± 0.1[#]	1.9 ± 0.1[*a]	2.1 ± 0.1[#]	1.4 ± 0.1[*a]	1.9 ± 0.2[#]	1.4 ± 0.1[*]
Olfactory system	3.0 ± 0.0	2.6 ± 0.1[#]	1.7 ± 0.1[#]	1.7 ± 0.1[*]	1.2 ± 0.1[#]	1.6 ± 0.1[*]	1.3 ± 0.1[#]	1.5 ± 0.1[*]
Brachial bars	3.6 ± 0.1	2.4 ± 0.1[#]	2.6 ± 0.1[#]	1.9 ± 0.1[*a]	2.1 ± 0.1[#]	1.4 ± 0.1[*a]	2.1 ± 0.1[#]	1.6 ± 0.1[*a]
Maxillary process	1.9 ± 0.1	1.4 ± 0.1[#]	1.3 ± 0.1[#]	1.0 ± 0.0[*a]	1.0 ± 0.0[#]	1.0 ± 0.0[*]	1.0 ± 0.0[#]	0.9 ± 0.1[*]
Mandibular process	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	0.9 ± 0.1	0.8 ± 0.1[#]	0.8 ± 0.1	0.9 ± 0.1	0.6 ± 0.1
Fore limb	3.0 ± 0.0	2.3 ± 0.1[#]	2.3 ± 0.1[#]	2.2 ± 0.1	2.4 ±0.1[#]	1.9 ± 0.1[*a]	2.4 ± 0.1[#]	1.9 ± 0.1[*a]
Hind limb	2.4 ± 0.1	1.8 ± 0.1[#]	1.5 ± 0.0[#]	1.5 ± 0.0[*]	1.1 ± 0.2[#]	1.0 ± 0.0[*]	1.0 ± 0.1[#]	1.0 ± 0.0[*]
Somites	4.0 ± 0.0	4.0 ± 0.0	3.0 ± 0.0[#]	3.0± 0.0[*]	2.9 ± 0.1[#]	3.0 ± 0.0[*]	1.9 ± 0.3[#]	2.7 ± 0.2[*a]

[†]Morphological features were scored according to Brown and Fabro (1981)
[‡]Values are means ± SEM
[#]Indicate that comparisons to Control are significantly different ($P < 0.05$)
[*]Indicate that comparisons to 18.75 G are significantly different ($P < 0.05$)
[a]Indicate that comparisons T_2 + 18.75G to T_2 are significantly different ($P < 0.05$)
[1]100 ng/ml
[2]1000 ng/ml
[3]5000 ng/ml

Table 4 . Effects of T_{12} and glucose (18.75 G) on growth parameters and morphological features in cultured rat embryos

	Dose							
Feature[†]	Control	18.75 G	T_{12}[1]	T_{12}[1]+18.75G	T_{12}[2]	T_{12}[2]+18.75G	T_{12}[3]	T_{12}[3]+18.75G
Number of embryos cultured	9	9	9	8	9	8	9	8
Yolk sac diameter (mm)	[‡]5.1 ± 0.1	4.8 ± 0.1[#]	4.5 ± 0.1	4.2 ± 0.1	4.4 ± 0.1[#]	3.9 ± 0.1	3.8 ± 0.1[#]	3.5 ± 0.1
Crown rump length (mm)	3.8 ± 0.1	3.3 ± 0.1[#]	3.6 ± 0.1	3.1 ± 0.1	3.5 ± 0.1	2.8 ± 0.1	2.7 ± 0.1	2.2 ± 0.1
Head length (mm)	2.4 ± 0.1	1.9 ± 0.1[#]	1.8 ± 0.1[#]	1.7 ± 0.1[*]	1.6 ± 0.1[#]	1.5 ± 0.1[*]	1.6 ± 0.1[#]	1.3 ± 0.1[*a]
Total morphological score	52.5 ± 0.4	43.6 ± 0.4[#]	40.6 ± 0.6[#]	40.1 ± 0.2[*]	38.3 ± 0.6[#]	35.9 ± 0.9[*a]	35.8 ± 0.5[#]	32.4 ± 0.7[*a]
Yolk sac circulation	3.7 ± 0.1	3.0 ± 0.0[#]	3.3 ± 0.1[#]	3.0 ± 0.0[a]	3.5 ± 0.0	2.9 ± 0.1[a]	3.2 ± 0.1[#]	2.5 ± 0.0[*a]
Allantois	3.0 ± 0.0	2.8 ± 0.1[#]	2.5 ± 0.0[#]	2.1 ± 0.1	2.5 ± 0.0[#]	2.3 ± 0.1	2.4 ± 0.1[#]	2.1 ± 0.1
Flexion	3.0 ± 0.0	3.0 ± 0.0	2.5 ± 0.0[#]	2.5 ± 0.0[*]	2.3 ± 0.1[#]	2.1 ± 0.1[*]	2.3 ± 0.1[#]	2.0 ± 0.0[*a]
Heart	3.6 ± 0.1	2.8 ± 0.1[#]	3.3 ± 0.1[#]	3.0 ± 0.0[*a]	3.1 ± 0.1[#]	3.0 ± 0.0[*]	3.0 ± 0.0[#]	2.6 ± 0.1[a]
Caudal neural tube	3.6 ± 0.1	3.0 ± 0.0[#]	3.4 ± 0.1[#]	3.3 ± 0.1[*]	2.9 ± 0.1[#]	2.8 ± 0.1	2.9 ± 0.1[#]	2.8 ± 0.1[a]
Hindbrain	3.3 ± 0.1	2.8 ± 0.1[#]	2.7 ± 0.1	2.9 ± 0.1	2.3 ± 0.1[#]	2.4 ± 0.1[*]	2.2 ± 0.1[#]	2.3 ± 0.1[*]
Midbrain	3.0 ± 0.0	3.0 ± 0.0	2.7 ± 0.1	2.5 ± 0.0[*a]	2.5 ± 0.0	2.3 ± 0.1[*a]	2.0 ± 0.0	2.2 ± 0.1[*a]
Forebrain	3.4 ± 0.1	2.8 ± 0.1[#]	2.8 ± 0.1[#]	2.7 ± 0.1	2.9 ± 0.1[#]	2.4 ± 0.1[*a]	2.0 ± 0.0[#]	2.0 ± 0.0[*]
Otic system	3.5 ± 0.0	2.6 ± 0.1[#]	1.3 ± 0.1[#]	1.4 ± 0.1[*]	1.1 ± 0.2[#]	1.3 ± 0.1[*a]	0.9 ± 0.1[#]	1.1 ± 0.1[*]
Optic system	3.4 ± 0.1	2.3 ± 0.1[#]	2.2 ± 0.1	2.2 ± 0.1	1.9 ± 0.1	1.8 ± 0.1[*]	1.8 ± 0.1	1.6 ± 0.1[*]
Olfactory system	3.0 ± 0.0	2.6 ± 0.1[#]	1.3 ± 0.1[#]	1.7 ± 0.1[*a]	1.0 ± 0.2[#]	1.2 ± 0.1[*]	1.3 ± 0.1[#]	1.4 ± 0.1[*]
Brachial bars	3.6 ± 0.1	2.4 ± 0.1[#]	2.4 ± 0.1[#]	2.1 ± 0.1[*]	2.6 ± 0.1[#]	2.1 ± 0.1[*a]	2.2 ± 0.1[#]	1.2 ± 0.1[*a]
Maxillary process	1.9 ± 0.1	1.4 ± 0.1[#]	1.7 ± 0.1[#]	1.5 ± 0.0	1.4 ± 0.1[#]	1.5 ± 0.1[*]	1.0 ± 0.0[#]	1.1 ± 0.1[*]
Mandibular process	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	0.7 ± 0.1[#]	0.5 ± 0.0[*a]	0.5 ± 0.0[#]	0.5 ± 0.0[*]
Fore limb	3.0 ± 0.0	2.3 ± 0.1[#]	2.8 ± 0.1	2.9 ± 0.1[*]	2.8 ±0.1	2.7 ± 0.1[*]	2.8 ± 0.1	2.2 ± 0.1[a]
Hind limb	2.4 ± 0.1	1.8 ± 0.1[#]	1.8 ± 0.1	2.0 ± 0.0	1.8 ± 0.1	1.9 ± 0.1	1.8 ± 0.1	1.7 ± 0.1[a]
Somites	4.0 ± 0.0	4.0 ± 0.0	3.0 ± 0.1[#]	3.0 ± 0.0	3.0 ± 0.0[#]	3.0 ± 0.0	3.0 ± 0.0[#]	3.0 ± 0.0

[†]Morphological features were scored according to Brown and Fabro (1981)
[‡]Values are means ± SEM
[#]Indicate that comparisons to Control are significantly different ($P < 0.05$)
[*]Indicate that comparisons to 18.75 G are significantly different ($P < 0.05$)
[a]Indicate that comparisons T_{12} + 18.75G to T_{12} are significantly different ($P < 0.05$)
[1]100 ng/ml
[2]1000 ng/ml
[3]5000 ng/ml

Table 5. Direct comparison of individual versus combined actual and observed versus predicted interactive effects of TOX, T_2, or T_{12} and hyperglycemia of malformations in cultured rat whole embryo

Treatment	CNS (%)	Limbs (%)	Flexion (%)
TOX[1] (100 ng/ml)	42.9	0	0
18.75 G	0	0	0
Combination	41.1	0	0
Predicted	42.9	0	0
TOX[2] (1000 ng/ml)	46.2	0	0
18.75 G	0	0	0
Combination	66.7	0	0
Predicted	46.2	0	0
TOX[3] (5000 ng/ml)	53.8	53.8	0
18.75 G	0	0	0
Combination	88.9	33.3	22.2
Predicted	53.8	53.8	0
T_2[1] (100 ng/ml)	47.1	17.6	0
18.75 G	0	0	0
Combination	25	0	0
Predicted	47.1	17.6	0
T_2[2] (1000 ng/ml)	46.7	26.7	6.7
18.75 G	0	0	0
Combination	75	12.5	0
Predicted	46.7	26.7	6.7
T_2[3] (5000 ng/ml)	50	37.5	15.5
18.75 G	0	0	0
Combination	87.5	25	0
Predicted	50	37.5	15.5
T_{12}[1] (100 ng/ml)	13.3	0	0
18.75 G	0	0	0
Combination	0	0	0
Predicted	13.3	0	0
T_{12}[2] (1000 ng/ml)	56.3	0	0
18.75 G	0	0	0
Combination	25	0	0
Predicted	56.3	0	0
T_{12}[3] (5000 ng/ml)	62.5	0	0
18.75 G	0	0	0
Combination	37.5	25	0
Predicted	62.5	0	0

Figure 1a

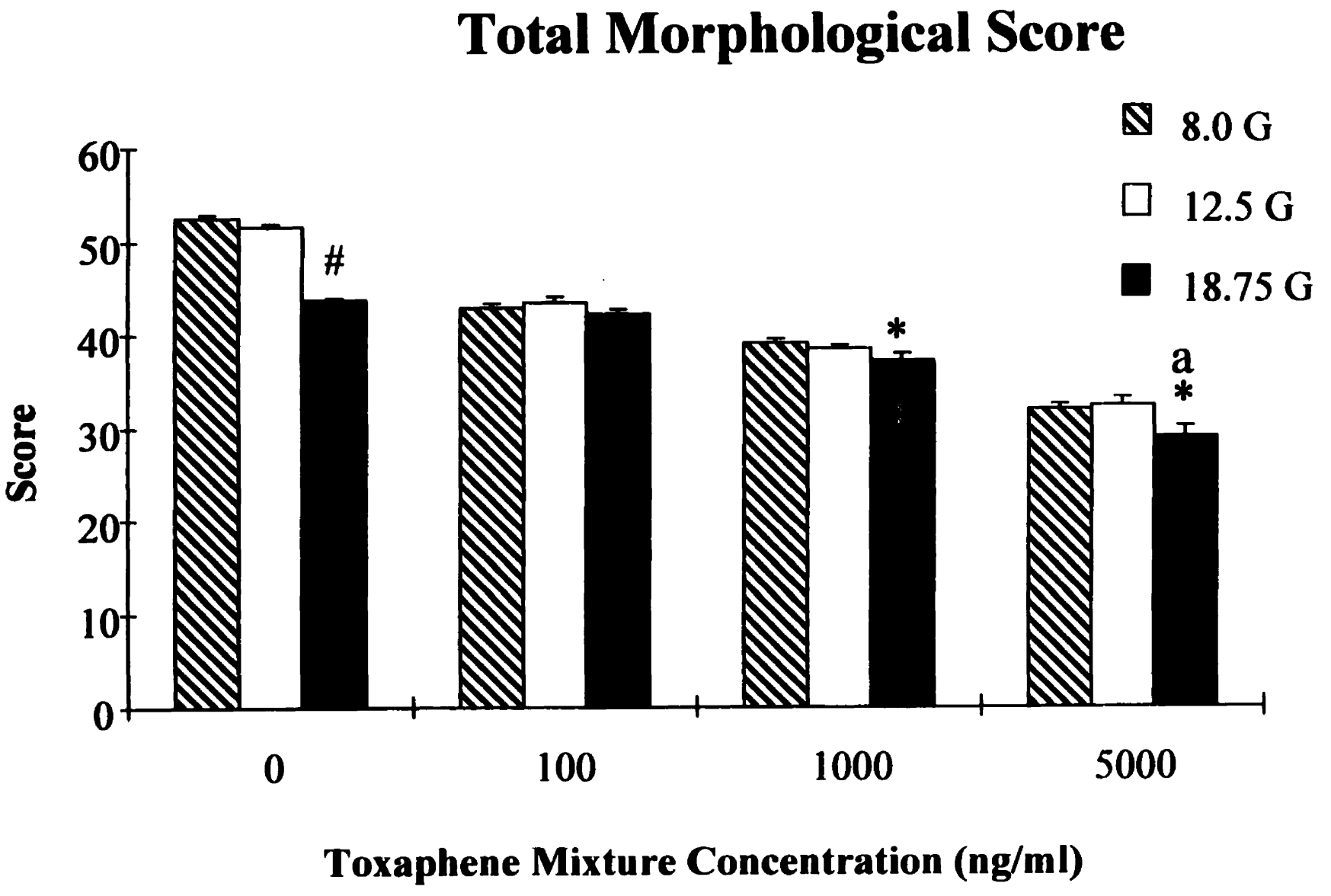

Total Morphological Score
8.0 G
12.5 G
18.75 G
Score
60
50
40
30
20
10
0
#
*
a
*
0
100
1000
5000
Toxaphene Mixture Concentration (ng/ml)

Figure 1b

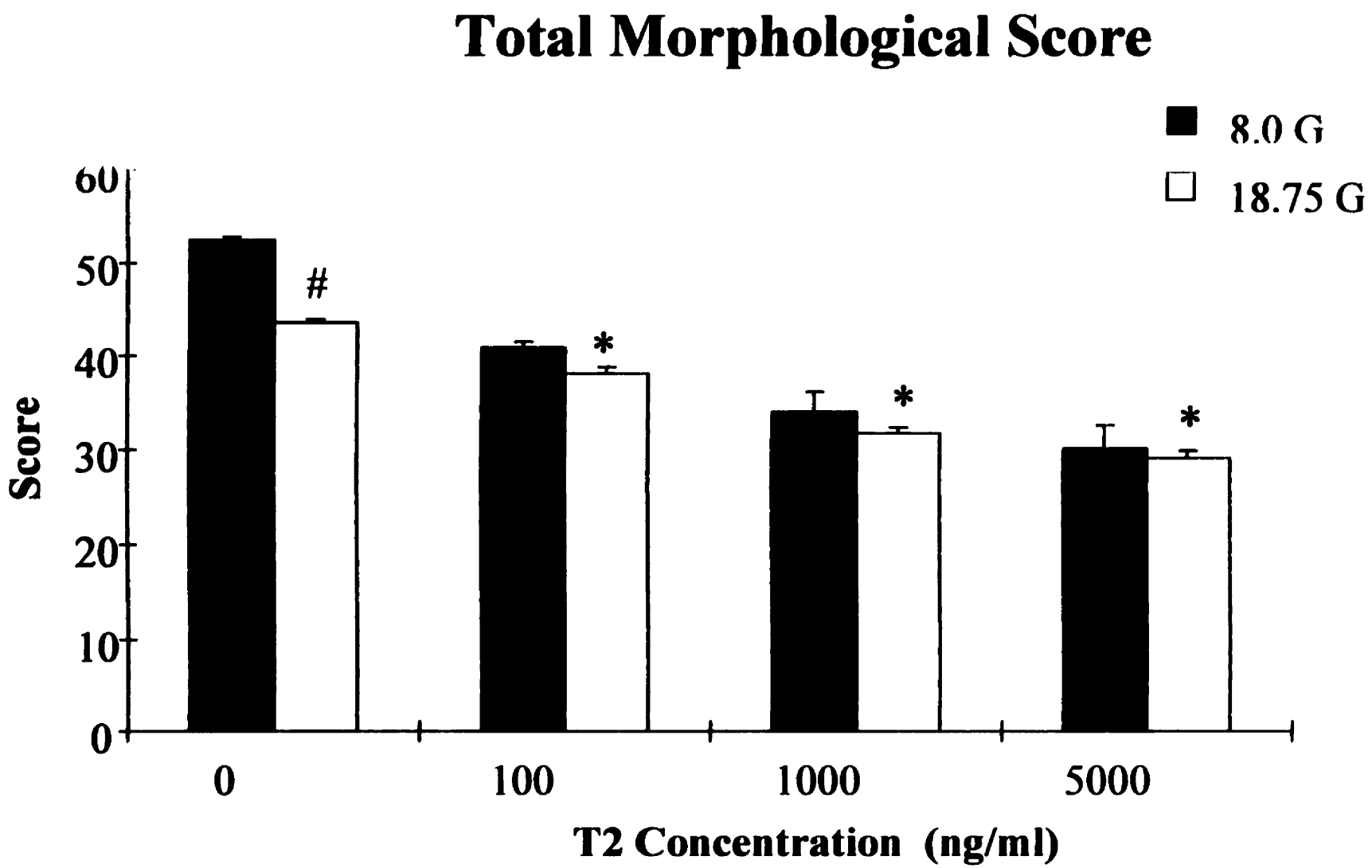

Total Morphological Score
8.0 G
18.75 G
Score
60
50
40
30
20
10
0
#
*
*
*
0
100
1000
5000
T2 Concentration (ng/ml)

Figure 1c

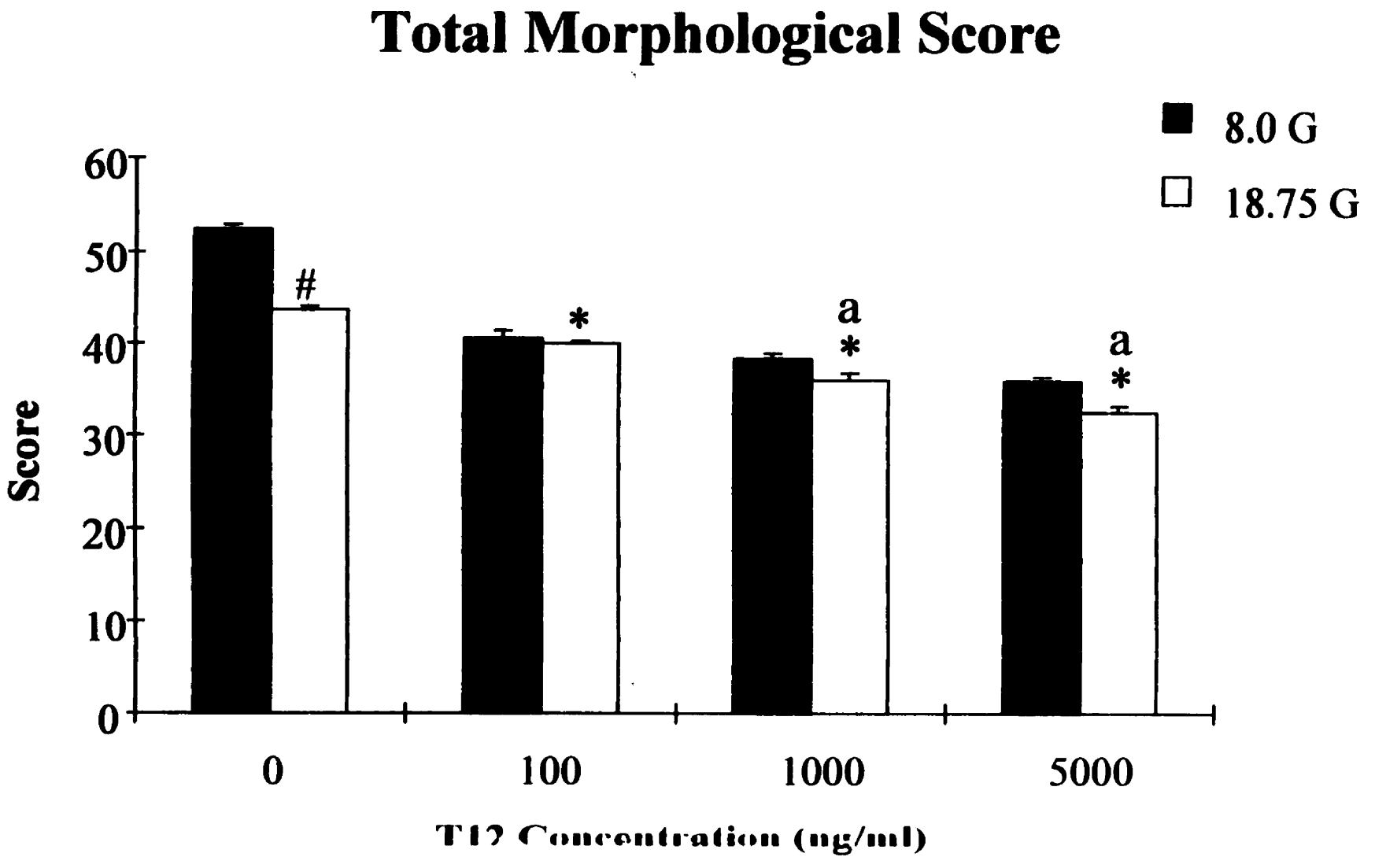

Total Morphological Score
8.0 G
18.75 G
Score
60
50
40
30
20
10
0
#
*
a
*
a
*
0
100
1000
5000
T12 Concentration (ng/ml)

Figure 2a

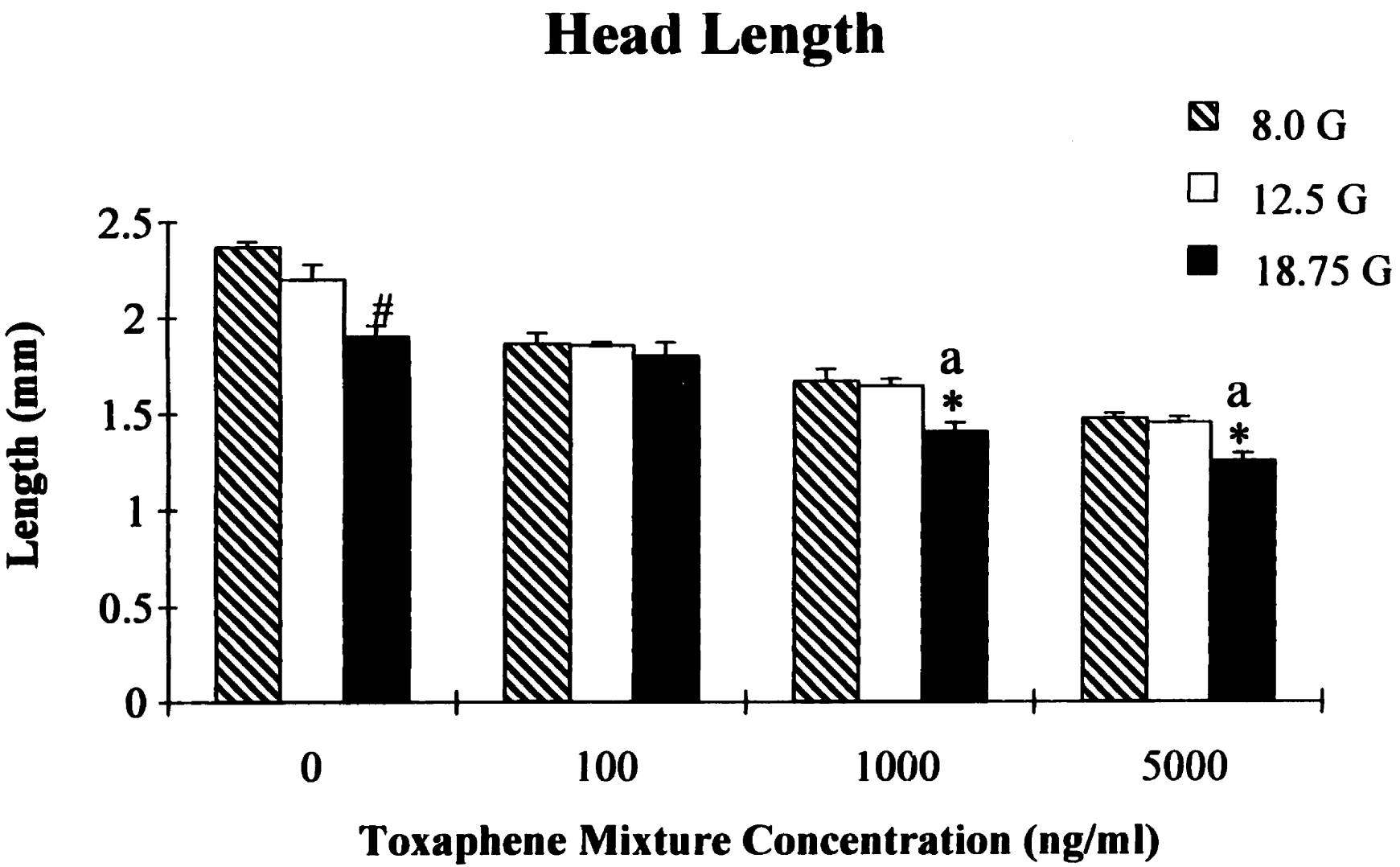

Head Length
8.0 G
12.5 G
18.75 G
Length (mm)
2.5
2
1.5
1
0.5
0
#
a
*
a
*
0
100
1000
5000
Toxaphene Mixture Concentration (ng/ml)

Figure 2b

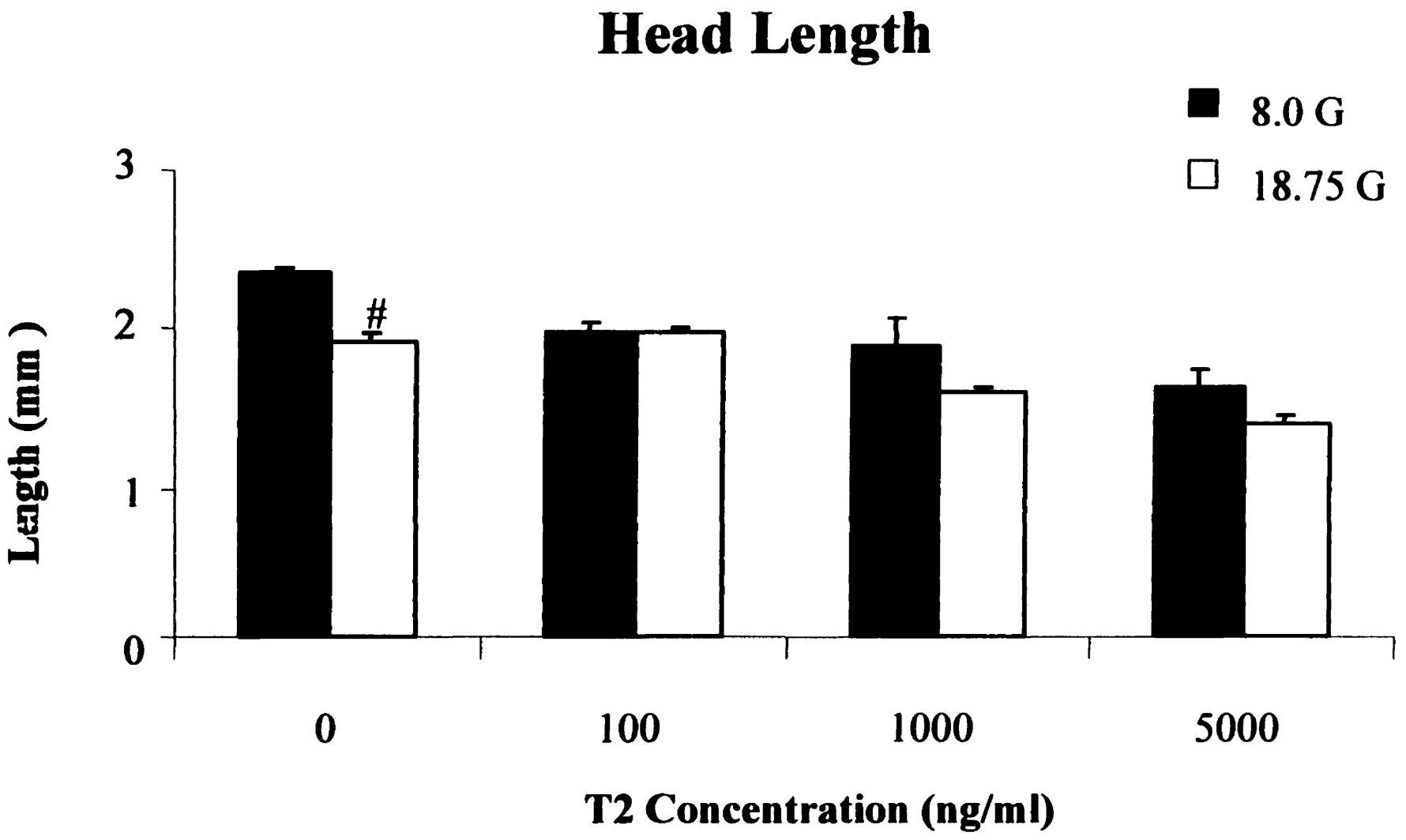

Head Length
8.0 G
18.75 G
Length (mm)
3
2
1
0
#
0
100
1000
5000
T2 Concentration (ng/ml)

Figure 2c

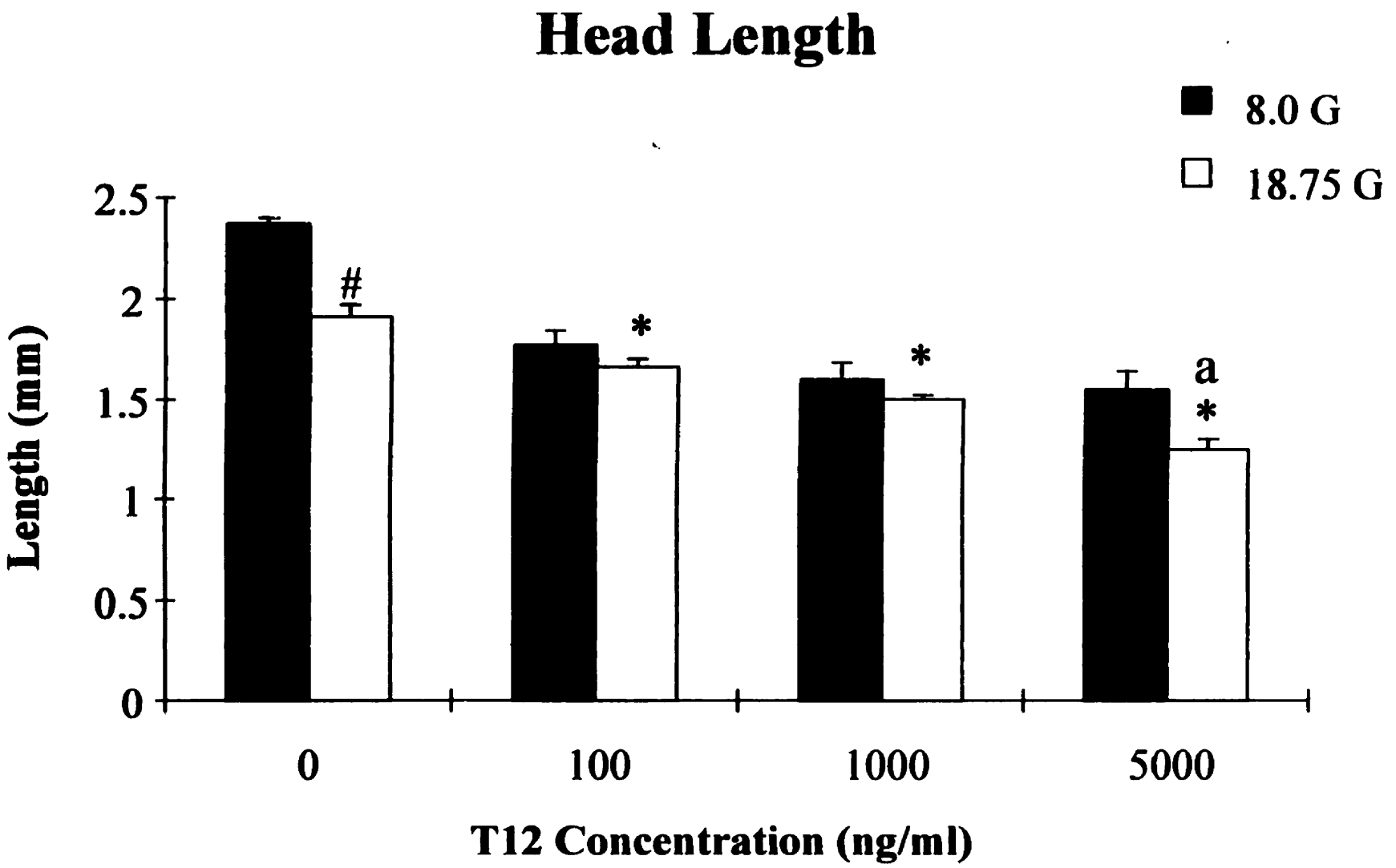

Head Length
8.0 G
18.75 G
Length (mm)
2.5
2
1.5
1
0.5
0
#
*
*
a
*
0
100
1000
5000
T12 Concentration (ng/ml)

Figure 3a

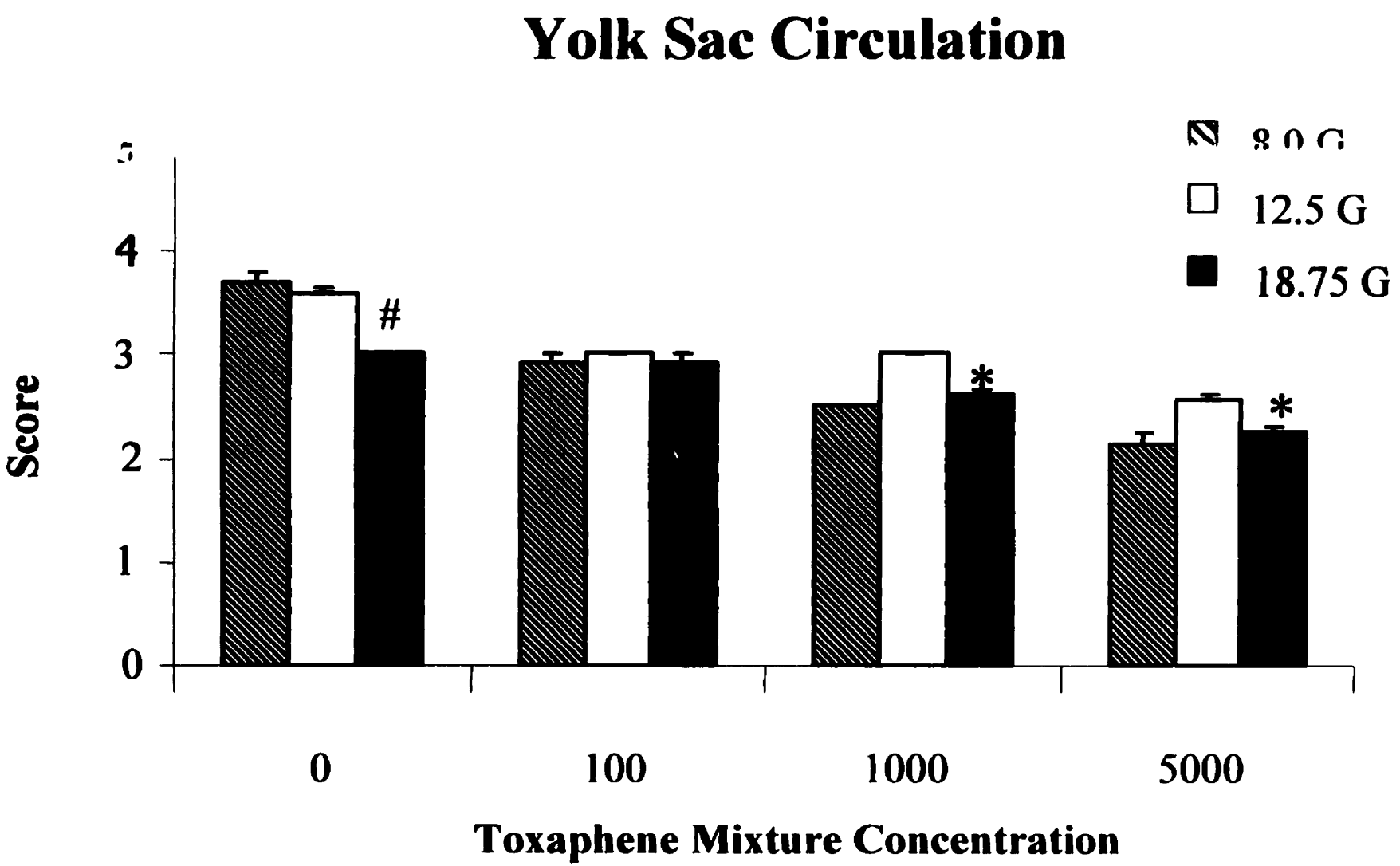

Yolk Sac Circulation
8.0 G
12.5 G
18.75 G
Score
5
4
3
2
1
0
#
*
*
0
100
1000
5000
Toxaphene Mixture Concentration

Figure 3b

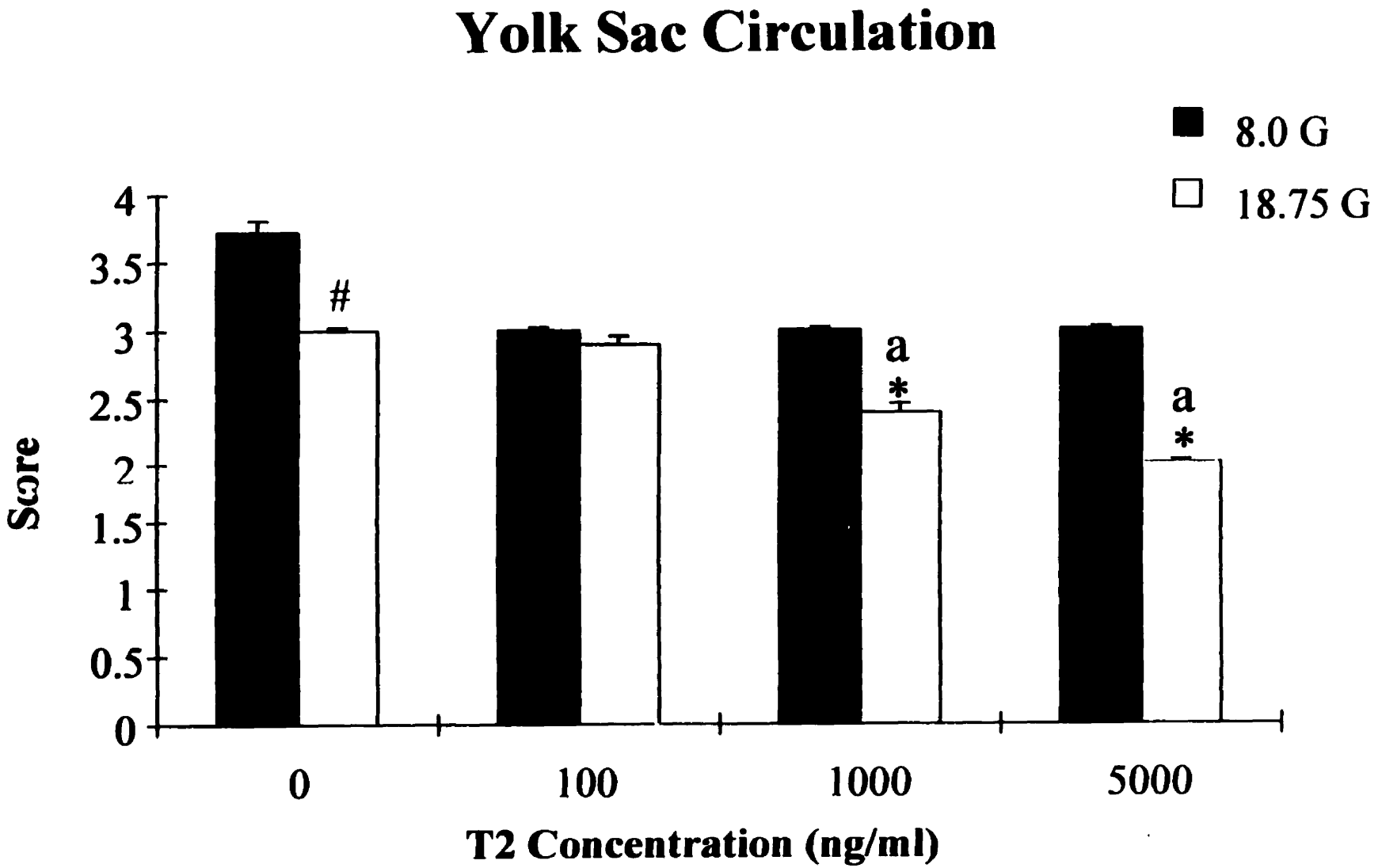
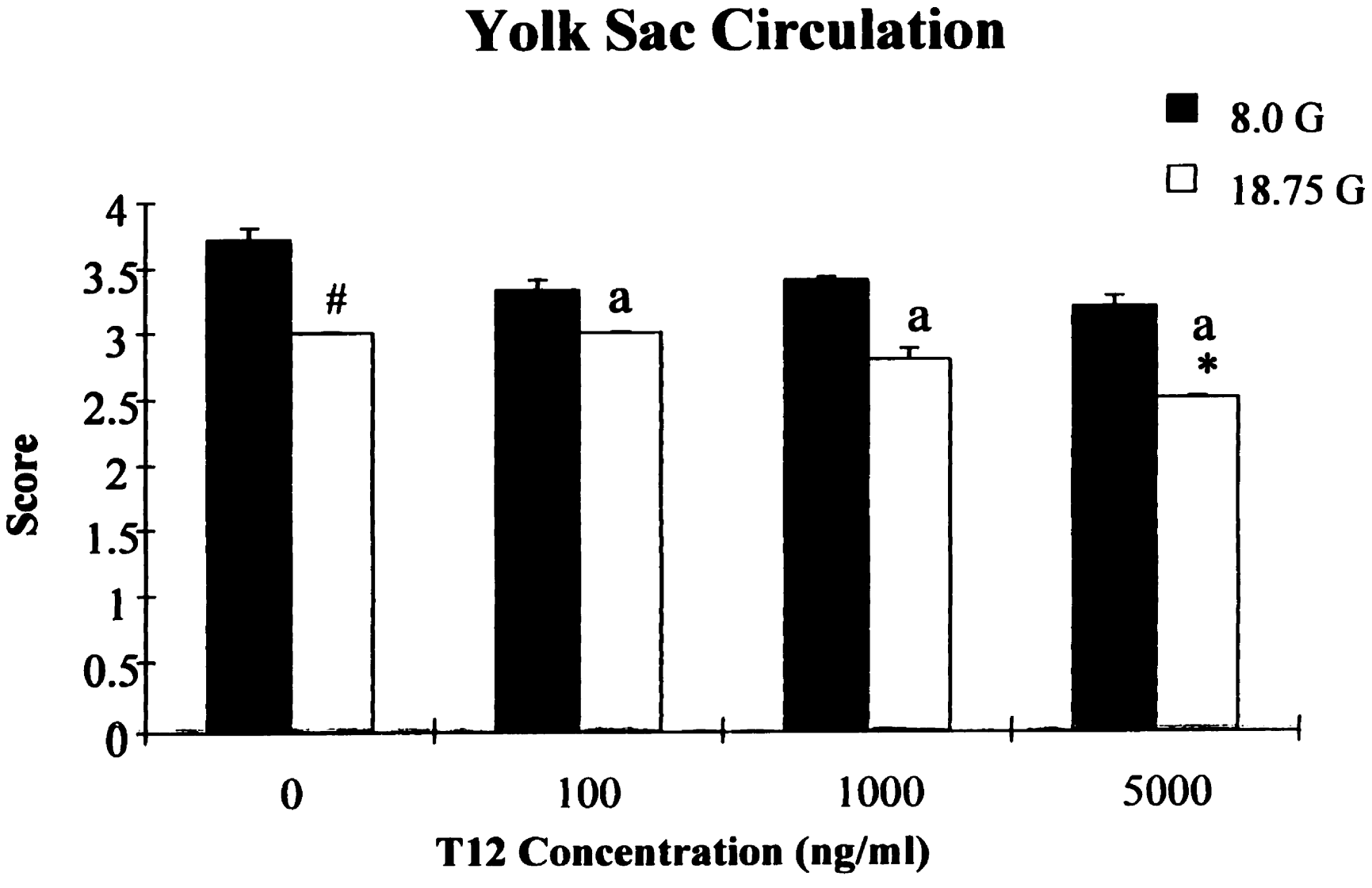

Yolk Sac Circulation
8.0 G
18.75 G
Score
4
3.5
3
2.5
2
1.5
1
0.5
0
#
a
*
a
*
0
100
1000
5000
T2 Concentration (ng/ml)

Figure 3c


Yolk Sac Circulation
8.0 G
18.75 G
Score
4
3.5
3
2.5
2
1.5
1
0.5
0
#
a
a
a
*
0
100
1000
5000
T12 Concentration (ng/ml)

LEGENDS TO FIGURES

Figure 1a

Total morphological score of embryos cultured for 48 h and dosed with 12.5 G, 18.75 G, TOX + 12.5 G, and TOX + 18.75 G.

[#]Indicate that comparisons to Control (8.0 G) are significantly different ($P < 0.05$).

[a]Indicate that comparisons TOX +18.75 G to TOX are significantly different ($P < 0.05$).

Figure 1b

Total morphological score of embryos cultured for 48 h and dosed with 18.75 G, T_2, and T_2 + 18.75 G.

[#]Indicate that comparisons to Control (8.0 G) are significantly different ($P < 0.05$).

*Indicate that comparisons to 18.75 G are significantly different ($P < 0.05$).

Figure 1c

Total morphological score of embryos cultured for 48 h and dosed with 18.75 G, T_{12}, and T_{12} + 18.75 G.

[#]Indicate that comparisons to Control (8.0 G) are significantly different ($P < 0.05$).

*Indicate that comparisons to 18.75 G are significantly different ($P < 0.05$).

[a]Indicate that comparisons T_{12} +18.75 G to T_{12} are significantly different ($P < 0.05$).

Figure 2a

Head length of embryos cultured for 48 h and dosed with 12.5 G, 18.75 G, TOX + 12.5 G, and TOX + 18.75 G.

#Indicate that comparisons to Control (8.0 G) are significantly different ($P < 0.05$).

*Indicate that comparisons to 18.75 G are significantly different ($P < 0.05$).

[a]Indicate that comparisons TOX +18.75 G to TOX are significantly different ($P < 0.05$).

Figure 2b

Head length of embryos cultured for 48 h and dosed with 18.75 G, T_2, and T_2 + 18.75 G.

#Indicate that comparisons to Control (8.0 G) are significantly different ($P < 0.05$).

Figure 2c

Head length of embryos cultured for 48 h and dosed with 18.75 G, T_{12}, and T_{12} + 18.75 G.

#Indicate that comparisons to Control (8.0 G) are significantly different ($P < 0.05$).

*Indicate that comparisons to 18.75 G are significantly different ($P < 0.05$).

[a]Indicate that comparisons T_{12} +18.75 G to T_{12} are significantly different ($P < 0.05$).

Figure 3a

Yolk sac circulation score of embryos cultured for 48 h and dosed with 12.5 G, 18.75 G, TOX + 12.5 G, and TOX + 18.75 G.

[#]Indicate that comparisons to Control (8.0 G) are significantly different ($P < 0.05$).

*Indicate that comparisons to 18.75 G are significantly different ($P < 0.05$).

Figure 3b

Yolk sac circulation score of embryos cultured for 48 h and dosed with 18.75 G, T_2, and T_2 + 18.75 G.

[#]Indicate that comparisons to Control (8.0 G) are significantly different ($P < 0.05$).

*Indicate that comparisons to 18.75 G are significantly different ($P < 0.05$).

[a]Indicate that comparisons T_2 +18.75 G to T_2 are significantly different ($P < 0.05$).

Figure 3c

Yolk sac circulation score of embryos cultured for 48 h and dosed with 18.75 G, T_{12}, and T_{12} + 18.75 G.

[#]Indicate that comparisons to Control (8.0 G) are significantly different ($P < 0.05$).

*Indicate that comparisons to 18.75 G are significantly different ($P < 0.05$).

[a]Indicate that comparisons T_{12} +18.75 G to T_{12} are significantly different ($P < 0.05$).

OVERALL CONCLUSIONS

These experiments were designed to study the dysmorphogenic effects of toxaphene or its two environmental prevalent congeners (T_2 and T_{12}) and the interactive effects of these compounds and high glucose concentrations in early stage embryos using rat whole embryo culture technique.

The first study showed that both toxaphene technical mixture and the two individual congeners had significant adverse effects on the total morphological score, somite number, head and crown rump length, and the CNS scores of embryos. All treatments caused a high incidence of CNS malformations. The toxicity of toxaphene technical mixture or the T_2 + T_{12} mixture was different than that of the individual congeners. This finding is unique since most information for the teratogenicity of toxaphene is based on studies using the toxaphene technical mixture of over 800 congeners. The only report on toxicity of individual congeners was by Matsumura et al. (1980) who demonstrated that there is a differential toxicity between two toxaphene congeners (Toxicant A and Toxicant B) versus the toxaphene technical mixture. However, the two congeners were not characterized. In this study, exposure of embryos to either T_2 or T_{12} treatments alone had less effect on growth than did the toxaphene technical mixture or the $T_2 + T_{12}$ mixture at the same dose. However, both T_2 and T_{12} had a stronger adverse effect on the otic system. Moreover, the two congeners differed in their target site as T_2 caused limb and flexion abnormalities that were not observed with

T_{12} exposure. These results suggest that the dysmorphogenic effects are congener specific.

The second study showed that toxaphene technical mixture or its two congeners (T_2 or T_{12}) and severe hyperglycemic conditions had a concentration-dependent adverse effect on total morphological score, somite number, head and crown rump length and the central nervous system scores. A lower glucose concentration (only twice compared with normal) can exacerbate dysmorphogenic effects of another teratogen such as toxaphene. The major malformations were abnormalities in the development of the CNS. In this study two glucose levels were chosen which normally are not associated with major malformations. For example, embryos exposed to 18.75 G did not show CNS abnormalities. Hyperglycemia, however, when combined with toxaphene technical mixture or T_2 (1000 ng/ml and 5000 ng/ml) showed synergistic adverse effects on CNS malformations. Moreover, the dysmorphogenic effects produced by TOX, or T_{12} were exacerbated by the addition of D-glucose. The action of toxaphene technical mixture and its congeners were also site-specific.

NOVEL FINDINGS

Novel findings in these two manuscripts are summarized below:

1. TOX, T_2, and T_{12} treatments had a significant adverse concentration-response effect on the total morphological score, crown rump length, head length and different features scores.

2. The embryotoxicity of the toxaphene technical mixture or the $T_2 + T_{12}$ mixture was different than of the congeners.

3. The target site and the type of toxicity are highly congener specific.

4. There is a synergistic effect on growth retardation between the two environmentally prevalent toxaphene congeners, T_2 and T_{12}.

5. Hyperglycemia exacerbated the effects of toxaphene, T_2 and T_{12} treatments on total morphological score.

6. There is a synergistic effect on CNS malformations between toxaphene or its congeners and hyperglycemic conditions.

GLOSSARY OF TERMINOLOGY

Acute – in toxicology, a single exposure or dose which is sufficient to cause an adverse reaction

Agenesis – congenital absence of an organ or part, usually caused by a lack of primordial tissue and failure of development in the embryo

Ames Test – a test which measures the potential of a chemical to cause mutations in bacteria; those causing mutations are probably capable of causing cancer in mammals

Anencephaly – congenital absence of the brain and spinal cord in which the cranium does not close and the vertebral canal remains a groove

Barbiturate – drug, a derivate of barbituric acid that acts as a sedative or hypnotic

Bioaccumulation – the build-up of a substance in an organism due to the presence of the chemical in the food supply; accumulation only occurs if the substance is slow to be excreted or metabolized

Biomagnification – the build-up of a chemical in a particular organism high on the food chain because of ingestion of prey containing the chemical

Carcinogen – an agent which can contribute to the development of cancer

Chronic toxicity – adverse effects manifested after a long period of uptake of small quantities of the toxicant in question

Contaminant – any undesirable substance in food, water or air

Decidua – the epithelial tissue of the endometrium lining the uterus

Dose – the amount of a substance taken into the body

Dysmorphogenesis – the development of ill-shaped or otherwise malformed body tissue

Enzyme – a chemical (protein) produced by a cell to act as a catalyst in a particular biochemical reaction

Exposure – the doses of a substance capable of causing toxic effects actually taken into the organism

Gavage – the process of feeding through a nasogastric tube

Homeostasis – a relative constancy in the internal environment of the body, naturally maintained by adaptive responses that promote healthy survival

Implantation – the process involving the attachment, penetration, and embedding of the blastocyst in the lining of the uterine wall during the early stages of prenatal development

In vitro – a biological reaction occurring in laboratory apparatus

In vivo - a biological reaction occurring in a living organism

LD_{50} – the dose of a toxin which kills half of a test population of a particular species; it can differ among species

Microsomal enzymes – a group of enzymes associated with a certain particulate fraction of liver homogenate that plays a role in the metabolism of many drugs

Mutagen – a substance that can cause a change or mutation in genetic material

Organogenesis – the formation and differentiation of organs and organ systems during embryonic development

Somatomedin – growth hormone, a single-chain peptide secreted by the anterior pituitary gland

Somite – any of the paired, segmented masses of mesodermal tissue that form along the length of the neural tube during the early stage of embryonic development; these structures give rise to the vertebrae and differentiate into various tissues of the body, including the voluntary muscle, bones, and the dermal layers of the skin

Spina bifida – congenital neural tube defect characterized by a developmental anomaly in the posterior vertebral arch

Synergism – the effect of two chemicals which, in combination, each act more strongly than either alone

Target organ – an organ most affected by a specific agent

Teratogen – a chemical that can cause birth defects when experienced by a female before or during pregnancy

Toxicology – the study of the adverse effects of chemicals on living organisms

Toxicity – the measure of the capacity of a chemical to harm an organism

Xenobiotics – a general term used to describe any chemical interacting with an organism that does not occur in the normal metabolic pathways of the organism

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