METALLOTHIONEIN PRE-INDUCTION BY ZINC AND ISOTRETINOIN TERATOGENICITY IN CD-1 MICE

DANIELLE BLAIN
SCHOOL OF DIETETICS AND HUMAN NUTRITION
MCGILL UNIVERSITY, MONTREAL

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ABSTRACT

Metallothionein (MT) pre-induction by Zn has been shown to provide protection against subsequent free radical (FR) exposure. This investigation evaluated the potential protective role of Zn, through modulation of MT, against the teratogenic effects of isotretinoin (ITR), a dermatological drug which causes oxidative damage, in CD-1 mouse embryos. Significant induction of MT by Zn supplementation was observed in mouse embryos both *in vivo* and *in vitro*. On gestational day (GD) 6.5, pregnant mice received a subcutaneous Zn injection of 0 (saline), 20 and 40 mg/kg, resulting in embryonic MT concentrations of 12.5, 54.5 and 93.4 µg/g protein, respectively, after 48h. Embryos were extracted at GD 8.5 and incubated with 0 (saline) and 15 µM Zn for 48h. There was a six-fold increase in MT expression in the Zn treatment group, resulting in MT concentration of 688 µg/g protein. Pre-induction of MT by Zn exposure was found to offer protection against subsequent exposure to ITR *in vivo* and *in vitro*. Zn injections of 40 and 20 mg/kg to pregnant mice on GD 8.5 and 9.5, respectively, alleviated fetal damage caused by three intragastric intubations of 100 mg ITR/kg on GD 10.5. Zn pre-treatment significantly increased fetal and placental masses, totally eliminated the incidence of cleft palate and lessened the frequency of *post-partum* mortality by 74%. Pre-treatment of GD 8.5 embryos with a 24h culture period with 15 µM Zn improved growth and totally restored normal embryonic development altered by a 24h culture with 17 µM ITR. Zn pre-treatment significantly lessened the reduction in crown-rump length induced by ITR, but not in the sum of scores. Zn decreased the frequencies of unfused mid brain and fore brain by 100%, and the incidence of abnormal flexion by 74%. A reduction in MT concentrations was observed in groups exposed to ITR, indicating that MT may have been oxidized by ITR-derived toxic FR. These results suggest that modulation of MT levels in mouse embryos by Zn can protect against FR toxicity of a teratogen such as ITR.

RÉSUMÉ

Il avait été démontré que l'induction de métallothionéine (MT) par le Zn protégeait contre l'exposition subséquente aux radicaux libres (RL). Cette recherche évalua le potentiel protecteur du Zn, par modulation de MT, contre les effets tératogènes de l'isotrétinoine (ITR), un médicament dermatologique nuisant, par oxidation, au développement embryonnaire des souris CD-1. Une supplémentation de Zn induisit la MT de façon significative chez les embryons de souris *in vivo* ainsi qu'*in vitro*. À 6.5 jours de gestation (JG), des souris gravides reçurent une injection sous-cutanée de Zn de 0 (saline), 20 et 40 mg/kg, résultant, après 48h, en des concentrations embryonnaires de MT de 12.5, 54.5 et 93.4 µg/g de protéines, respectivement. Des embryons furent extraits à 8.5 JG et incubés avec 0 (saline) et 15 µM Zn pendant 48h. Le groupe traité au Zn contint six fois plus de MT, résultant en une concentration de 688 µg/g de protéines. La pré-induction de MT par une exposition au Zn offrit une protection contre l'exposition subséquente à l'ITR, *in vivo* ainsi qu'*in vitro*. L'injection de 40 et 20 mg Zn/kg à des souris gravides à 8.5 et 9.5 JG, respectivement, remédia aux dommages foetaux causés par trois intubations intragastriques de 100 mg ITR/kg à 10.5 JG. Le pré-traitement au Zn accrut de façon significative les masses foetale et placentale, élimina totalement l'incidence de fissure palatine et réduisit de 74% la fréquence de mortalité *post-partum*. Le pré-traitement d'embryons à 8.5 JG par une culture de 24h avec 15 µM Zn améliora la croissance et rétablit totalement le développement embryonnaire altérés par une culture de 24h avec 17 µM ITR. Le pré-traitement au Zn amoindrit de façon significative la réduction de la taille tête-à-croupe induite par l'ITR, mais non celle de la somme des points. Le Zn diminua de 100% la fréquence des cerveaux médian et antérieur non-fusionnés et de 74% l'incidence de flexion anormale. Chez les groupes exposés à l'ITR une réduction des concentrations de MT fut observée, indicant que la MT aurait pu être oxidée par les RL toxiques dérivés de l'ITR. Ces résultats suggèrent que la modulation des niveaux de MT par le Zn chez les embryons de souris peut protéger contre la toxicité des RL dérivés d'un tératogène tel que l'ITR.

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ASA : Acetylsalicylic Acid

CAT : Catalase

FR : Free Radical

4-oxo-ITR : 4-oxo-Isotretinoin

4-oxo-RA : 4-oxo-Retinoic Acid

GD : Gestational Day

GPX : Glutathione Peroxidase

GSH : Glutathione

H_2O_2 : Hydrogen Peroxide

ITR : 13-cis-Retinoic Acid, Isotretinoin

MDA : Malondialdehyde

MT : Metallothionein

$O_2^{-\bullet}$: Superoxide Anion

·OH : Hydroxyl Radical

PGG2 : Prostaglandin G2

PGH2 : Prostaglandin H2

PGS : Prostaglandin Synthase

PUFA : Polyunsaturated Fatty Acid

RA : Retinoic Acid

s.c. : Sub Cutaneous

SOD : Superoxide Dismutase

ssb : Single Strand Break

1. INTRODUCTION

1.1. ISOTRETINOIN

1.1.1. Historical Background and Human Teratogenicity

Retinoic acid analogs were developed in the late 1960's by the pharmaceutical company Hoffman-Laroche (Basel, Switzerland). It was discovered that one of them, isotretinoin (13-cis-retinoic acid, ITR), was a potent reductor of sebum secretion through the decrease of sebaceous gland size and inhibition of sebaceous gland differentiation (Chalmers, 1992). Released into the American market as Accutane® in 1982, ITR was prescribed for the treatment of severe inflammatory, cystic and recalcitrant acne (Canadian Pharmaceutical Association, 1994). The results seemed miraculous and represented a major advance in skin disease therapy.

However, ITR treatment also caused important side effects characteristic of hypervitaminosis A such as alterations in skin, hair and mucous membranes, alteration in circulating lipid levels and high dosage could induce abnormal new bone formation (Hummler and Schüpbach, 1981). Furthermore, ITR proved to be a highly potent human teratogen when used at therapeutic doses. In a prospective study following 65 pregnancies where the mother had taken ITR during the first trimester, Lammer et al. (1988) estimated a 40% increase in risk of miscarriage. In those pregnancies reaching 20 or more weeks of gestation, there was a one in four risk of major malformation. The manufacturers of Accutane®, Hoffman-Laroche, stated that 160 000 females of childbearing age

took the drug from 1982 until 1985, and that 426 cases of exposed pregnancies were reported to them from 1982 to 1989. In contrast with these reported cases, the U.S. FDA estimated that 900-1300 babies were born with severe birth defects and that 700-1000 spontaneous abortions and 5000-7000 induced abortions which occurred from 1982 to 1987 were accountable to Accutane® treatment (Schardein, 1993). ITR-induced abnormalities include craniofacial (anotia, microtia, small or absent auditory canals, microphtalmia, cleft palate, facial dysmorphia), cardioaortic (septal defects, aortic arch abnormalities, tetralogy of Fallot), central nervous system (hydrocephaly, microcephaly, posterior fossa cyst, cortical blindness, retardation) and skeletal birth defects as well as thymic hypoplasia and parathyroid deficiency (Rosa et al., 1986; Canadian Pharmaceutical Association, 1994).

Because of the severity of ITR teratogenicity, Accutane® is contraindicated in females of childbearing potential, unless patients have failed to respond to any other treatment. In such cases: 1) a pregnancy test must be performed within two weeks prior to starting Accutane® treatment, 2) treatment should start on the second or third day of the next normal menstrual period, following a negative pregnancy test, 3) effective contraception must be used for at least four weeks before, during, and four weeks after treatment. It is strongly recommended that patients have regular monthly pregnancy tests throughout. If a pregnancy does occur during treatment or the post-treatment period, termination of the pregnancy may be advised (Canadian Pharmaceutical Association, 1994).

1.1.2. Animal Studies

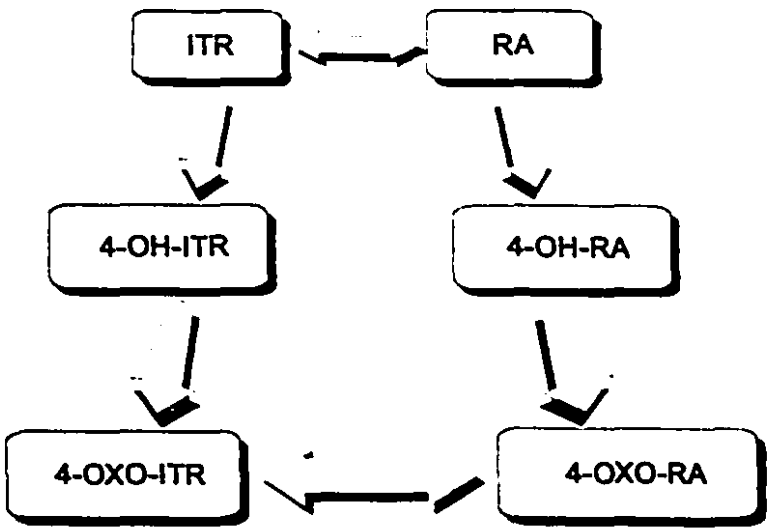
In vivo rodent exposure to ITR during organogenesis has shown to be embryotoxic only at extremely high or at multiple and regular doses since placental transfer of 13-cis-retinoic acid to the mouse embryo is limited (Creech-Kraft et al., 1991). Abnormalities observed in several mouse strains include mandible, eye, middle ear (Louryan et al., 1992), palate and skull defects (Yuschak and Gautieri, 1993), malformed branchial arches, tail irregularities (Creech-Kraft and Juchau, 1991) as well as skeletal alterations such as delayed ossification, limb bone and paw defects and fused or misaligned sternebrae (Yuschak and Gautieri, 1993). *In vitro* studies indicate a lower viability of mouse embryos and a high incidence of craniofacial, limb and tail malformations after ITR exposure (Ritchie and Webster, 1991).

1.1.3. Mechanisms of Teratogenicity

A. BIOTRANSFORMATION

It has been suggested that ITR exerts a teratogenic effect through biotransformation into parent compounds (Fig.1). ITR is known to isomerize normally to the active form of vitamin A, all-trans-retinoic acid (tretinoin, RA). Both of these isomers are further metabolized to give rise to 4-oxo-ITR and 4-oxo-RA, respectively. It has been shown that RA (Creech-Kraft et al., 1991) and 4-oxo-RA (Sartre et al., 1989) were six-fold, and 4-oxo-ITR (Kochhar and Penner, 1987) four-fold more potent teratogens than ITR. These metabolites are efficiently transported across the placenta and their half-life is longer than that of ITR (Creech-Kraft et al., 1991; Sartre et al., 1989; Kochhar and Penner, 1987).

Fig.1: Retinoid Biotransformation[1,2]



1: Adapted from Kochhar and Penner (1987).
2: Isotretinoin (ITR) isomerizes to all-trans-retinoic acid (RA). Both these isomers are further metabolised to give rise to 4-oxo-ITR and 4-oxo-RA, respectively.

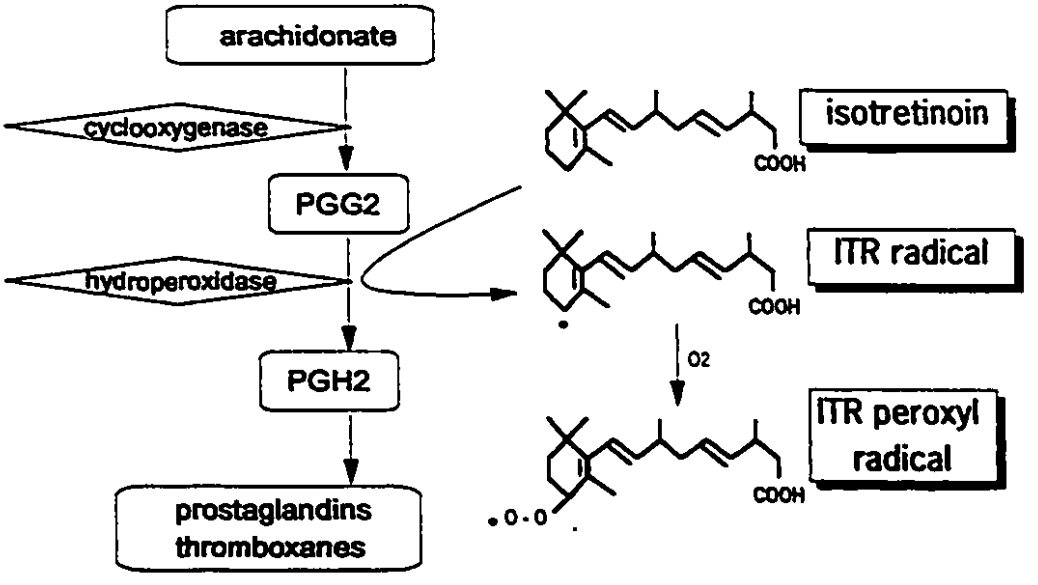
Additionally, rat conceptuses in culture have been shown to be capable of retinoid biotransformation (Creech-Kraft and Juchau, 1992).

B. FREE RADICAL GENERATION

Retinoids may also induce embryonic malformations by generation of toxic free radicals (FR). Davis et al. (1990) found that culture of chick neural crest cells with 13-cis or 4-oxo-RA both increased the liberation of oxygen reactive species superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH). Addition of scavenging enzymes superoxide dismutase (SOD) and catalase (CAT) to the culture medium reduced the quantity of FR generated and increased cell viability.

A postulated mechanism for FR formation is through the co-oxidation of ITR by hydroperoxidase in the prostaglandin synthase (PGS) pathway (Samokyszyn et al., 1984; Kubow, 1992) (Fig. 2). During prostaglandin and thromboxane synthesis, arachidonic acid is first oxidized to prostaglandin G2 (PGG2) by the cyclooxygenase component of PGS. PGG2 is then reduced by the hydroxyperoxidase component of PGS to form prostaglandin H2 (PGH2), which is further metabolized into prostaglandins and thromboxanes. This latter reaction requires non-specific reducing cofactors. Consequently, numerous endogenous compounds and drugs may serve as electron donors, thus generating electron-deficient metabolites (Siedlick and Marnett, 1984). It was shown that ITR could be co-oxidized *in vitro* by the hydroxyperoxidase component of PGS to form an ITR-peroxyl radical which may oxidize biomolecules and produce more radical species (Samokyszyn et al., 1984). In

Fig.2 : Prostaglandin Synthase Mediated Mechanism of Free Radical Bioactivation of Isotretinoin[1,2]



1: Adapted from Kubow (1992).
2: Postulated co-oxidation of isotretinoin to a teratogen free radical by the hydroperoxidase catalytic component of the prostaglandin synthase. PGG2: prostaglandin G2; PGH2: prostaglandin H2.

mice, pre-treatment with acetylsalicylic acid (ASA), an inhibitor of the cyclooxygenase component of PGS, provided protection against ITR teratogenicity (Kubow, 1992). ASA caused 75% and 26% reductions in embryonic posterior and anterior defects, respectively. Since embryos demonstrate high PGS activity (Mitchell et al., 1985), embryonic PGS metabolism of ITR into ITR-peroxyl FR could contribute to the teratogenicity of the drug.

1.2. FREE RADICALS

Free radicals are atoms or molecules with an unpaired electron, which leads to a very high chemical reactivity (Slater, 1984). They are necessary for the normal operation of a wide spectrum of biological processes where their reactivity is targeted towards a specific reaction. Some examples of normal production and utilization of FR include oxygen metabolism, inflammatory response, and oxygenase and oxidase enzyme activity. Free radicals may also be formed as by-products of the metabolism of exogenous compounds such as drugs, herbicides, solvents and other chemicals, or from exposure to certain physical stresses (Pryor, 1986).

The most important FR in biological systems are often named reactive oxygen species, since some of these compounds, such as H_2O_2, are not radicalic in nature. Reaction of $O_2^{\cdot-}$ and H_2O_2 with (eqs.1, 2, 3) or without (eq.4) transition metals, such as iron or copper, form the short-lived and highly reactive ·OH (Rice-Evans and Burdon, 1993).

(1) $H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + {\cdot}OH$ (Fenton reaction)

(2) $H_2O_2 + Cu^{+} \longrightarrow Cu^{2+} + OH^- + {\cdot}OH$

(3) $O_2^{\cdot-} + H_2O_2 \xrightarrow{Fe^{2+}/Cu^{+}} OH^{-} + O_2 + {\cdot}OH$ (Harber-Weiss reaction)

(4) $O_2^{\cdot-} + H_2O_2 + H^{+} \longrightarrow O_2 + H_2O + {\cdot}OH$

Tissue injury may be due to increased FR formation and/or decreased resistance to them. FR may oxidize virtually any molecule, leading to metabolic and structural modifications which can ultimately cause cell death (Freeman and Crapo, 1982). These modifications include: protein denaturation and cross-linking, enzyme inhibition, DNA strand scission, base modifications and mutations, cell surface receptor changes, cholesterol and fatty acid oxidation, lipid cross-linking and organelle and cell permeability changes (Freeman and Crapo, 1982).

Lipid peroxidation is particularly damaging because it proceeds as a self-perpetuating chain-reaction. Oxidation of a polyunsaturated fatty acid (PUFA) generates a fatty acid radical (L·) (eq.5) that is rapidly transformed into a fatty acid peroxyl radical (LOO·) by addition of oxygen (eq.6). Peroxyl radicals are the propagators of the chain reaction, oxidizing other PUFAs and producing lipid hydroperoxydes (LOOH) (eq.7). In the presence of iron, lipid hydroperoxyde can break down into further radical species LOO· and alkoxyl radical (LO·). They can also undergo cleavage of carbon bonds and form reactive aldehyde compounds, such as malondialdehyde (MDA) and 4-hydroxynonenal (Cheeseman and Slater, 1993).

(5) $LH + R{\cdot} \longrightarrow L{\cdot} + RH$

(6) $L{\cdot} + O_2 \longrightarrow LOO{\cdot}$

(7) $LOO{\cdot} + L'H \longrightarrow LOOH + L'{\cdot}$

The enzymes superoxide dismutase (SOD) (eq.8), catalase (CAT) (eq.9) and glutathione peroxidase (GPX) (eq.10 and 11) as well as non-enzymatic antioxidants, such as vitamin C, vitamin E and beta-carotene, can scavenge FR before they cause damage to various biomolecules or prevent oxidative stress from spreading (deGroot, 1994).

(8) $2\,O_2^{-\cdot} + 2\,H^+ \xrightarrow{\textit{superoxide dismutase}} H_2O_2 + O_2$

(9) $2\,H_2O_2 \xrightarrow{\textit{catalase}} 2\,H_2O + O_2$

(10) $H_2O_2 + 2\,G\text{-}SH \xrightarrow{\textit{glutathione peroxidase + Se}} 2\,H_2O + G\text{-}S\text{-}S\text{-}G$

(11) $LOOH + 2\,G\text{-}SH \xrightarrow{\textit{glutathione peroxidase + Se}} LOH + H_2O + G\text{-}S\text{-}S\text{-}G$

Intracellular thiol groups, most in the form of glutathione (GSH), are effective FR stabilizers because of their labile hydrogen atom. ·OH (eq.12) and $O_2^{-\cdot}$ (eq.13) may interact with a thiol, producing a poorly reactive thiyl radical (RS·). RS· reacts rapidly with another thiol, generating the reducing species $RSSR^{-\cdot}$ (eq.14) which may then be transformed into $O_2^{-\cdot}$ and the stable disulfide RSSR by addition of oxygen (eq.15). Reaction of RS· radicals with one another result in the formation of RSSR (eq.16) (deGroot, 1994; Slater, 1984; Thomas et al., 1986).

(12) $\cdot OH + R\text{-}SH \longrightarrow H_2O + RS\cdot$

(13) $O_2^{-\cdot} + RS^- + 2H^+ \longrightarrow RS\cdot + H_2O_2$

(14) $RS\cdot + RS^- \longleftrightarrow RSSR^{-\cdot}$

(15) $RSSR^{-\cdot} + O_2 \longrightarrow O_2^{-\cdot} + RSSR$

(16) $2\,RS\cdot \longrightarrow RSSR$

1.3. METALLOTHIONEIN

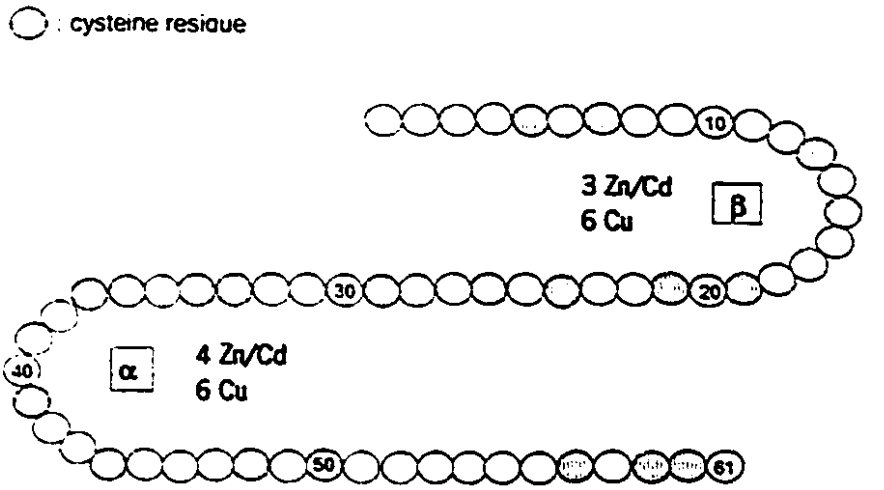
1.3.1. Characteristics

Metallothionein (MT) is a single-chain polypeptide of low molecular weight (6000-8000 Da), containing 61 amino acids with 20 cysteine residues (Fig.3). In animals, the protein is most abundant in parenchymal tissues, i.e., liver, kidney, pancreas and intestines (Kagi and Schaffer, 1988). The wide variation in concentrations in different species and tissues reflects the effects of age, stage of development, nutrition and other factors not yet fully identified (Kagi and Schaffer, 1988). Functions of MT include homeostasis of essential minerals such as Zn and Cu and detoxification of heavy metals such as Cd and Hg (Cherian and Chan, 1993). The affinity of metals for the protein binding sites is in the order of Zn< Cd< Cu and Hg (Otvos et al., 1993). MT contains two separate metal-thiolate clusters. Cluster A contains 11 cysteines and may bind a maximum of 4 atoms of Zn and/or Cd or 5-6 of Cu, or any combination thereof. Cluster B has 9 cysteines and may bind 3 atoms of Zn and/or Cd or 6 atoms of Cu, or any combination thereof (Hamer, 1986). Synthesis of MT is induced by other metals as well as by other factors such as physical stress, glucocorticoids and cytokines involved in inflammatory response, by radiation and by organic substances generating FR such as alkylating agents, drugs, herbicides, solvents and producers of hydroperoxide (Sato and Bremner, 1993).

1.3.2. Metallothionein Against Oxidative Stress

The induction of MT synthesis by FR generating compounds and the high number of thiol groups the protein contains have led to the speculation that MT may be involved in cellular defense against oxidative stress by acting as a radical

Fig.3 : Metallothionein[1]



1: From Sato and Bremner (1993).

scavenger (Templeton and Cherian, 1991). Thornalley and Vasak (1985) demonstrated that MT was more effective in scavenging ·OH than O_2^{-}· *in vitro*. FR have been shown to cause subsequent metal loss from MT and thiolate oxidation (Thornalley and Vasak, 1985; Fliss and Ménard, 1992). However, it has been suggested that FR induction of MT, and the FR-scavenging ability of MT are two independent events, as shown in oxidative stress induced in rats by paraquat (Sato and Sazaki, 1991), and in mice by menadione or CCl_4 (Min et al., 1992). In both experiments, pre-treatment with vitamin E, although protective against oxidative stress induced by these chemicals, did not affect the levels of MT found in the liver. It was hypothesized that cytokines are an important mediating factor involved in the induction of MT by FR (Min et al., 1992, 1993 ; Sato et al., 1993).

Nevertheless, appreciable evidence has accumulated which indicates that MT induction by pre-treatment with Zn provides protection against subsequent *in vitro* and *in vivo* FR exposure. As reviewed in Table 1, studies performed with different models such as cell lines (Ochi, 1988; Greenstock et al., 1987; Mello-Filho et al., 1988), rat liver slices (Chan et al., 1992) or live mice (Matsubara, 1987) exposed to various types of oxidative stress have shown that Zn pre-treatment significantly induced MT expression and decreased toxicity.

Other investigations went a step further. As shown in Table 2, experiments performed with different models such as cell membrane (Thomas et al., 1986), cell lines (Chubatsu and Meneghini, 1993), hepatocytes (Coppen et al., 1988) or live rats (Yang et al., 1991) exposed to various types of oxidative

Table 1: Protective Effect of Zn-MT

AUTHOR	SYSTEM	TREATMENT	INDICES
Ochi (1988)	cell lines	*t*-butyl hydroperoxide	-cell survival -colony forming ability
Greenstock et al. (1987)		irradiation	
Mello-Filho et al. (1988)		-H_2O_2 -xanthine-xanthine oxidase	
Chan et al. (1992)	rat liver slices	menadione	membrane intergrity
Matsubara (1987)	mice	irradiation	survival

Table 2 : Zn-MT Mitigation of Free Radical Toxicity

AUTHOR	SYSTEM	TREATMENT	INDICES
Thomas et al. (1986)	cell membranes	xanthine-xanthine oxidase	-malondialdehyde -SH and Zn
Chubatsu and Meneghini (1993)	cell lines	H_2O_2	DNA single strand break
Coppen et al. (1988)	hepatocytes	-*t*-butyl hydroperoxide -methylindole	-malondialdehyde -free radicals
Yang et al. (1991)	rats	gentamicin	serum malondialdehyde

stress have also shown that Zn pre-treatment significantly induced MT expression and reduced toxicity. Furthermore, these studies have demonstrated that Zn pre-treatment specifically inhibited oxidative damage as measured by decreased levels of MDA (Thomas et al., 1986; Coppen et al., 1988; Yang et al., 1991) or DNA single strand break (ssb) formation (Chubatsu and Meneghini, 1993). Possible mechanisms of inhibition of oxidative damage by Zn-MT include:

(a) Thiolate oxidation by FR leading to loss of thiol content of the protein with concomitant metal displacement (Thornalley and Vasak, 1985; Fliss and Ménard, 1992). Zn release and subsequent uptake by membranes would cause dislocalization or destabilization of membrane-bound Fe, thus suppressing the production of reactive •OH involving Fe catalyzed reactions (Thomas et al., 1986; Ochi, 1988). Furthermore, upon release, Zn could act as a "compensatory messenger" of oxidative stress by a feed-back mechanism, stimulating a factor in the promoter region of the MT gene, resulting in enhanced expression of Zn-MT under conditions of oxidative stress (Thomas et al., 1986).

(b) MT could also act as a reducing agent of radiation-induced FR. As suggested by Greenstock et al. (1987), MT may not only scavenge FR, but also donate a hydrogen atom to a cellular target radical on a DNA molecule, restoring it to an undamaged state after radiation exposure.

Some experiments found that MT induction by pre-treatment with Zn did not offer protection against FR toxicity. However, it can be argued that the experimental protocols played a role in these findings. The use of low Zn or Cd concentrations may not have induced sufficient levels of MT to scavenge all FR

generated (Lorher and Robson, 1989 ; Kaina et al., 1990). Also, the use of Cu-containing MT may increase lipid peroxidation by allowing Cu to participate in the Fenton or the Harber-Weiss reactions (Arthur et al., 1987). Ochi and Cerutti (1989) observed a protective effect of Zn against FR cytotoxicity, but did not find Zn-MT suppression of DNA ssb formation. However, Chubatsu and Meneghini (1993) found a protective effect of Zn-MT on DNA ssb by a longer Zn pre-exposure (16h vs 10-12h) and shorter FR treatment (30 min. vs 1h).

The relative importance of MT to act as a scavenger is dependent on: 1) the presence of MT in sufficient concentration in specific cellular compartments, 2) the relative activity or concentration of the enzymes SOD, GPX and CAT, and of other cytosolic thiols, namely glutathione, and 3) the amount of metal co-enzyme, Fe or Cu, involved in the redox cycling. In summary, there is much evidence from both *in vitro* and *in vivo* experiments (Tables 1 and 2) to support the hypothesis that preinduced Zn-MT can act as a radical scavenger and prevent oxidative damage.

1.3.3. Metallothionein Induction by Zinc During Mouse Gestation

Levels of MT mRNA are low in preimplantation mouse embryos but increase significantly at the blastocyst stage (gestational day (GD) 4). From implantation (GD 5) to late gestation, the embryo is surrounded by cells of the yolk sac, of the deciduum and, later (from GD 12) of the placenta, that actively express MT genes (Andrews et al., 1993).

Following 5h *in vitro* Zn exposure, MT mRNA induction has been detected at the blastocyst stage by Andrews et al. (1991). De et al. (1990) found similar results with GD 10 embryos cultured 5h with Zn or collected 5h following

maternal Zn injection. Vidal and Hidalgo (1993) also reported that MT synthesis in its protein form was increased significantly by a 24h *in vitro* Zn exposure at the blastocyst stage. With the development of the fetal liver, hepatic MT concentrations increase dramatically from GD 16, following Zn supplementation to pregnant rats, to reach a maximum at birth (Charles-Shannon et al., 1981). Hepatic MT levels in neonates decrease gradually from day 7 to stabilize at adult levels between days 28 and 35, while liver MT mRNA remain constant (Klaassen and Lehman-McKeeman, 1989). The effect of *in vivo* and *in vitro* Zn supplementation during the critical period of organogenesis (GD 8 to 10) on embryonic MT in its protein form has not been determined.

The use of whole mouse embryo culture permits the study of direct mechanisms, independent of maternal metabolism and of placental permeability characteristics. Embryonic development *in vitro* resembles *in vivo* development, with similar organ formation, but with a slower rate of growth (New, 1978).

2. RATIONALE, HYPOTHESIS AND OBJECTIVES

2.1. RATIONALE

Experimental evidence has shown that MT pre-induction by Zn protects against subsequent oxidative stress. Although the protective effect of Zn-MT against oxidative damage has never been studied with an *in vivo* and *in vitro* embryonic model, it has been demonstrated that MT mRNA is inducible at different stages of mouse embryonic development and that MT synthesis is increased at the blastocyst stage following Zn exposure. MT induction in its protein form has never been shown during the critical period of mouse organogenesis (GD 8 to 10). Because of the low activity of antioxidant enzymes in mid-gestational embryos and low glutathione concentration in differentiating cells (Allen and Balin, 1989), MT may play a more important role against FR oxidation.

It is now known that ITR is a potent teratogen for humans and animals. Furthermore, ITR may induce birth defects through FR generation, irreversibly damaging the embryo which does not yet possess the metabolic capacity to protect itself from such an insult. The present investigation was designed to evaluate the potential role of MT modulation by Zn in the protection against the teratogenic effects of ITR in CD-1 mouse embryos.

2.2. HYPOTHESIS

The hypotheses of this investigation were the following:

1. mid-gestational (GD 8 to 10) mouse embryos are capable of MT synthesis after Zn exposure and,

2. Zn treatment during mouse gestation could protect against ITR teratogenicity, by modulating embryonic MT synthesis.

2.3. OBJECTIVES

The overall objective of this investigation was to identify a possible protective role of *in vitro* and *in vivo* Zn supplementation, by inducing MT expression, specifically on growth and development of mid-gestational (GD 8 to 10) CD-1 mouse embryos exposed to ITR.

The specific objectives of this study were the following:

1. to evaluate the capacity of mid-gestational mouse embryos to synthesize MT after various routes of Zn exposure. Hence, the aim was to quantify embryonic MT expression following Zn exposure *in vitro*, *in vivo* and *in vivo* followed by a 48h whole embryo culture period;

2. to evaluate *in vitro* and *in vivo* ITR teratogenicity following Zn supplementation during gestation. Maternal influence, if any, on embryonic MT synthesis and protection against ITR was to be identified. Hence, growth and development of embryos were to be quantified following different combinations of Zn and ITR exposures, i.e. exposure to both Zn and ITR *in vitro*, exposure to both Zn and ITR *in vivo*, and exposure to Zn *in vivo* followed by exposure to ITR *in vitro*;

3. to identify a protective effect of embryonic MT against ITR teratogenicity. Hence, embryonic MT was to be quantified in relation to embryonic growth and morphological features as affected by ITR.

3. MATERIALS AND METHODS

3.1. EXPERIMENTAL DESIGN

This investigation was conducted in two parts. Part I investigated mid-gestational MT expression in mouse embryos, following various Zn exposures. Part II studied the protective effect of Zn supplementation during mouse gestation against subsequent ITR exposure.

3.1.1. PART I: Embryonic Metallothionein Induction by Zinc

Three experiments were conducted to test the hypothesis that mid-gestational embryos are capable of MT synthesis. Embryonic MT was assessed following exposure to Zn: 1) *in vitro*, 2) *in vivo*, and 3) *in vivo* followed by a 48h whole embryo culture period.

A. EXPERIMENT 1: EMBRYONIC EXPOSURE TO ZINC IN VITRO

On GD 8.5, five pregnant mice were killed and embryos containing four to six somites were randomly allocated to five culture groups: 1) 24h with saline (control) (n=4), 2) 24h with Zn 15 µM (1 µg/ml) (n=4), 3) 48h with saline (control) (n=9), 4) 48h with Zn 15 µM (n=4) and 5) 48h with ITR 17 µM (5 µg/ml) (n=9). At the end of the culture period, embryonic and yolk sac protein, MT and Zn concentrations were measured. Intrinsic Zn level of the culture medium was found to be 40 µM. The addition of 15 µM Zn was chosen because it is considered to be an effective *in vitro* physiological concentration to support

normal embryonic development (Mieden et al., 1986). ITR has been identified as inducing birth defects *in vitro* at a threshold concentration of 0.5 µg/ml (1.7 µM) (Ritchie and Webster, 1991). Based on previous preliminary results, a teratogenic dosage of 5 µg/ml iTR was chosen for this investigation.

B. EXPERIMENT 2: EMBRYONIC EXPOSURE TO ZINC IN VIVO

In previous experiments involving pre-induction of MT by Zn injection, administered dosages of Zn varied from 2mg/kg for four days (Wormser and Ben Zakine, 1989), to 10 and 20 mg/kg 48 and 24h prior to the toxic insult, respectively (Chan and Cherian, 1992; Chan et al., 1992a). Bay and Sit (1992) found that the LD_{50} for $ZnCl_2$ was approximately 50mg/kg when injected sub cutaneously (s.c.) five times per week, for three and a half weeks, in C57/6J mice. Concentrations of 20 and 40 mg Zn/kg, 48h prior to MT measurement were chosen in this study to investigate their potency on embryonic MT induction. On GD 6.5, 12 pregnant mice were randomly allocated to three concentration groups and were injected s.c. with 0 (saline) (n=4), 20 (n=4) or 40 (n=4) mg Zn per kg body mass. On GD 8.5, mice were killed and protein and MT concentrations were measured in embryos containing four to six somites and their Reichert's membrane. Retained Zn was measured in decidua and maternal livers.

C. EXPERIMENT 3: EMBRYONIC EXPOSURE TO ZINC IN VIVO *FOLLOWED BY A 48h WHOLE EMBRYO CULTURE PERIOD*

On GD 6.5, eight pregnant mice were randomly allocated to two concentration groups and were injected s.c. with 0 (saline) (n=4) or 40 (n=4) mg

Zn per kg body mass. On GD 8.5, half the mice from each group (n=4) were killed and the embryos containing four to six somites were cultured for 48h. The remaining mice were kept alive for the same period of time. On GD 10.5, cultured and live embryos were recovered and embryonic and yolk sac protein, MT and Zn concentrations were measured.

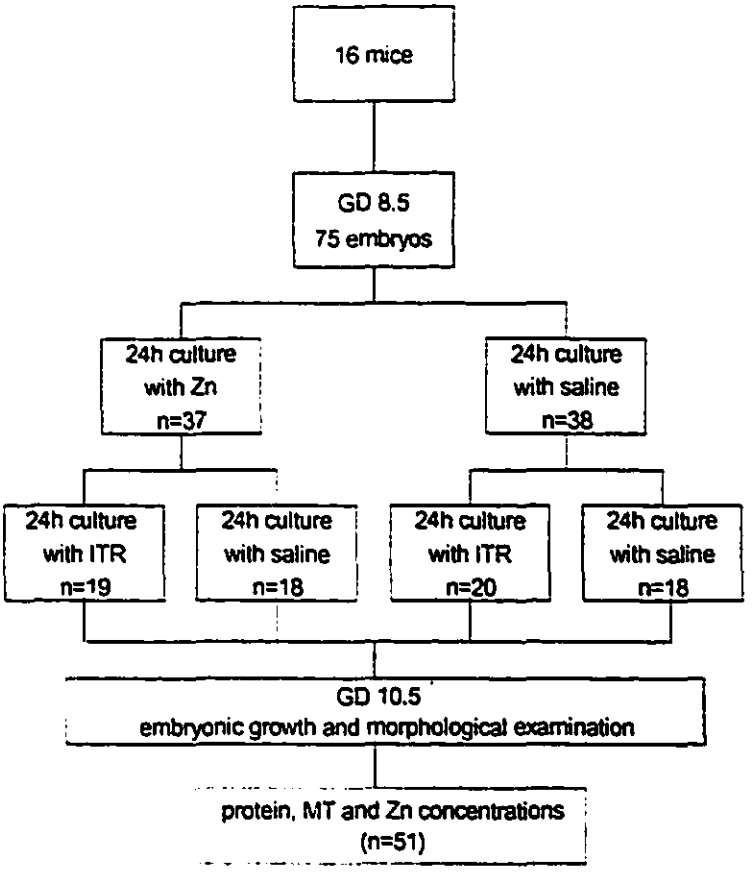
3.1.2. PART II: Protective Effect of Zinc-Metallothionein Against Isotretinoin Teratogenicity

Three experiments were conducted to test the hypothesis that Zn supplementation during mouse gestation could provide protection against subsequent ITR exposure. Embryonic growth and development was assessed following exposure to: 4) both Zn and ITR *in vitro*, 5) both Zn and ITR *in vivo*, and 6) Zn *in vivo* and ITR *in vitro*.

A. EXPERIMENT 4: IN VITRO *ZINC AND ISOTRETINOIN EXPOSURES*

On GD 8.5, 16 pregnant mice were killed and embryos of four to six somites were randomly allocated to the pre-treatment groups. The Zn group (n=37) was exposed *in vitro* to Zn 15 µM for 24h, and the control group (n=40) was exposed to saline under the same conditions. On GD 9.5, embryos were then cultured for 24h with either saline (control) or 17 µM ITR treatments. At the end of the culture period, embryos were examined for growth and development and embryonic and yolk sac protein, MT and Zn concentrations were measured (Fig. 4).

Fig.4 : Exp.4
In vitro Zn and ITR exposures



B. EXPERIMENT 5: IN VIVO *ZINC AND ISOTRETINOIN EXPOSURES*

Eight pregnant mice were randomly allocated to pre-treatment groups. On GD 8.5 and 9.5, the Zn group (n=4) received s.c. injections of 40 and 20 mg Zn per kg body mass, respectively, and the control group (n=4) was exposed to saline under the same conditions. On GD 10.5, all animals received three intragastric intubations of 100 mg ITR/kg body mass each, in the morning (11 A.M.), in the afternoon (3 P.M.) and in the evening (7 P.M.). This dose regime was selected based on a study previously conducted by Kubow (1992). On GD 18.5, mice were killed and fetuses recovered and examined for growth and development (Fig.5).

C. EXPERIMENT 6: IN VIVO *ZINC AND* IN VITRO *ISOTRETINOIN EXPOSURES*

Twenty pregnant mice were randomly allocated to pre-treatment groups. On GD 6.5 and 7.5, the Zn group (n=12) received s.c. injections of 40 and 20 mg Zn per kg body mass, respectively, and the control group (n=8) was exposed to saline under the same conditions. On GD 8.5, mice were killed and embryos of 4 to 6 somites from each litter were exposed *in vitro* to either 17 µM ITR or to control (saline) treatments. After 48h culture, embryos were examined for growth and development and embryonic protein, MT and Zn concentrations were measured (Fig. 6).

3.2. ANIMALS AND MATING PROCEDURES

CD-1 mice (Charles River Canada, St-Constant, Québec) were housed in

Fig.5: Exp.5
In vivo Zn and ITR exposures

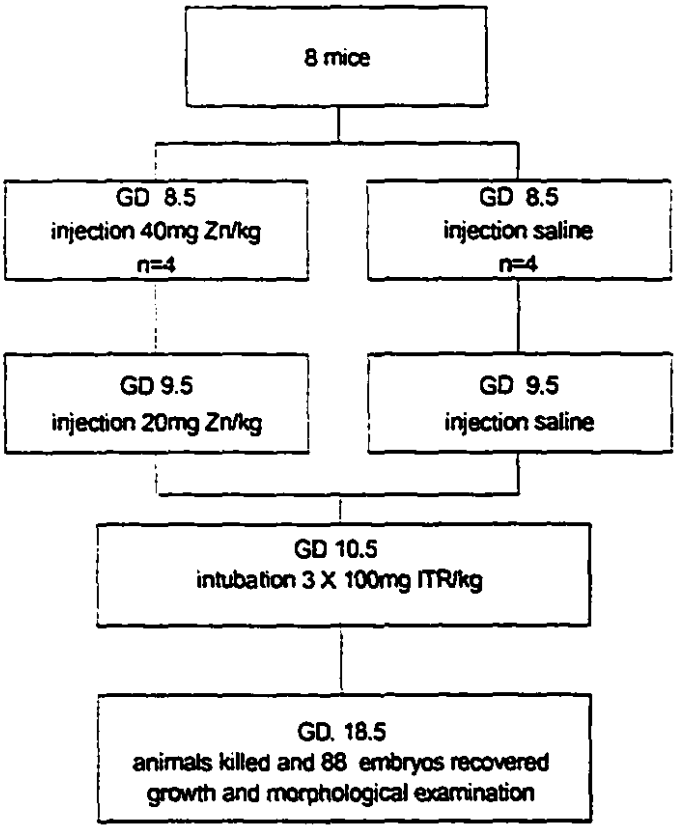
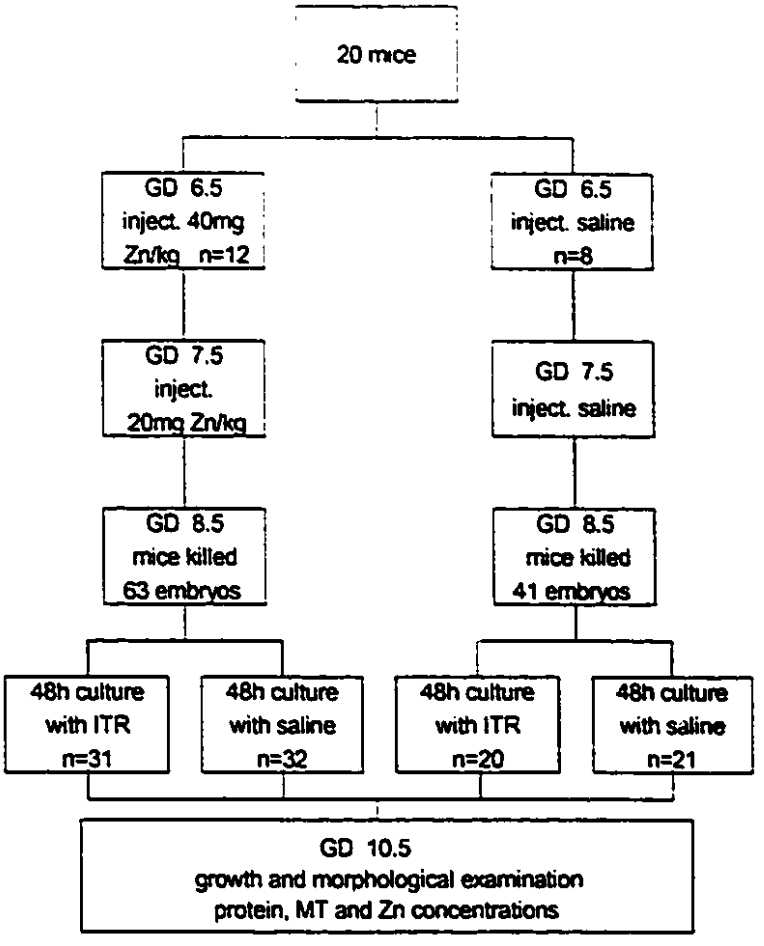


Fig.6: Exp.6
In vivo Zn and *in vitro* ITR exposures



a temperature-controlled room (24 ± 1 °C) with a 12 hr light-dark cycle (light from 8:00 to 20:00). The animals were kept in plastic shoe box cages with Beta-Chip bedding (Beta-Chip, Northeastern products Corp., Warrensburg, NY, USA). Purina Mouse Chow® (Ren's Feed and Supply, Oakville, Ontario, Canada) and tap water were fed *ad libitum*. Females were housed three or four per cage and males were caged individually.

For mating purposes, three or four females were housed with a male, at random, from 9:00 to 11:00. The presence of a vaginal plug indicated insemination and this day was considered as GD 0.5.

3.3. MOUSE EMBRYO CULTURE

The techniques for explanting and culturing embryos followed the methods described by New (1978). Mouse embryos were cultured in rat serum, provided by exsanguinated male Sprague-Dawley rats (Charles River Canada, St-Constant, Quebec), under halothane anaesthesia. The blood was collected through cardiac puncture and was centrifuged after clotting. The clot was removed and the serum was recentrifuged. Serum was pooled and heat-inactivated at 56°C for 20 min. and filter-sterilized using a 0.45 micron mesh filter (Millipore). The serum was stored at -80°C and thawed before use in embryo culture.

On GD 8.5, mice were killed by cervical dislocation and the uteri excised immediately. Using aseptic techniques, the decidua were dissected from uteri in Hank's Balanced Salt Solution (HBSS) (Gibco, Burlington, Ontario) under a

stereomicroscope. Embryos were exposed by removing the decidual tissue and Reichert's membrane and embryos containing four to six somites were chosen for culture. The ectoplacental cone, amnion and visceral yolk sac were left intact. Embryos were placed into 60 ml glass bottles containing 1.55 ml of warm sterile male rat serum per embryo. Streptomycin sulfate and penicillin G potassium were added to the medium at 5 µl per ml serum. For control treatment, sterile saline 0.9% was added for a final volume of 1.6 ml of culture medium per embryo. For Zn treatment, $ZnCl_2$ was dissolved in sterile 0.9% saline to prepare a stock solution of 69.4 µg $ZnCl_2$/ml, and was added to the medium for a final concentration of 1 µg Zn/ml (15µM). Isotretinoin was dissolved in 70% ethanol to prepare a stock solution of 1.25 mg ITR/ml and was added to the medium for a final concentration of 5 µg / ml (17 µM). All solutions were prepared immediately prior to addition to the culture medium.

The bottles were gassed with 5% O_2 - 5% CO_2 - 90% N_2 at the start of the culture, and with 20% O_2 - 5% CO_2 -75% N_2 and 40% O_2 - 5% CO_2 - 45% N_2 at 18 and 26 hr of culture, respectively. They were placed in a 37°C incubator and rotated at 30 rpm for a culture period of 24 or 48h.

3.4. GROWTH AND MORPHOLOGICAL ASSESSMENT OF CULTURED EMBRYOS

After culture, embryos were transferred in 0.9% saline solution and examined under a dissecting microscope. Yolk sac diameter, crown-rump length and head diameter of live embryos were measured using an eyepiece micrometer. Somite number was also counted. Morphological assessment was done following the scoring system of Brown and Fabro (1981) under which 13

morphological criteria are each given a score ranking from zero to five, corresponding to a given stage of differentiation. The sum of the scores for each individual feature gives a total morphological score that is indicative of the stage of development of each embryo (See Appendix A for scoring sheet). In the present investigation, the following features were considered abnormal for mid-gestational (GD 8 to 10) embryos: unfused neural tube, hind brain, mid brain or fore brain, malrotated, kinked or ventrally convex flexion of the tail, and missing forelimb. Although not included in the scoring system, enlarged pericardium and bleb were also recorded.

3.5. GROWTH AND MORPHOLOGICAL ASSESSMENT OF LIVE EMBRYOS

On GD 18.5, mice were weighed, killed by cervical dislocation and their uteri excised immediately. The number and mass of resorptions, the number of implantation sites, pup location and viability were recorded. Placentas were also matched to the pups and weighed. Fetuses were weighed, sexed, and examined for external malformations. Animals were examined for craniofacial defects, such as microagnathia, eye, ear or nose underdevelopment or malformation and cleft palate, and for skeletal defects, such as limb and tail underdevelopment or malformation. *Post-partum* mortality was recorded after a 2h period following fetus recovery.

3.6. INJECTIONS

$ZnCl_2$ was dissolved in sterile saline 0.9% to prepare stock solutions of

9.38 and 18.75 mg $ZnCl_2$ per ml and was immediately injected s.c. to mice according to their mass. Animals thus received doses of 20 and 40 mg Zn/kg body mass. Total volume injected did not exceed 0.2 ml. Control mice received injection of sterile saline 0.9% only.

3.7. INTRAGASTRIC INTUBATION

Isotretinoin was suspended in food grade safflower oil (ICN Biochemicals Ltd., cat.102888, St-Laurent, Que.) to obtain a stock solution of 8 mg/ml. The solution was sonicated and vortexed to obtain a uniform suspension, and was immediately administered to mice according to their mass. Animals thus received a dose of 100 mg ITR per kg body mass, three times, as described in section 3.1.2.B. Total volume administered did not exceed 0.57 ml.

3.8. PROTEIN ANALYSIS

Embryos, Reichert's membranes and yolk sacs were homogenized in sterile saline 0.9% with a Polytron® homogenizer (Brinkman, PT 3000). Total protein content was measured following the Bradford Microprotein Assay (Bradford, 1976), with the Bio-Rad Assay Kit II (Bio-Rad, no. 500-0002, Hercules, CA), using bovine serum albumin as the standard protein (see Appendix B.1 for calibration curve). Saline 0.9% was added to 0.1 ml of the homogenized samples to a volume of 0.8 ml and mixed with 0.2 ml of Bio-Rad dye reagent concentrate. Absorbance was read at 595 nm (Beckman, DU-40 spectophotometer, Fullerton, CA) in 1 ml cuvettes.

3.9. ZINC ANALYSIS

Zn concentrations were measured with flame atomic absorption spectrophotometry (Hitachi, Polarized Zeeman AAS, Z-8200) using a mixture of air and acetylene. Deciduas and maternal livers were first digested with 70-71% nitric acid. Embryos, Reichert's membranes and yolk sacs were homogenized as previously described. Absorbance was read at 213.9 nm (see Appendix B.2 for calibration curve).

3.10. METALLOTHIONEIN ANALYSIS

Total MT concentration in embryos, Reichert's membranes and yolk sacs were measured using an enzyme-linked immunosorbant assay (ELISA), as described by Chan et al. (1992b). Samples were homogenized in sterile saline 0.9% and reacted overnight with a 1:1000 dilution of an isolated IgG fraction of a rabbit antiserum to rat liver Cd-MT-2 polymer. Samples were then transferred to MT-2 (1mg/ml) coated plates and allowed to incubate for 30 min. at room temperature. After washing with a phosphate buffer saline/Tween (PBS/T) solution, the MT coated plates were incubated in turn with: 1) a 1:2000 dilution of biotinylated goat anti-rabbit IgG (Vector Lab Inc., no.BA-1000, Burlingame, CA) in PBS/T for 30 min., 2) a 1: 3000 dilution of horseradish peroxidase conjugated Avidin D (Vector Lab Inc., no. A-2004, Burlingame, CA) in PBS/T for 30 min., and 3) a 1:10 000 dilution of 1,2-phenylenediamine dihydroxide (Sigma Chemical Co., no. P-3804, St-Louis, MO) in PBS/T with 0.003% hydrogen peroxide (Sigma Chemical Co., no. H-1009, St-Louis, MO) until color development. Each step was preceded by three washes with PBS/T. The enzyme reaction was terminated by

adding 50 µl of 4N sulfuric acid, and absorbance was read at 490 nm (Molecular Devices. Vmax Kinetic Microplate Reader). MT concentration of the samples was determined by linear regression of the inhibition curve (see Appendix B.3) after logit Y transformation.

3.11. CHEMICALS

$ZnCl_2$ (Sigma Chemical Co., no. Z-3500, St-Louis, MO) was kept in a desiccator, at room temperature, for the time of the experiments. ITR (Isotretinoin, 13-cis retinoic acid, Sigma Chemical Co., no. R-3255, St-Louis, MO) was stored at -20°C in the dark in a sealed, lightproof container. ITR was handled in subdued light.

3.12. STATISTICAL ANALYSIS

Embryonic and yolk sac MT of 24h culture groups of Exp.1 and pregnancy outcome indices of Exp.5 were compared with a one-tailed unpaired t-test to identify any significant difference between treatment groups.

Yolk sac Zn of Exp.3 as well as fetal and placental weights of Exp.5 were compared with a one-tailed unpaired nested t-test to identify a significant difference between treatments, nested for dams. Dams were included in the analyses to control for different mice behaviors and metabolic responses to injection.

Embryonic MT (Exp.1- 48h and Exp.2), Reichert's membrane MT (Exp.2), as well as decidual and maternal liver Zn (Exp.2) were compared with a one-way

analysis of variance (ANOVA) to determine if there was a main effect of treatment on these parameters. Dams, nested within injection treatments were included in the analyses of Exp.2.

Growth parameters (Exps.4 and 6), embryonic MT and Zn (Exps.3, 4 and 6), Yolk sac MT (Exps.3, 4 and 6) and Zn (Exps.4 and 6), as well as mean number of malformations per embryo (Exp.4 and 6) were compared with a two-way ANOVA to identify the main effect of pre-treatment, treatment, and the interaction of pre-treatment and treatment on these parameters. For Exp.3, dams, nested within injection pre-treatment , were included in the analyses.

When a significant main effect was observed, one-way and two-way ANOVAs were all followed by least-significant difference multiple comparison test to identify group mean differences.

Frequency of abnormal embryos (Exps.4 and 6) as well as frequency of cleft palate and *post-partum* mortality (Exp.5) were compared with chi-square statistics.

Analyses were performed using SPSS software for Windows, version 5.0 (1992), except for nested analyses which were conducted using version 6.04 (1992) of SAS software. Model statements, t, F and p values from the statistical analysis of each dependent variable are listed in the Appendix C. A probability of $p<0.05$ was accepted as the minimal level of significance for all analyses.

4. RESULTS

4.1. EXPERIMENT 1:
Embryonic exposure to zinc *in vitro*

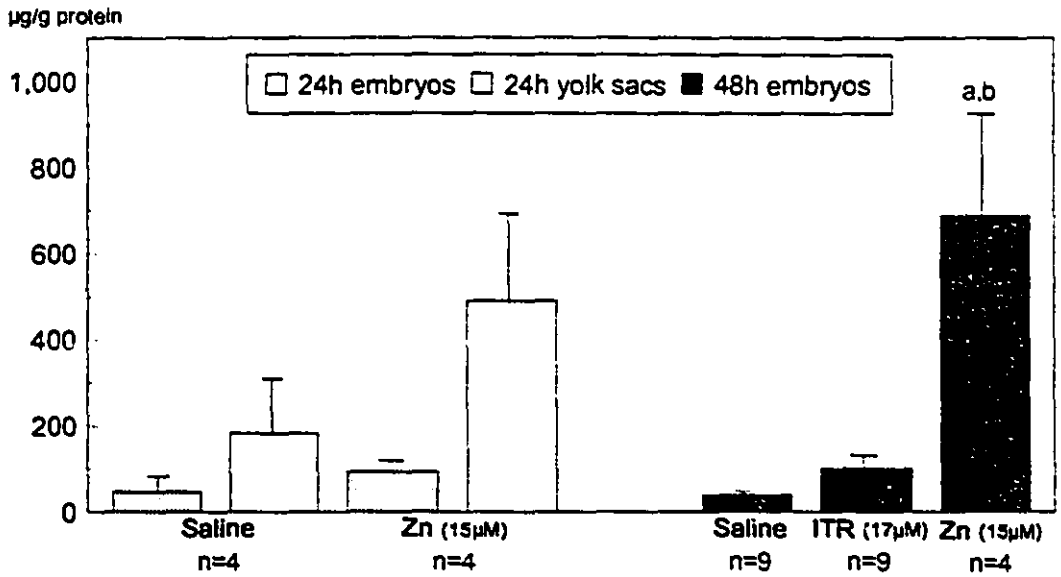
As illustrated in Fig. 7, *in vitro* exposure to 15 µM Zn for 24 and 48h resulted in mean embryonic MT concentrations of 93.9 and 688 µg/g protein, respectively. MT expression was significantly induced ($p<0.05$) within the 48h culture period, resulting in a six-fold increase in concentration, when compared to saline and ITR groups (40.3 and 102 µg/g protein, respectively). The mean MT concentrations in embryos and yolk sacs were higher but not statistically different from control of the 24h culture groups, possibly the result of large sample variations.

Following 24h *in vitro* exposure to saline or Zn, embryos contained similar amounts of Zn (411±10 (standard error) and 468±40 ng/embryo, respectively). However, yolk sac Zn was significantly higher ($p<0.05$) in the saline group (400±20 ng/yolk sac) than in the Zn group (357±10 ng/yolk sac). Zn concentrations after 48h exposure to saline, ITR or Zn could not be measured because of a lack of sufficient sample.

4.2. EXPERIMENT 2:
Embryonic exposure to zinc *in vivo*

Maternal injection of 0, 20 and 40 mg Zn/kg resulted in mean embryonic MT concentrations of 12.5, 54.5 and 93.4 µg/g protein, respectively (Fig. 8).

Fig.7 : Exp.1- Mean MT Concentrations in Embryos and Yolk Sacs Following Culture with Zn or ITR on GD 8.5 [1,2]



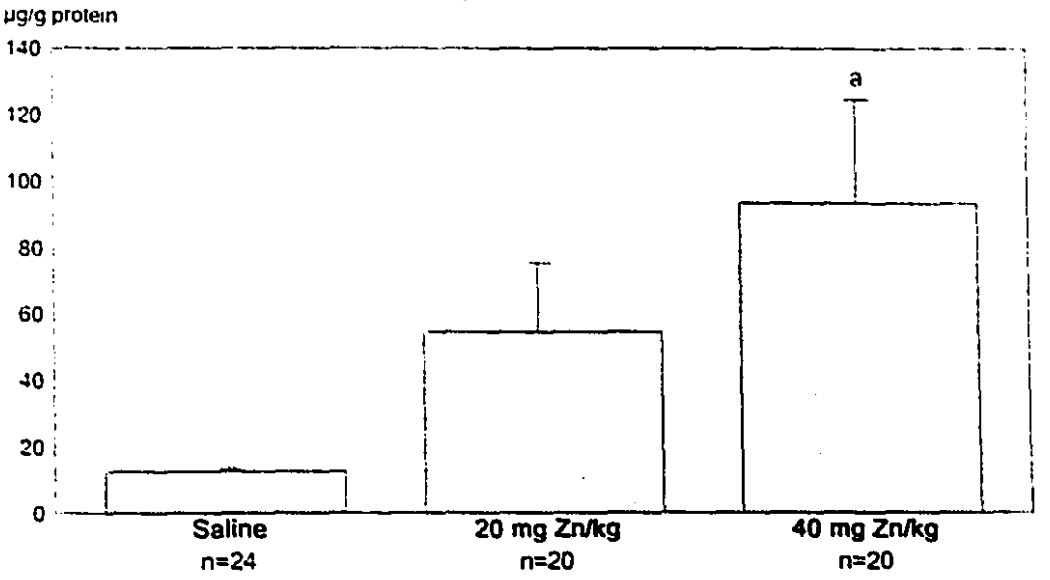
1: Embryos were cultured with 15 µM Zn for 24 and with 15 µM Zn or 17 µM ITR for 48h. MT was measured on GD 9.5 (24h) and on GD 10.5 (48h).

2: Values are expressed as mean + SEM. See Table C.1a for t-test t and p values (for 24h). See Table C.1b for ANOVA F and p values (for 48h).

a: Significantly different from Saline (48h), $p<0.05$.

b: Significantly different from ITR 17µM (48h), $p<0.05$.

Fig.8 : Exp.2- Mean MT Concentrations in Embryos Following Maternal Zn Injection on GD 6.5[1,2]



1: Dams were injected with 0, 20 or 40 mg Zn/kg. MT was measured on GD 8.5.
2: Values are expressed as mean + SEM. See Table C.2 for nested ANOVA F and p values.
a: Significantly different from saline, $p<0.05$.

Mean Reichert's membrane MT concentrations were 0.85, 0.64 and 7.09 mg/g protein, respectively (Fig. 9). Treatment with Zn significantly induced MT in the Reichert's membranes, but not in the embryos (See Table C.2 for ANOVA). However, the highest dosage significantly induced ($p<0.05$) MT in both embryos and Reichert's membranes when compared to the saline-injected group.

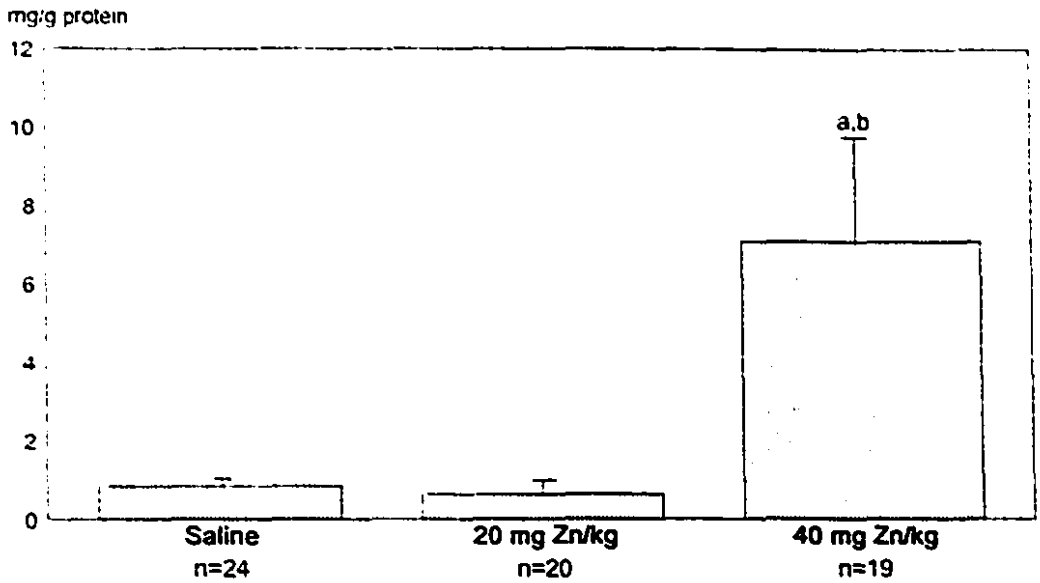
Decidual tissue of all three groups contained similar amounts of Zn, ranging from 0.700 to 0.776 ng/decidua.

Indicative that the treatments were properly administered, maternal liver Zn reflected the injected doses, resulting in levels of 34.2±0.64 (standard error), 48.1±2.77 and 53.6±6.97 µg/g wet mass, for the saline, 20mg Zn/kg and 40 mg Zn/kg treatment groups, respectively. The highest dose of Zn led to a significant increase ($p<0.05$) in liver Zn content, when compared to the saline-injected group.

4.3. EXPERIMENT 3 : Embryonic exposure to zinc *in vivo* followed by culture

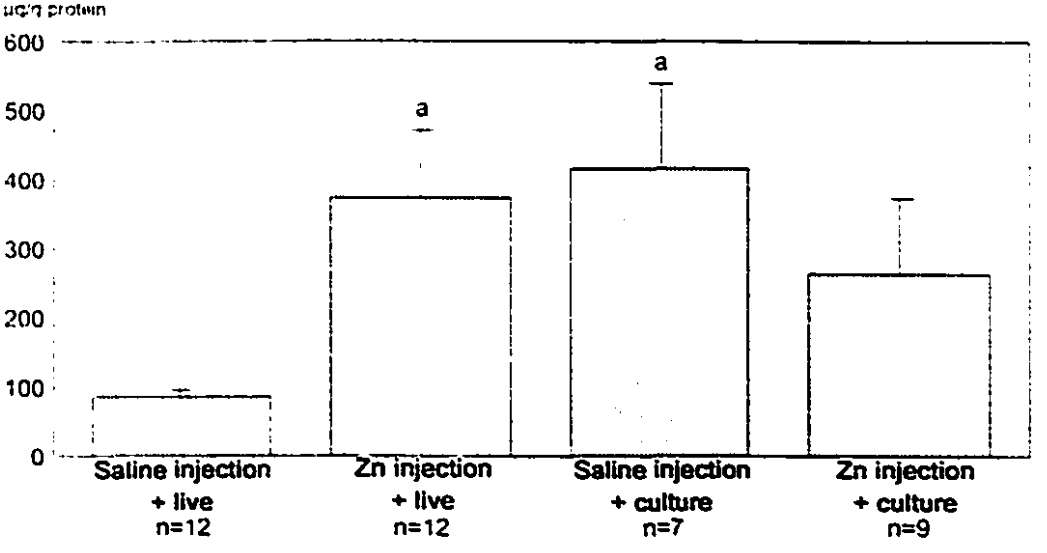
After dams were injected with Zn or saline, half of the embryos were recovered and cultured for 48h, whereas the other half was maintained *in vivo* for the same period. Four experimental groups were thus obtained: saline injection+culture, Zn injection+culture, saline injection+live and Zn injection+live. Similar to Experiment 2, a Zn injection on GD 6.5 resulted in a significant increase (375 µg/g protein) ($p<0.05$) in mean embryonic MT concentration on GD 8.5 when compared to a saline injection (86.7 µg/g protein) (Fig.10). Further

Fig.9 : Exp.2- Mean MT Concentrations in Reichert's Membranes Following Maternal Zn Injection on GD 6.5[1,2]



1: Dams were injected with 0, 20 or 40 mg Zn/kg. MT was measured on GD 8.5.
2: Values are expressed as mean + SEM. See Table C.2 for nested ANOVA F and p values.
a: Significantly different from saline, $p<0.05$.
b: Significantly different from 20 mg Zn/kg, $p<0.05$.

Fig.10 : Exp.3 - Mean MT Concentrations in Embryos Following Maternal Zn Injection on GD 6.5 and Culture on GD 8.5[1,2]



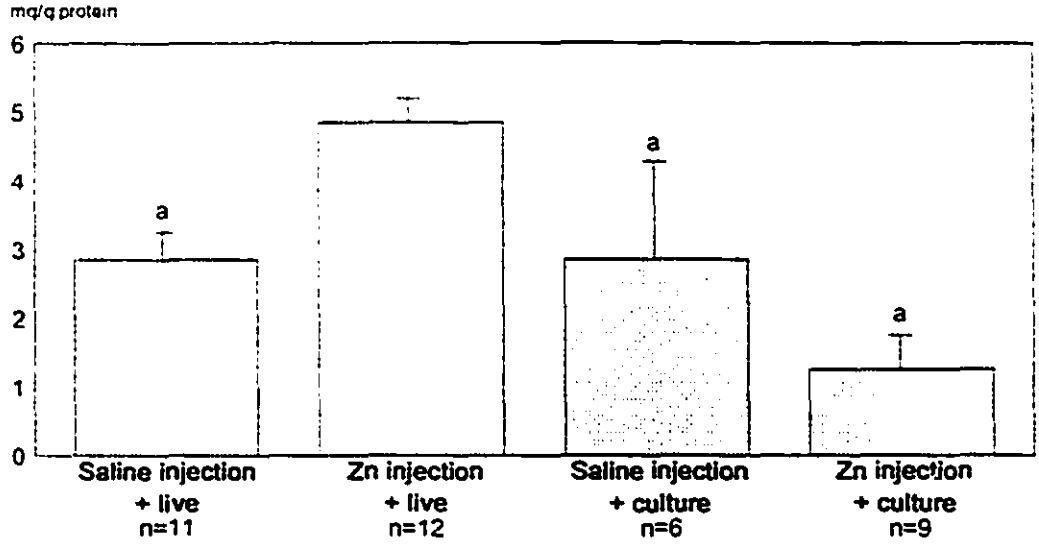
1: Dams were injected with 40 mg Zn/kg on GD 6.5. Half of the embryos were recovered on GD 8.5 and cultured for 48h, whereas the other half was maintained in vivo. MT was measured on GD 10.5.
2: Values are expressed as mean + SEM. See Table C.3a for nested ANOVA F and p values.
a: Significantly different from saline injection+live, $p<0.05$.

culture of the Zn-treated group for 48h did not affect the MT concentration (264 µg/g protein). However, culture of the saline-treated group for 48h resulted in a significantly higher ($p<0.05$) MT concentration (416 µg/g protein). This level was comparable to that of the Zn-treated group.

Embryonic Zn levels were 453±70 (star.dard error), 401±37, 299±13 and 383±30 ng/embryo, for the saline injection+live, Zn injection+live, saline injection+culture and Zn injection+culture groups, respectively. Saline injection+live treatment resulted in a significantly higher ($p<0.05$) embryonic Zn level, when compared to the saline injection+culture treatment. The fact that embryonic Zn concentrations are comparable in both the Zn- and saline-injected groups indicates that transfer of the metal to the embryo may be highly regulated.

Mean yolk sac MT was significantly higher ($p<0.05$) in the Zn injection+live group (4.85 mg/g protein) when compared to the two culture groups (1.26 and 2.86 mg/g protein for the Zn injection+culture and the saline injection+culture groups, respectively) (Fig.11). Saline-injected groups, either followed by culture or maintained *in vivo*, resulted in a similar yolk sac MT concentration of 2.86 mg/g protein. However, the yolk sac MT level of the saline injection+live group was significantly lower ($p<0.05$) than that of the Zn injection+live group. Yolk sac Zn levels were measured in the culture groups and were 410±19 (standard error) and 437±27 ng/yolk sac for the saline injection and the Zn injection groups, respectively. Yolk sac Zn concentrations of the two live groups could not be measured because of a lack of sufficient sample.

Fig.11 : Exp.3 - Mean MT Concentrations in Yolk Sacs Following Maternal Injection on GD 6.5 and Culture on GD 8.5[1,2]



1: Dams were injected with 40 mg Zn/kg on GD 6.5. Half the embryos were recovered on GD 8.5 and cultured for 48h, whereas the other half was maintained in vivo. MT was measured on GD 10.5.
2: Values are expressed as mean + SEM. See Table C.3a for nested ANOVA F and p values.
a: Significantly different from Zn injection + live, $p<0.05$.

4.4. EXPERIMENT 4:
In vitro zinc and isotretinoin exposures

As presented in Table 3, similar growth parameters were observed in the saline+saline and the Zn+saline culture groups. Treatment with ITR resulted in a decrease in crown-rump length and in the sum of scores, but the difference was not significant. However, Zn pre-treatment before drug exposure improved the reduction in crown-rump length, but not the reduction in the sum of scores. Both ITR treatment groups had a significantly ($p<0.05$) lower sum of scores and the saline+ITR group had a significantly ($p<0.05$) smaller crown-rump length when compared to the Zn+saline group.

Types and frequencies of embryonic abnormalities are listed in Table 4. Major embryonic malformations following the culture period included unfused neural tube, hind brain, mid brain and fore brain as well as abnormal flexion, missing forelimb, and enlarged pericardium. Similar frequencies of abnormalities were observed in the saline+saline and the Zn+saline groups, except for a significanlty lower ($p=0.03$) incidence of abnormal flexion in the group cultured with Zn+saline. Saline+ITR treatment significanlty increased ($p=0.04$) the incidence of unfused mid brain, when compared to the saline+saline treatment and raised, though not significantly, the frequencies of unfused neural tube, hind brain and fore brain, of abnormal flexion and of missing forelimb. Zn pre-treatment before drug exposure led to a significant reduction in the frequencies of unfused mid brain ($p=0.04$), fore brain ($p=0.04$) and abnormal flexion ($p=0.04$), when compared to the saline+ITR culture group, and in a trend towards a reduction in the frequencies of unfused neural tube, hind brain

Table 3 : Exp.4 - Mean Embryonic Growth Parameters After Culture With Saline or Zn, Followed by Culture With Saline or ITR[1,2]

	Crown rump length (mm)	Head diameter (mm)	Somite number	Sum of scores[3]	Embryonic protein (µg)	Yolk sac protein (µg)
Saline+ Saline (n=18)	2.64 ± 0.12	1.29 ± 0.09	28.76 ± 0.95	39.86 ± 2.03	325.59 ± 26.06	19.29 ± 1.75
Zn+ Saline (n=18)	2.89 ± 0.07	1.41 ± 0.07	29.50 ± 0.64	43.78 ± 1.03	320.76 ± 31.91	16.79 ± 1.46
Saline+ ITR (n=20)	2.41 ± 0.12 (a)	1.28 ± 0.06	28.75 ± 0.66	35.80 ± 1.71 (a)	324.82 ± 15.45	17.13 ± 1.20
Zn+ ITR (n=19)	2.58 ± 0.13	1.26 ± 0.08	28.71 ± 0.77	37.08 ± 1.67 (a)	360.44 ± 33.58	19.29 ± 0.96

1: GD 8.5 embryos were cultured for 24h with 15 µM Zn or saline. At GD 9.5, saline or 17 µM ITR was added to the culture medium for the next 24h. Embryos were examined on GD 10.5.
2: Values are expressed as mean ± SEM. Sample size is expressed in parenthesis. See Table C.4a for ANOVA F and p values.
3: Morphological assessment was done following a scoring system under which the sum of the scores for each individual feature gives a total score that is indicative of the stage of development of each embryo. See section 3.4.
a: Significantly different from Zn+ Saline, $p<0.05$.

Table 4 : Exp.4 - Types and Frequencies of Embryonic Abnormalities After Culture With Saline or Zn, Followed by Culture With Saline or ITR[1,2]

	Unfused neural tube	Unfused hind brain	Unfused mid brain	Unfused fore brain	Abnormal flexion	Missing fore limb	Enlarged pericardium	Bleb
Saline + Saline (n=18)	0	0	0	5.6	22.2	5.6	11.1	16.7
Zn + Saline (n=18)	0	0	11.1	16.7	0 (a)	0	5.6	5.6
Saline + ITR (n=20)	5.0	5.0	20.0 (a)	20.0	40.0 (b)	15.0	10.0	5.0
Zn + ITR (n=19)	0	0	0 (c)	0 (c)	10.5 (c)	5.3	15.8	36.8 (b,c)

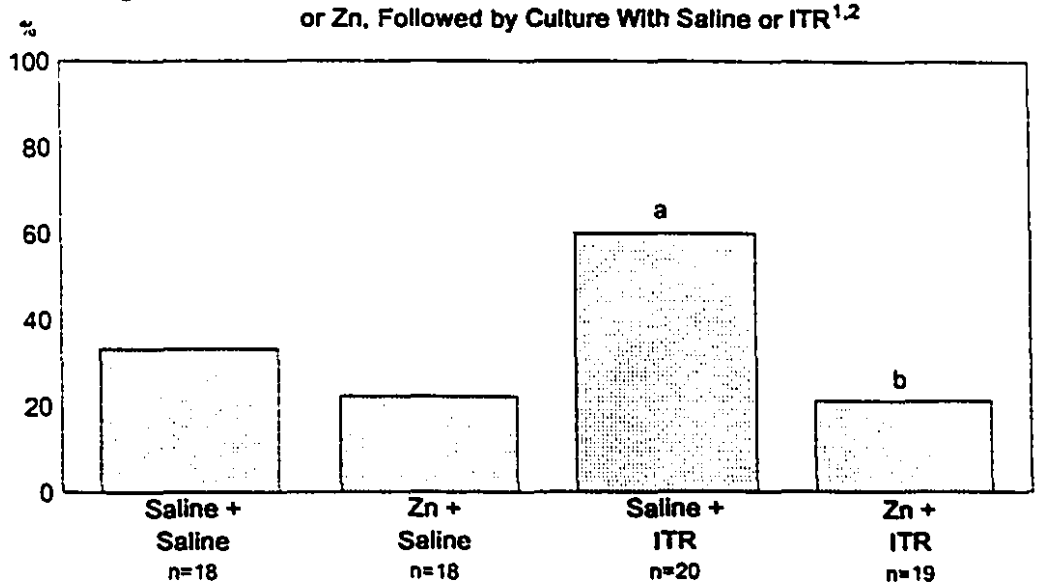
1: GD 8.5 embryos were cultured fro 24h with saline or 15 µM Zn At GD 9.5 saline or 17 µM ITR was added to the culture medium for the next 24h. Embryos were examined on GD 10.5.
2: Values are expressed in %.
a: Significantly different from Saline + Saline, $p<0.05$.
b: Significantly different from Zn + saline, $p<0.05$.
c: Significantly different from Saline + ITR, $p<0.05$.

and missing forelimb. Bleb frequency was significantly higher in the Zn+ITR culture group, when compared to the saline+ITR ($p=0.01$) and the Zn+saline ($p=0.02$) groups. Although plasma membrane blebbing is a common event in the progression of toxic injury, blebs appear before alterations in plasma membrane permeability characteristics and seem to be initially reversible (Orrenius et al.,1992). In the present experiment, small blebs were observed on the tail of some embryos, and were not considered as a major malformation.

For the purpose of calculating overall incidence of malformations, embryos with any major abnormality were counted once (Fig.12). For the mean number of malformations per embryo, the number of defects were counted separately, therefore the sum of abnormalities per embryo may exceed one (Fig.13). The frequency of malformed embryos was similar in the saline+saline (33%) and the Zn+saline (22%) groups. A higher frequency, though not significant, was observed in the saline+ITR culture group (60%) when compared to the saline+saline group. Zn+ITR treatment alleviated ITR teratogenicity by resulting in a significantly lower (21%) ($p=0.01$) incidence of malformed embryos than that of the saline+ITR treatment.

The mean number of defects per embryo was similar in the saline+saline (0.44) and the Zn+saline (0.33) groups. A significantly higher ($p<0.05$) mean was observed in the saline+ITR culture group (1.15) when compared to the saline+saline group. However, Zn+ITR treatment reduced ITR teratogenicity, as it led to a significantly lower (0.32) ($p<0.05$) number of defects than that of the saline+ITR treatment. Both the frequency of malformed embryos and the

Fig.12 : Exp.4 - Frequency of Malformed Embryos After Culture With Saline or Zn, Followed by Culture With Saline or ITR[1,2]



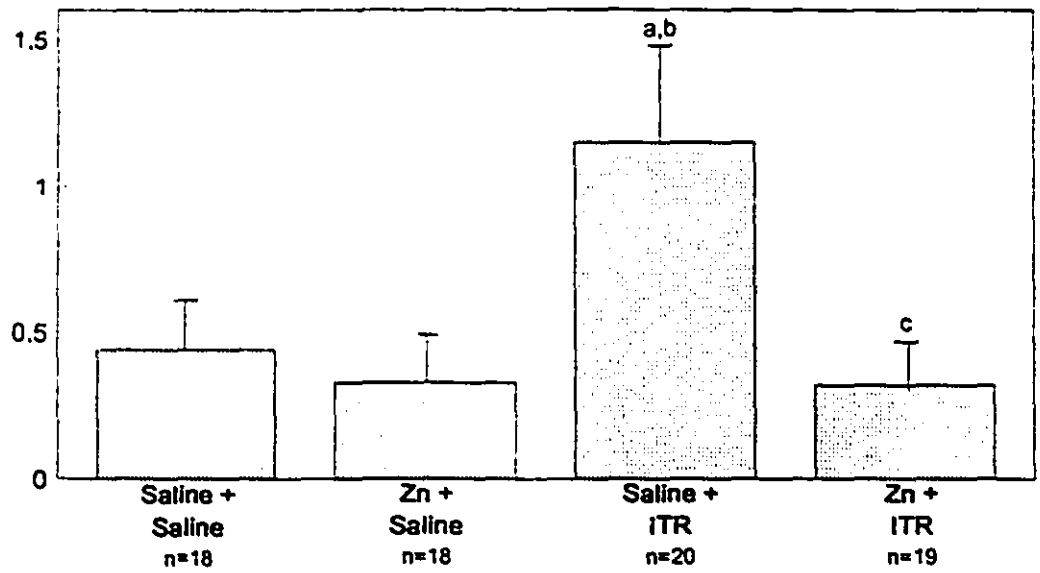
1: GD 8.5 embryos were cultured for 24h with saline or 15 µM Zn. At GD 9.5, saline or 17 µM ITR was added to the culture medium for the next 24h. Embryos were examined on GD 10.5.

2: Was considered malformed any embryo having at least one of the following abnormalities: unfused neural tube, hind brain, mid brain or fore brain, abnormal flexion, missing fore limb and/or enlarged pericardium. See Table C.4b for chi square F and p values.

a: Significantly different from Zn + Saline, $p<0.05$.

b: Significantly different from Saline + ITR, $p<0.05$.

Fig. 13 : Exp.4 - Mean Number of Malformations per Embryo After Culture With Saline or Zn, Followed by Culture With Saline or ITR[1,2]



1: GD 8.5 embryos were cultured for 24h with saline or 15 µM Zn. On GD 9.5, saline or 17 µM ITR was added to the culture medium for the next 24h. Embryos were examined on GD 10.5.

2: Values are expressed as mean + SEM. Was considered a malformation any of the following abnormalities: unfused neural tube,hind brain, mid brain or fore brain, abnormal flexion, missing fore limb and/or enlarged pericardium. See Table C.4a for ANOVA F and p values.

a: Significantly different from Saline+Saline, $p<0.05$.

b: Significantly different from Zn+Saline, $p<0.05$.

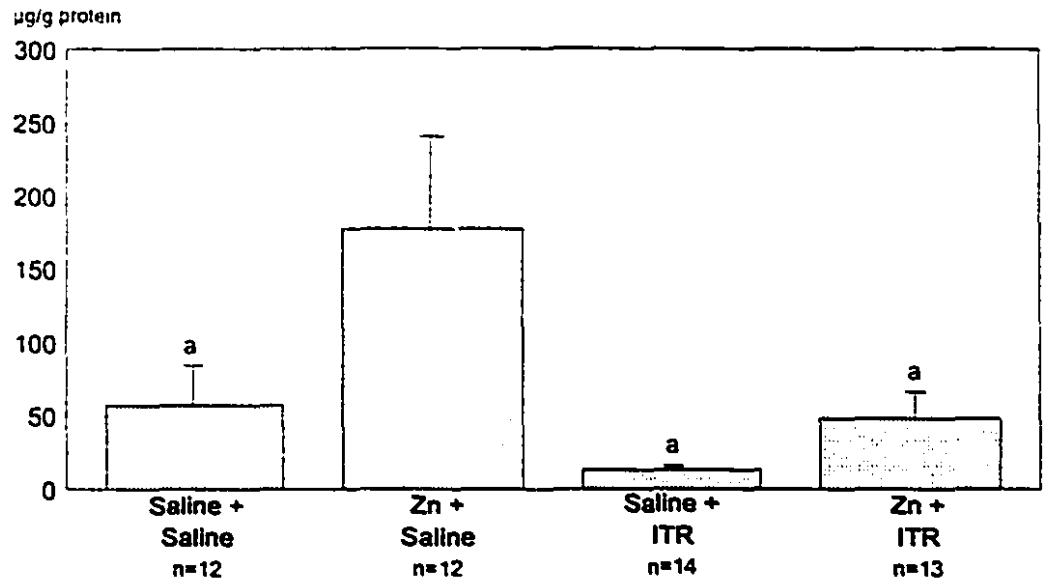
c: Significantly different from Saline+ITR, $p<0.05$.

mean number of malformations per embryo observed in the Zn + ITR culture group were not significantly different from those observed in the Zn and saline groups that had not been not exposed to ITR.

Mean embryonic MT concentrations following culture were 57.4, 178, 13.2 and 47.9 µg/g protein for the saline+saline, Zn+saline, saline+ITR and Zn+ITR culture groups, respectively (Fig.14). Zn+saline treatment resulted in a significantly higher ($p<0.05$) embryonic MT concentration when compared to the other treatments. Although not significant, the saline+ITR group resulted in a lower MT concentration than that of the saline+saline and the Zn+ITR groups. The reduction in MT levels in the ITR-treated groups indicates that the protein may have been oxidized by ITR-derived toxic FR. Embryonic Zn levels were 728±70 (standard error), 717±70, 680±0 and 769±20 ng/embryo for the saline+saline, Zn+saline, saline+ITR and Zn+ITR, respectively, without significant difference among groups.

Yolk sac MT concentrations were 142±37, 180±53, 110±44 and 161±74 mg/g protein, for the saline+saline, Zn+saline, saline+ITR and Zn+ITR groups, respectively. No statistical difference in MT concentrations were observed in yolk sacs. Yolk sac Zn levels were 439 ±30, 468±20, 396±20 and 452±10 ng/yolk sac, for the saline+saline, Zn+saline, saline+ITR and Zn+ITR groups, respectively. Yolk sac Zn was significantly lower ($p<0.05$) in the saline+ITR group when compared to the Zn+saline group.

Fig.14 : Exp.4 - Mean MT Concentrations in Embryos After Culture With Saline or Zn, Followed by Culture With Saline or ITR[1,2]



1: GD 8.5 embryos were cultured for 24h with saline or 15 µM Zn. On GD 9.5, saline or 17 µM ITR was added to the culture medium for the next 24h. MT was measured on GD 10.5.
2: Values are expressed as mean + SEM. See Table C.4a for ANOVA F and p values.
a: Significantly different from Zn+Saline. $p<0.05$.

4.5. EXPERIMENT 5:
In vivo zinc and isotretinoin exposures

Pre-treatment with saline or Zn injections before ITR gavages did not affect pregnancy outcome, as both groups resulted in comparable indices of maternal mass gain, implantation sites, live fetuses and resorptions (Table 5). However, mean fetal body mass ($p=0.0001$) and placental mass ($p=0.0006$) were significantly increased, and the frequencies of cleft palate ($p=0.02$) and of *post-partum* mortality ($p=0.01$) were significantly reduced when Zn was injected to dams before ITR exposures (Table 6). Cleft palates were observed only in fetuses from mice who received saline injections before ITR treatments. Microagnathia, ear, eye or nose defects as well as skeletal defects of limbs or tail were not seen in either treatment groups.

4.6. EXPERIMENT 6:
In vivo zinc and *in vitro* isotretinoin exposures

As shown in Table 7, similar growth parameters were observed in the saline+saline and the Zn+saline groups. However, all growth parameters of the two ITR-treated groups were significantly lower ($p<0.05$) than those of the control groups, which were also pre-treated with Zn or saline injections but exposed *in vitro* to saline only. These results indicate that considerable growth retardation resulted from *in vitro* drug exposure, in spite of an *in vivo* Zn pre-treatment. Zn pre-treatment before exposure to ITR did not increase any growth parameter and resulted in a significantly lower ($p<0.05$) mean sum of morphological scores

Table 5 : Exp.5 - Pregnancy Outcome After Saline or Zn Injections, Followed by ITR Gavages[1,2]

	Saline injections + ITR gavages		Zn injections + ITR gavages	
	Total	Per litter (n=4)	Total	Per litter (n=4)
Maternal weight gain (g)		21.5 ± 1.65		19.7 ± 2.08
Implantation sites	53	13.3 ± 1.03	46	11.5 ± 0.65
Live fetuses	45	11.3 ± 0.85	43	10.8 ± 0.63
Live fetus incidence (%)*		85.4 ± 5.00		93.5 ± 2.00
Resorptions	8	2.00 ± 0.71	3	0.75 ± 0.25
Resorption incidence (%)*		14.6 ± 5.00		6.51 ± 2.00

1: Dams were injected with saline or with 40 and 20 mg Zn/kg on GD 8.5 and 9.5, respectively. On GD 10.5, animals received three intragastric intubations of 100 mg ITR3kg. Pregnancy outcome was assessed on GD 18.5.
2: Values are expressed as mean ± SEM. See Table C.5a for t-test t and p values.
* : Incidences are calculated on an implantation site basis.

Table 6 : Exp.5 - Fetal Indices After Saline or Zn Injections, Followed by ITR gavages[1,2]

	Saline injections + ITR gavages (n=45)	Zn injections + ITR gavages (n=43)
Fetal body weight (g)	1.35 ± 0.03	1.41 ± 0.01[a]
Placental weight (mg)	93.9 ± 2.38	106 ± 3.15 [a]
Frequency of cleft palate (%)	11.1	0[a]
Frequency of post-partum mortality (%)	26.7	7[a]

1: Dams were injected with saline or 40 and 20 mg Zn/kg on GD 8.5 and 9.5, respectively. On GD 10.5, animals received three intragastric intubations of 100 mg ITR/kg. Fetuses were examined on GD 18.5.
2: Weight values are expressed as mean ± SEM. See Table B.5b for nested t-test t and p values. See Table C.5c for chi square F and p values.
a: Significantly different from Saline injections + ITR gavages, $p<0.05$.

Table 7 : Exp.6 - Mean Embryonic Growth Parameters After Saline or Zn Injections, Followed by Culture With Saline or ITR[1,2]

	Crown rump length (mm)	Head diameter (mm)	Somite number	Sum of scores[3]	Embryonic protein (µg)
Saline + Saline (n=20)	3.20 ± 0.06	1.71 ± 0.15	32.1 ± 0.40	47.6 ± 0.65	248 ± 26.1
Zn + Saline (n=32)	3.14 ± 0.16	1.59 ± 0.05	32.4 ± 0.40	47.1 ± 0.48	305 ± 26.1
Saline + ITR (n=21)	2.29 ± 0.05 (a,b)	1.16 ± 0.05 (a,b)	27.1 ± 0.50 (a,b)	35.0 ± 1.34 (a,b)	219 ± 29.2 (a,b)
Zn + ITR (n=31)	2.28 ± 0.06 (a,b)	1.06 ± 0.03 (a,b)	26.8 ± 0.49 (a,b)	31.1 ± 1.23 (a,b,c)	219 ± 22.8 (b)

1: Dams were injected with saline or with 40 and 20 mg Zn/kg on GD 6.5 and 7.5, respectively. On GD 8.5, embryos were cultured with saline or 17 µM ITR for 48h. Embryos were examined on GD 10.5.
2: Values are expressed as mean ± SEM. See Table C.6a for ANOVA F and p values.
3. Morphological assessment was done following a scoring system under which the sum of the scores for each individual feature gives a total score that is indicative of the stage of development of each embryo. See section 3.4.
a: Significantly different from Saline+ Saline, $p<0.05$.
b: Significantly different from Zn + Saline, $p<0.05$.
c: Significantly different from Saline + ITR, $p<0.05$.

than the one obtained from a saline pre-treatment followed by culture with ITR.

Types and frequencies of embryonic abnormalities are listed in Table 8. Saline+saline and Zn+saline groups resulted in comparable incidence of abnormalities. The saline+ITR treatment significantly increased the incidence of unfused mid brain ($p=0.04$), abnormal flexion ($p=0.001$), missing forelimb ($p=0.0003$) and enlarged pericardium ($p=0.002$) when compared to the saline+saline treatment and increased, though not significantly, the frequencies of unfused neural tube, hind brain and fore brain. All frequencies of unfused brain parts, as well as frequencies of abnormal flexion, missing forelimb and enlarged pericardium were significantly higher ($p<0.05$) in the Zn+ITR culture group when compared to the two control groups. There was no difference in the frequencies of all types of abnormalities between the two groups exposed to ITR, except for a significant reduction ($p=0.03$) in enlarged pericardium in the Zn-pre-treated group when compared to the saline pre-treated group. Again, these results indicate that considerable embryonic malformations resulted from *in vitro* ITR exposure, in spite of an *in vivo* Zn pre-treatment. This is more clearly illustrated in Figs.15 and 16, which show that the overall incidence of embryonic malformations, and the mean number of defects per embryo are significantly higher in the ITR-treated groups than in the control groups, regardless of a Zn pre-treatment *in vivo*. Similar frequencies of malformed embryos and similar numbers of malformations per embryo were observed in the saline+saline and the Zn+saline groups.

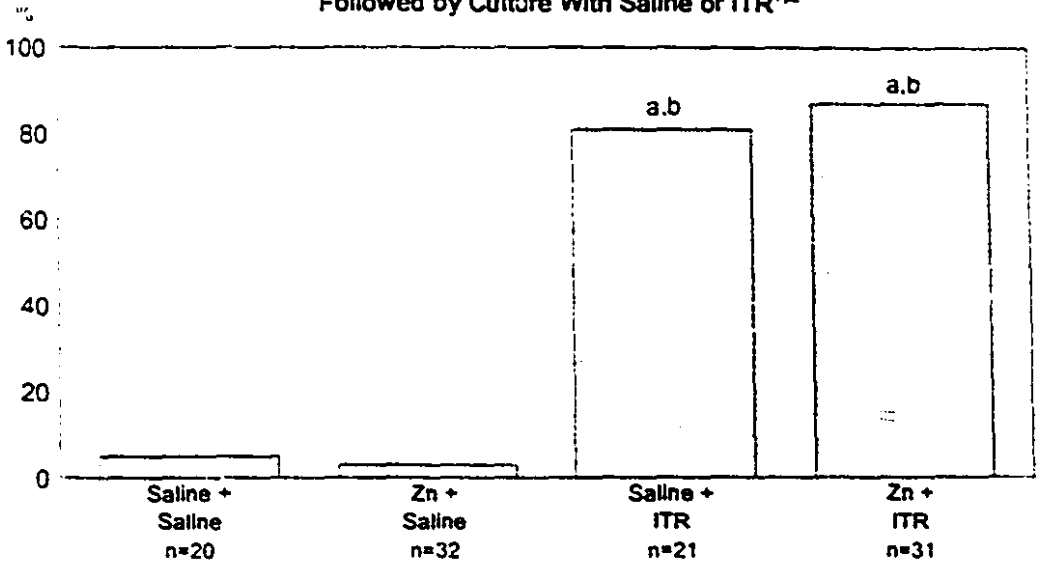
Mean embryonic MT concentrations after 48h culture were 220, 155, 54.9 and 40.8 µg/g protein for the saline+saline, Zn+saline, saline+ ITR, and Zn+ITR

Table 8 : Exp.6 - Types and Frequencies of Embryonic Abnormalities After Zn or Saline Injections, Followed by Culture With Saline or ITR[1,2]

	Unfused neural tube	Unfused hind brain	Unfused mid brain	Unfused fore brain	Abnormal flexion	Missing fore limb	Enlarged pericardium	Bleb
Saline + Saline (n=20)	0	0	5.0	0	0	0	0	0
Zn + Saline (n=32)	0	0	3.1	3.1	0	0	0	3.1
Saline + ITR (n=21)	4.8	4.8	28.6 (a,b)	14.3	42.9 (a,b)	47.6 (a,b)	38.1 (a,b)	9.5
Zn + ITR (n=31)	16.1 (b)	16.1 (b)	51.6 (a,b)	38.7 (a,b)	54.8 (a,b)	61.3 (a,b)	12.9 (b,c)	9.7

1: Dams were injected with saline or 40 and 20 mg Zn/kg on GD 6.5 and 7.5, respectively. On GD 8.5, embryos were cultured with saline or 17 µM ITR for 48h. Embryos were examined on GD 10.5.
2: Values are expressed in %.
a: Significantly different from Saline+Saline, $p<0.05$.
b: Significantly different from Zn+ Saline, $p<0.05$.
c: Significantly different from Saline+ITR, $p<0.05$.

Fig.15 : Exp.6 - Frequency of Malformed Embryos After Saline or Zn Injections, Followed by Culture With Saline or ITR[1,2]



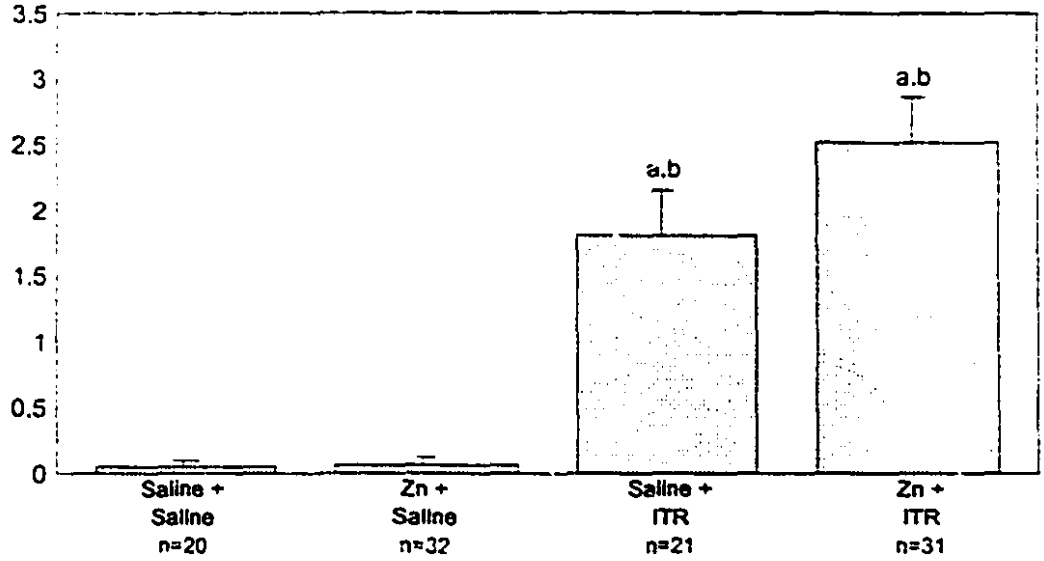
1: Dams were injected with saline or with 40 and 20 mg Zn/kg on GD 6.5 and 7.5, respectively. On GD 8.5, embryos were cultured with saline or 17 µM ITR for 48h. Embryos were examined on GD 10.5.

2: Was considered malformed any embryo having at least one of the following abnormalities: unfused neural tube, hind brain, mid brain or fore brain, abnormal flexion, missing fore limb and/or enlarged pericardium. See Table C.6b for chi square F and p values.

a: Significantly different from Saline + Saline, $p<0.05$.

b: Significantly different from Zn + Saline, $p<0.05$.

Fig.16 : Exp.6 - Mean Number of Malformations per Embryo After Saline or Zn Injections, Followed by Culture With Saline or ITR[1,2]



1: Dams were injected with saline or with 40 and 20 mg Zn/kg on GD 6.5 and 7.5, respectively. On GD 8.5, embryos were cultured with saline or with 17 µM ITR for 48h. Embryos were examined on GD 10.5.

2: Values are expressed as mean + SEM. Was considered a malformation any of the following abnormalities: unfused neural tube,hind brain, mid brain or fore brain, abnormal flexion, missing fore limb and/or enlarged pericardium. See Table C.6a for ANOVA F and p values.

a: Significantly different from Saline + Saline, $p<0.05$.

b: Significantly different from Zn + Saline, $p<0.05$.

groups, respectively (Fig.17). The significant reduction ($p<0.05$) in MT levels in ITR-treated groups indicates that the protein may have been oxidized by ITR-derived toxic free radicals. Embryonic Zn levels were significantly higher ($p<0.05$) in the Zn injections+saline culture group (394 ng/embryo) than in the other groups (302 to 314 ng/embryo).

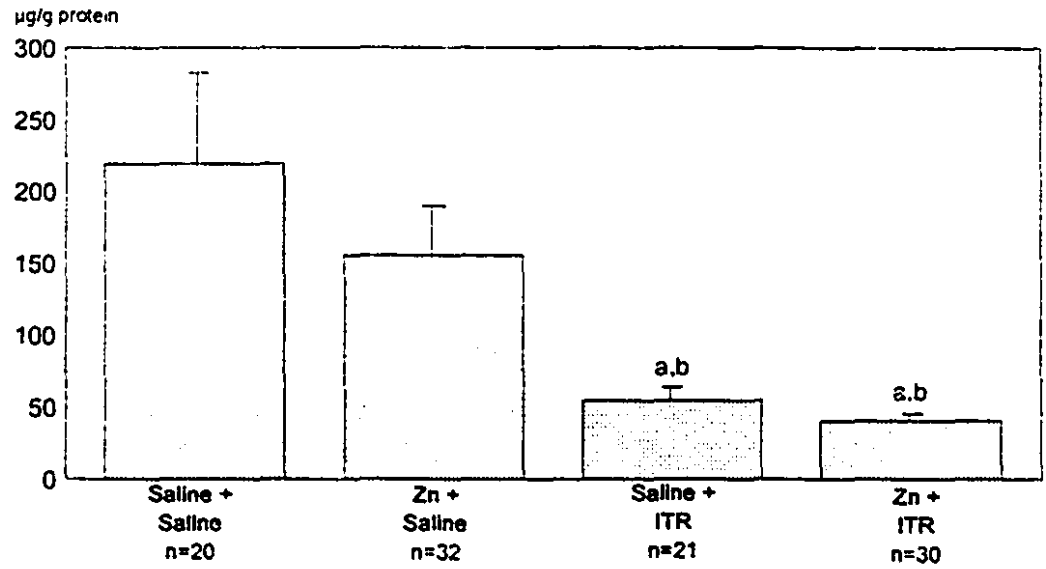
4.7. COMPARISON OF EMBRYONIC METALLOTHIONEIN CONCENTRATIONS ON GESTATIONAL DAY 10.5

In order to put the effect of culture on embryonic MT induction in perspective, mean embryonic MT concentrations of experimental groups assessed on GD 10.5 were compared.

Fig.18 illustrates the mean embryonic MT of control groups that had been exposed to saline only under various conditions. Saline injection (group1) or culture with saline for 48h (groups 2) resulted in similar embryonic MT levels (86.7 and 76.5 µg/g protein, respectively). Combination of saline injection and culture with saline, as in group 3, resulted in a significant increase in mean embryonic MT levels (271 µg/g protein). These results indicate that injection followed by 48h culture induces MT synthesis in embryos.

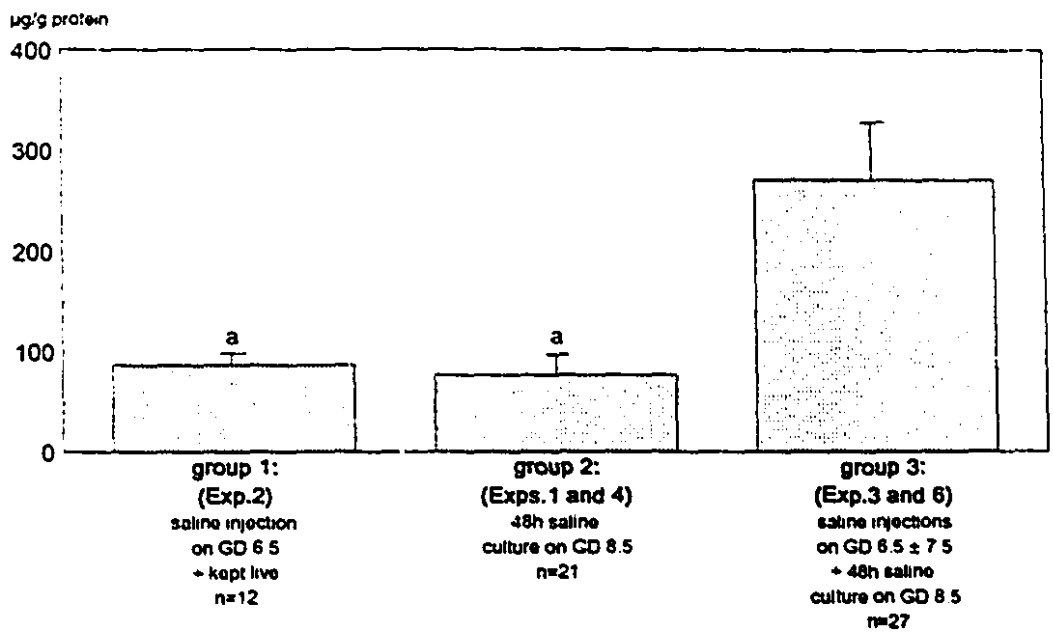
Fig.19 illustrates mean embryonic MT of groups that were exposed to Zn under various conditions. Zn injection (group1) or culture with Zn for 24 or 48h (groups 2 and 3), resulted in mean embryonic MT levels of 374, 178 and 688 µg/g protein, respectively. Combination of Zn injection and 48h culture with saline resulted in a mean embryonic MT level of 179 µg/g protein (group 4). These results indicate that Zn dosage *in vitro* for 48h is a more effective MT inducer

Fig.17 : Exp.6 - Mean MT Concentrations After Saline or Zn Injections, Followed by Culture With Saline or ITR[1,2]



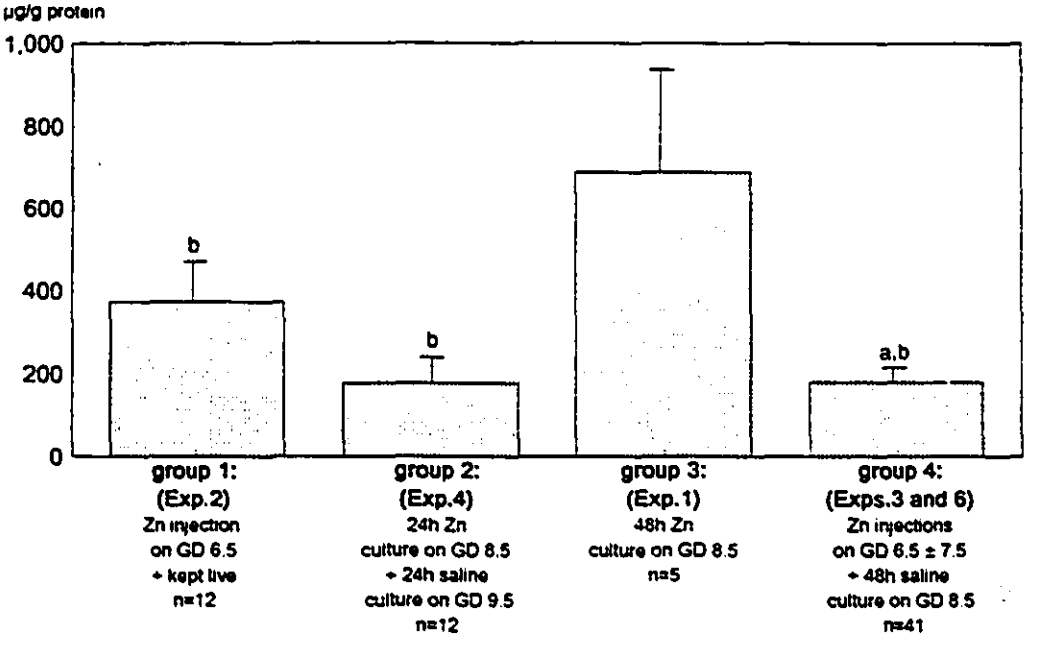
1: Dams were injected with saline or with 40 and 20 mg Zn/kg on GD 6.5 and 7.5, respectively. On GD 8.5, embryos were cultured with saline or with 17 µM ITR for 48h. Embryos were examined on GD 10.5.
2: Values are expressed as mean + SEM. See Table C.6a for ANOVA F and p values.
a: Significantly different from Saline + Saline, $p<0.05$.
b: Significantly different from Zn + Saline, $p<0.05$.

Fig. 18 : Mean MT Concentrations in Embryos on GD 10.5, After Saline Treatments[1]



1: Values are expressed as mean + SEM. See Table C.7 for ANOVA F and p values.
a: Significantly different from group 3, $p<0.05$.

Fig. 19: Mean MT Concentrations in Embryos on GD 10.5, After Zn Treatments[1]



1: Values are expressed as mean + SEM. See Table C.7 for ANOVA F and p values.
a: Significantly different from group 1, $p<0.05$.
b: Significantly different from group 3, $p<0.05$.

than *in vivo* exposure to Zn and 24h culture with Zn. Zn injection followed by 48h culture showed no significant increase in MT concentration indicating that once MT is induced by a Zn injection, incubation does not induce MT any further.

4.8. SUMMARY OF RESULTS

In the first part of this study, MT induction was demonstrated in mid-gestational mouse embryos after Zn exposure, both *in vivo* and *in vitro*.

- Experiment 1 showed that MT was significantly induced following a 48h culture period (GD 8.5 to 10.5) with 15 μM Zn, but not following a 24h incubation period.
- In Experiment 2, a maternal s.c. injection of 40 mg Zn/kg on GD 6.5 resulted in significantly elevated embryonic MT levels after 48h.
- Experiment 3 revealed that mice injection with 0 or 40 mg Zn/kg on GD 6.5 followed or not by a 48h whole embryo culture period on GD 8.5 led to significant induction of MT after Zn injection alone and after saline injection followed by culture.

In the second part of this investigation, protective effects of Zn, through modulation of MT, against ITR toxicity was demonstrated.

- In Experiment 4, pre-treatment of GD 8.5 embryos with a 24h culture period with Zn improved growth and totally restored normal development altered by exposure to 17 μM ITR for the next 24h.
- In Experiment 5, Zn injections of 40 and 20 mg/kg to pregnant mice on GD 8.5 and 9.5, respectively, alleviated fetal damage

caused by three intragastric intubations of 100 mg ITR/kg on GD 10.5.

- In Experiment 6, Zn injections of 40 and 20 mg/kg to pregnant mice on GD 6.5 and 7.5, respectively, did not improve embryonic growth retardation and developmental malformations induced by a 48h *in vitro* exposure to 17 µM ITR on GD 8.5.

5. DISCUSSION

Increased MT expression during fetal development is a well known phenomenon (Webb, 1987). It is therefore suspected that MT may play a role for Zn metabolism during development (Cherian and Chan, 1993). Experiments 1 and 2 demonstrate that mid-gestational mouse embryos (GD 8 to 10) can synthesize MT in its protein form and that embryonic MT expression is enhanced by *in vivo* and *in vitro* Zn supplementation during this gestational period. This is the first time that MT inducibility by Zn is shown in mouse embryos during the critical period of organogenesis. Most of the previous studies reported MT induction in its mRNA form (De et al., 1990; Andrews et al., 1991). The only studies showing MT induction in its protein form was on GD 4 (Vidal and Hidalgo, 1993) and in fetal liver from GD 16 (Charles-Shannon et al., 1981). The ability to synthesize MT during the critical period of organogenesis suggests that protection of embryos against the toxicity of exogenous compounds can be provided using MT inducers such as Zn.

The degree of MT expression varied in different experiments. The level of MT induction depended on the Zn dosage, the length of incubation time and the gestational day. The highest embryonic MT concentration was induced by a 48h culture period with 15 µM Zn, which resulted in a six-fold increase in MT levels compared to a 24h culture period with Zn. Exposure of GD 8.5 embryos to Zn *in vitro* for 48h might be a more potent MT inducer than a 24h *in vitro* exposure for one or more of the following three reasons: 1) a longer Zn exposure could result

in higher MT expression; 2) during organogenesis, the metabolic capacities of the embryo vary extremely rapidly and on GD 9.5 the embryo could have developed a greater capacity to synthetize MT in response to a Zn exposure than on GD 8.5; and 3) on GD 9, the circulatory system of the embryo becomes functional with the onset of umbilical and yolk sac blood circulation (Theiler, 1972). Hence, Zn may become more accessible to the embryo. Alternatively, since our results showed that yolk sacs have a higher MT inducibility than do embryos from GD 8.5 to 10.5 (Exps. 3 and 4), MT may be transferred from the yolk sac to the embryo via the newly functional vascular system.

Zn dosage given *in vivo* induced MT on GD 6.5 (Fig.8). However, the magnitude of the induction is less than that of the *in vitro* 48h treatment. This might be explained two ways: 1) mice were injected on GD 6.5 and MT was measured on GD 8.5. At this stage of development, MT inducibility in embryos may be low; 2) the embryonic circulatory system is not developed yet and extra-embryonic tissues may act as barriers, thus limiting the amount of Zn reaching the embryo. Our results show that Zn in embryos remained almost constant under all experimental conditions. Similarly, Reis et al. (1988) found that Zn concentrations in mouse conceptuses did not change during the first ten days of gestation, despite a Zn supplementation to dams prior to mating. These results suggest that Zn transfer to the embryo is a well regulated process. Zn transfer may be regulated by extra-embryonic tissues, as the embryo is surrounded by, in turn, the Reichert's membrane, the deciduum, the trophoblast cells, the maternal endometrium and utero (Theiler, 1972). Andrews et al. (1993) found that from the time of implantation to late in gestation, MT mRNA is highly expressed in

decidual cells, trophoblasts and the visceral endoderm of the yolk sac. Our results indicated that following a maternal injection of 40 mg Zn/kg, MT concentrations were increased in the Reichert's membrane as well as in the yolk sac. Therefore, extra-embryonic tissues may bind the maternal Zn through the newly synthetized MT and therefore serve as a barrier, similar to the role of the placenta in later developmental stages (Goyer and Cherian, 1992).

MT induction was observed in Zn-treated embryos although no significant increase in Zn concentrations was observed. These results suggest that Zn accumulation in the embryo may be compartmentalized and MT may be induced by an increase in Zn availability in certain sites. Immunochemistry studies are required to study the localization of MT within the embryo.

To better understand maternal influence on embryonic MT synthesis, mice were injected with Zn on GD 6.5 followed by a 48h culture period (Experiment 3). MT concentrations in cultured embryos were comparable to those found in the embryos kept *in vivo* after Zn injection (Fig.10). These results suggest that MT synthesis may have reached a maximum within 48h. Similar findings were reported by Nordberg and Kojima (1979). However, the saline-treated group followed by culture resulted in MT expression as high as the two Zn-treated groups, suggesting that the process of embryo culture could induce MT, possibly due to stress (Oh et al., 1978).

To study the induction of MT due to experimental procedures, MT levels from control groups assessed on GD 10.5 were compared (Fig.18). The results show that injection or culture on their own represent comparable stressful events, resulting in similar levels of embryonic MT. However, when injection is

followed by culture, the stress effect seems to be a cumulative or synergistic MT inducer, resulting in MT levels equal to the sum of that induced by each single stress. Considering the observations made from comparing the previous control groups, we would expect the same cumulative effect on the Zn-treated groups. However, the MT concentration of embryos that were injected with Zn and cultured with saline was not higher than those induced by Zn injection alone or by culture alone (Fig.19). These results suggest that the potential for MT induction may be limited in the embryos. Once MT was induced by Zn treatment, the stress factors involved in culture would not induce MT in embryos any further. These results show that the handling of animals, culture, developmental stage and duration of Zn dosage all influence MT induction.

Exposure of mid-gestational mouse embryos to ITR proved to be teratogenic *in vitro* and *in vivo*. Whole embryo culture for 24h with 17 μM ITR (Exp.4) reduced crown-rump length and the sum of scores (Table 3). It led to a significant increase in the frequencies of unfused mid brain and abnormal flexion (Table 4). Ritchie and Webster (1991) found comparable results in Sprague-Dawley rats after a 24h *in vitro* exposure to 0.3 or 0.6 μM ITR.

Extending the treatment period for 48h showed an increase in ITR toxicity. Crown-rump length, head diameter, somite number, sum of scores and embryonic protein were all significantly decreased (Table 7). Embryos also showed significantly higher frequencies of unfused mid brain, abnormal flexion, missing forelimb and enlarged pericardium (Table 8). Similarly, whole rat embryo culture for 48h with 0.3 or 0.6 μM ITR was shown to reduce growth and alter morphological development (Ritchie and Webster, 1991). Forelimb bud culture of

ICR mouse embryos for 6 days with 0.3 or 0.6 μM ITR (Kochhar and Penner, 1987), or for 72h with 1.5 or 7.4 μM ITR (Kwasigroch and Bullen, 1991) were found to totally inhibit cartilage and skeletal development.

In vivo exposure to ITR through three intragastric intubations of 100 mg/kg at 4h intervals on GD 10.5 (Exp.5) did not alter pregnancy outcome (Table 5). However, it markedly affected fetal growth and development, as observed by a significant reduction in fetal and placental weights, and a significant increase in the frequencies of cleft palate and post-partum mortality (Table 6). Following the same protocol, Kubow (1992) found similar results in CD-1 mouse fetuses, in addition to microagnathia, limb and tail defects. Creech-Kraft et al. (1991) also observed skull abnormalities in NMRI mouse fetuses under these experimental conditions. Therefore, the use of CD-1 mouse embryos, both *in vivo* and *in vitro*, as an effective model for ITR teratogenicity is demonstrated.

Pre-treatment of GD 8.5 embryos over a 24h culture period with Zn induced significantly higher levels of MT (Fig.14). Zn pre-treatment prior to addition of ITR to the culture medium improved growth and totally restored normal embryonic development (Exp.4). Zn pre-treatment reduced the frequencies of unfused mid brain and fore brain by 100% and the frequency of abnormal flexion by 74% (Table 4). Overall, Zn pre-treatment before ITR exposure decreased the frequency of abnormal embryos by 65% (Fig.12) and the mean number of malformations per embryo by 72% (Fig.13). Similar protection was observed *in vivo* (Exp.5). Zn pre-treatment of pregnant mice on GD 8.5 and 9.5 alleviated fetal damage caused by three intragastric intubations of ITR on GD 10.5. Zn pre-treatment increased fetal and placental masses, totally eliminated

incidence of cleft palate and reduced the frequency of *post-partum* mortality by 74% (Table 6).

However, *in vivo* Zn pre-treatment of pregnant mice on GD 6.5 and 7.5 did not offer embryos protection against a 48h *in vitro* teratogenic insult on GD 8.5 (Exp.6). Crown-rump length, head diameter, somite number, sum of scores and embryonic protein were all affected by ITR, despite Zn pre-treatment (Table 7). Zn injections before culture with ITR reduced the frequency of enlarged pericardium by 66%, but did not lessen the incidences of unfused brain parts, abnormal flexion or missing forelimb (Table 8). Overall, ITR treatment *in vitro* resulted in a significant increase in the frequency of abnormal embryos (Fig.15) and in the mean number of malformations per embryo (Fig.16), regardless of a previous Zn pre-treatment *in vivo*.

The lack of protection may be explained the following ways: 1) Zn is less accessible to the embryo *in vivo*, where maternal transfer of the metal is highly regulated (Reis et al., 1988), than *in vitro*; 2) MT inducibility in live embryos may be lower on GD 6.5 and 7.5 than on GD 8.5 and 9.5; 3) a 48h *in vitro* exposure to ITR may generate more FR than a 24h *in vitro* exposure; 4) a 48h *in vitro* dose of ITR may be a more potent teratogenic insult than the actual *in vivo* internal dose, and the damage is beyond any protective effect offered by Zn.

The results of Experiments 4 and 5 clearly demonstrate that Zn pre-treatment can offer protection against ITR teratogenicity, possibly through MT modulation. Moreover, MT levels in embryos were significantly decreased after ITR treatment (Figs.14 and 17). This indicates that MT may have been oxidized by ITR-derived toxic FR, as the ELISA used in this study is not able to

measure oxidized MT (Chan et al., 1992b). It has been demonstrated that ITR may induce birth defects through FR generation (Kubow, 1992), and FR were shown to cause metal loss from the MT molecule and thiolate oxidation (Thornalley and Vasak, 1985; Fliss and Ménard, 1992). MT induced by Zn may act as a radical scavenger, hence alleviating the teratogenic effects of ITR.

Independently of its role as an MT inducer, Zn may alleviate ITR teratogenicity through other protective mechanisms. Zn has important biological roles in membrane structure and function, as it is an integral part of membrane phospsolipids, lipoproteins and proteins associated with biomembranes (Bettger et al., 1981). Zn interacts with enzymes controlling the integrity of the membrane, such as phospholipase A_2 inhibition (Cunnane, 1988) which could indirectly inhibit the prostaglandin synthase cascade by reducing substrate availability. Zn interferes with macromolecular components of the membrane, changing their conformation or enzyme-substrate specificity, and it interferes with metal-catalysed peroxidation (Bettger et al., 1981). Zn was also shown to reduce lipid peroxidation through a mechanism involving a decrease in the oxidation of NADPH (Chapvil et al., 1976) and through inhibition of malondialdehyde formation (Chapvil et al., 1974). Furthermore, Zn supplementation, either by pre-treating mice with $ZnSO_4$ or adding Zn^{2+} to the media of cell culture, can prevent apoptotic death, as induced by physical, chemical or immunological stimuli, possibly by modulating the activation or activity of endonucleases (Sunderman, 1995). These findings may be of importance when considering that retinoids may exert a teratogenic effect through induction of excessive cellular necrosis in areas associated with programmed cell death, especially those

involved in craniofacial and mesomelic chondrogenic development (Alles and Sulik, 1989). Some researchers have suggested that a nuclear retinoic acid receptor may play a role in the morphogenetic activities of retinoids, activating apoptosis by the cleaving of DNA into small fragments by an endonuclease (Petkovich et al., 1987). Zn may directly offer protection against excessive programmed cell death, or indirectly, through MT modulation. The presence of MT in the cell nucleus during cell proliferation has been associated with resistance to anti-cancer drugs (Cherian and Chan, 1993). It could be hypothesized that nuclear MT could protect the developing embryo against ITR, which may exert a teratogenic effect through different mechanisms including action on the genome. The cytosolic distribution of MT during organogenesis has yet to be confirmed. Whether it is Zn or MT which is conferring the protective effect against ITR teratogenicity can also be confirmed using the MT knockout transgenic mice model (Iszard et al., 1995).

6. CONCLUSION

This investigation has shown, for the first time, that mid-gestational mouse embryos (GD 8 to 10) are capable of MT synthesis *in vivo* and *in vitro*, under Zn supplementation. MT inducibility in its protein form was highest after a 48h whole embryo culture period with 15 µM Zn, on GD 8.5. This treatment led to a sixfold increase in embryonic MT concentration, when compared to a 24h culture period with 15 µM Zn on GD 8.5, and to a maternal Zn injection of 40 mg/kg on GD 6.5. *In vivo* transfer of Zn to the embryo may be well regulated by extra-embryonic tissues and maternal metabolism. Induction of MT may be partially due to handling of animals or administration of treatments. We also observed that culture and injection resulted in an increase in embryonic MT synthesis.

ITR teratogenicity was found to be comparable to previous findings. *In vitro* exposure of embryos to 17 µM ITR for 24 or 48h affected embryonic growth and development, causing brain, flexion, limb and pericardium defects. *In vivo* exposure of embryos to ITR through three maternal intragastric intubations of 100 mg/kg affected growth and development as well, inducing cleft palate and increasing *post-partum* mortality.

We found that embryonic exposure to Zn alleviated the subsequent teratogenic effects of ITR *in vivo* and *in vitro*. The protection against the toxicity of retinoids by Zn supplementation is a novel finding. Zn pre-treatment prior to ITR exposure resulted in embryonic growth and development comparable to control groups that were not exposed to ITR. Embryos cultured with ITR showed

a decrease in MT concentrations, suggesting that MT may have been oxidized by ITR-derived toxic FR. Our results suggest that modulation of MT levels in mouse embryos by Zn offers protection against ITR teratogenicity, possibly through the scavenging of FR by MT.

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APPENDIX A: SCORING SYSTEM FOR CULTURED EMBRYOS

Species_____ Media Used _____

Stage of Gestation (Day)_____ (no. somites)_____

Time in Time out_____

Scoring Date _____

Treatment_____

		0	1	2	3	4	5	A	B	C	D	E	F
A													
B													
C													
D													
E													
F													
G													
H													
J													
K													
L													
M													
N													
P													
Q													
R													
S													

Comments_____

SUM

SOMITES

YS

C-R

Hd

APPENDIX B : STANDARD CURVES

Fig. B.1 :
Standard Curve for Protein Analysis

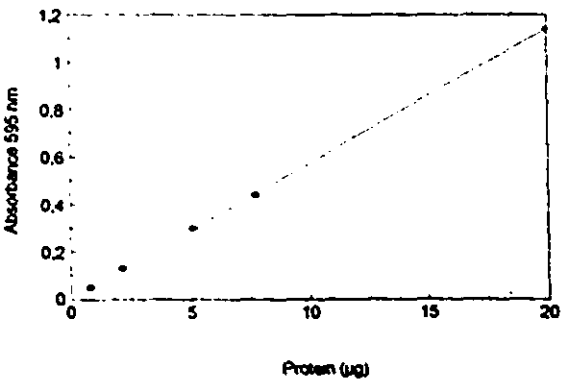


Fig. B.2 :
Standard Curve for Zinc Analysis

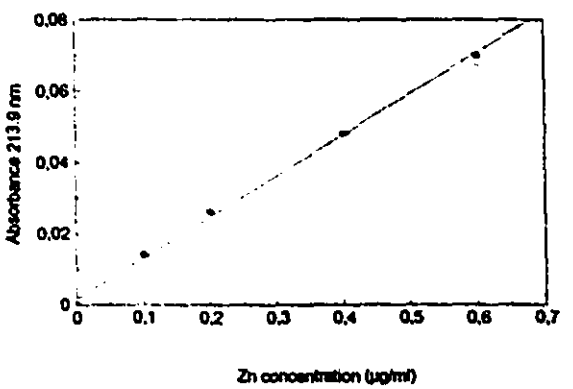
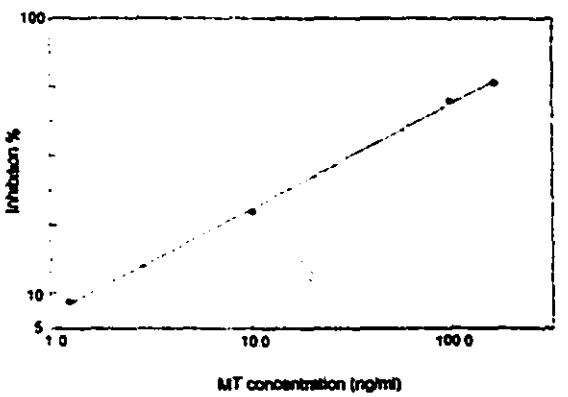


Fig. B.3 :
Standard Curve for Metallothionein Analysis (logit-log plot)



APPENDIX C : STATISTICAL ANALYSES

Appendix C.1 : Experiment 1

Table C.1a:
One-Tailed t-test for MT and Zn Levels After 24h Culture Between Saline and Zn Groups

dependent variable	t-value	p>T
Embryonic MT	1.23	0.132
Yolk sac MT	1.37	0.110
Embryonic Zn	1.25	0.129
Yolk sac Zn	2.24	0.034

Table C.1b:
One Way ANOVA for the Effect of 48h Culture With Saline, ITR or Zn on Embryonic MT

dependent variable	F-value	p>F
Embryonic MT	11.38	0.0005

Appendix C.2 : Experiment 2

Table C.2:
One Way ANOVA for the Effect of Saline, 20mg Zn/kg or 40mg Zn/kg Injection on MT and Zn Levels [1]

dependent variable	source of effect	F-value	p>F
Embryonic MT	group dam(group)	1.81	0.1241
	group	0.70	0.5016
	dam(group)	0.83	0.4814
Reichert's membrane MT	group dam(group)	15.33	0.0001
	group	7.78	0.0010
	dam(group)	18.10	0.0001
Decidual Zn	group dam(group)	6.71	0.0001
	group	1.59	0.2140
	dam(group)	9.31	0.0001
Maternal liver Zn	group	5.24	0.0310

1: For each variable the F and p values for the model are listed on the first line, followed by the F and p values for individual terms in the model.
Dams, nested within group is indicated by dam(group).

Appendix C.3 : Experiment 3

Table C.3a:
Two Way ANOVA for the Effects of Injection (Zn or saline) and Culture (+ or -) on MT and Zn Levels [1]

dependent variable	source of effect	F-value	p>F
Embryonic MT	injec. cult. injec.*cult. dam(injec.)	2.99	0.0243
	injection	0.46	0.5005
	culture	2.52	0.1216
	injection*culture	2.70	0.1094
	dam(injection)	2.59	0.0894
Yolk Sac MT	injec. cult. injec.*cult. dam(injec.)	7.88	0.0001
	injection	8.09	0.0077
	culture	12.24	0.0014
	injection*culture	2.17	0.1505
	dam(injection)	6.14	0.0056
Embryonic Zn	injec. cult. injec.*cult. dam(injec.)	2.16	0.0825
	injection	6.03	0.0195
	culture	0.01	0.9260
	injection*culture	4.14	0.0499
	dam(injection)	3.02	0.0624

1: For each variable the F and p values for the model are listed on the first line, followed by the F and p values for individual terms in the model.
Interaction of terms is indicated by *
Dams, nested within group is indicated by dam(group).

Table C.3b:
One-Tailed Nested t-test for Cultured Yolk Sac Zn Between Saline and Zn Groups[1]

dependent variable	t-value	p>T
Yolk sac Zn	0.0311	0.181

1: Mean yolk sac Zn values were compared per dam, nested within treatment.

Appendix C.4 : Experiment 4

Table C.4a:
Two Way ANOVA for the Effects of Pre-treatment (Zn or saline) and Treatment (saline or ITR) on Growth Parameters, MT and Zn Levels and Number of Malformations per Embryo [1]

dependent variable		source of effect	F-value	p>F
Crown rump lenght	pre-treat	treatment pre-treat.*treatment	3.17	0.0290
		pre-treatment	3.63	0.0610
		treatment	5.71	0.0200
		pre-treatment*treatment	0.13	0.7200
Head diameter	pre-treat	treatment pre-treat.*treatment	0.88	0.4580
		pre-treatment	0.51	0.4780
		treatment	1.29	0.2610
		pre-treatment*treatment	0.89	0.3480
Somite number	pre-treat	treatment pre-treat.*treatment	0.25	0.8590
		pre-treatment	0.21	0.6500
		treatment	0.29	0.5950
		pre-treatment*treatment	0.27	0.6080
Sum of scores	pre-treat.	treatment pre-treat.*treatment	4.52	0.0060
		pre-treatment	2.45	0.1220
		treatment	10.51	0.0020
		pre-treatment*treatment	0.63	0.4290
Embryonic protein	pre-treat.	treatment pre-treat.*treatment	0.47	0.7080
		pre-treatment	0.32	0.5750
		treatment	0.51	0.4790
		pre-treatment*treatment	0.55	0.4620
Embryonic MT	pre-treat.	treatment pre-treat.*treatment	4.43	0.0080
		pre-treatment	5.27	0.0260
		treatment	6.63	0.0130
		pre-treatment*treatment	1.60	0.2120
Yolk sac MT	pre-treat.	treatment pre-treat.*treatment	0.91	0.4440
		pre-treatment	1.68	0.2010
		treatment	0.22	0.6380
		pre-treatment*treatment	0.72	0.4000
Embryonic Zn	pre-treat.	treatment pre-treat.*treatment	0.64	0.5960
		pre-treatment	0.69	0.4120
		treatment	0.002	0.9620
		pre-treatment*treatment	1.11	0.2970
Yolk sac Zn	pre-treat.	treatment pre-treat.*treatment	2.23	0.0970
		pre-treatment	4.03	0.0510
		treatment	1.90	0.1750
		pre-treatment*treatment	0.45	0.5070
Mean number of malformations per embryo	pre-treat.	treatment pre-treat.*treatment	3.26	0.0260
		pre-treatment	4.42	0.0390
		treatment	2.34	0.1300
		pre-treatment*treatment	2.59	0.1120

1: For each variable the F and p values for the model are listed on the first line, followed by the F and p values for individual term in the model. Interaction of terms is indicated by *.

Appendix C.4 : Experiment 4 (cont'd)

Table C.4b:
Chi Square Statistics for the Frequency of Abnormal Embryos Between Groups

dependent variable	comparison	F-value	p>F
Frequency of abnormal embryos	(24h Zn + 24h Saline) + (24h Saline + 24h Saline)	0.55	0.457
	(24h Zn + 24h Saline) + (24h Zn + 24h ITR)	0.007	0.931
	(24h Zn + 24h Saline) + (24h Saline + 24h ITR)	5.55	0.019
	(24h Saline + 24h Saline) + (24h Zn + 24h ITR)	0.71	0.401
	(24h Saline + 24h Saline) + (24h Saline + 24h ITR)	2.70	0.100
	(24h Zn + 24h ITR) + (24h Saline + 24h ITR)	6.11	0.013

Appendix C.5 : Experiment 5

Table C.5a:
One-Tailed t-test for Pregnancy Outcome Indices Between Saline and Zn Groups

dependent variable	t-value	p>T
Maternal weight gain	0.67	0.263
Implantation sites	1.44	0.100
Live fetuses	0.47	0.327
Live fetus incidence	1.38	0.109
Resorptions	1.67	0.074
Resorption incidence	1.38	0.109

Table C.5b:
One-Tailed Nested t-test for Fetal and Placental Weights Between Saline and Zn Groups[1]

dependent variable	t-value	p>T
Fetal body weight	3.15	0.0001
Placental weight	12.28	0.0006

1: Mean weight values were compared per dam, nested within treatment.

Table C.5c:
Chi Square Statistics for Frequencies of Cleft Palate and Post-Partum Mortality Between Saline and Zn Groups

dependent variable	F-value	p>F
Cleft palate	5.065	0.024
Post-partum mortality	6.029	0.014

Appendix C.6 : Experiment 6

Table C.6a:
Two Way ANOVA for the Effects of Injection (Zn or saline) and Culture (saline or ITR) on Growth Parameters, MT and Zn Levels and Number of Malformations per Embryo [1]

Dependent variable	Source of effect	F-value	p>F
Crown rump lenght	injection culture injection*culture	65.01	0.0001
	injection	1.60	0.2090
	culture	182.67	0.0001
	injection*culture	0.19	0.6650
Head diameter	injection culture injection*culture	51.42	0.0001
	injection	5.55	0.0200
	culture	144.12	0.0001
	injection*culture	0.05	0.8290
Somite number	injection culture injection*culture	45.71	0.0001
	injection	0.01	0.9220
	culture	128.40	0.0001
	injection*culture	0.31	0.5800
Sum of scores	injection culture injection*culture	76.28	0.0001
	injection	4.75	0.0320
	culture	203.73	0.0001
	injection*culture	2.83	0.0960
Embryonic protein	injection culture injection*culture	2.70	0.0500
	injection	0.96	0.3290
	culture	4.33	0.0400
	injection*culture	1.38	0.2430
Embryonic MT	injection culture injection*culture	6.05	0.0010
	injection	1.34	0.2500
	culture	17.03	0.001
	injection*culture	0.55	0.4610
Embryonic Zn	injection culture injection*culture	10.44	0.0001
	injection	10.25	0.0020
	culture	10.66	0.0010
	injection*culture	5.78	0.0180
Mean number of malformations per embryo	injection culture injection*culture	25.33	0.0001
	injection	1.89	0.1720
	culture	64.88	0.0001
	injection*culture	1.76	0.1880

1: For each variable the F and p values for the model are listed on the first line, followed by the F and p values for individual terms in the model.
Interaction of terms is indicated by *.

Appendix C.6 : Experiment 6 (cont'd)

Table C.6b:
Chi Square Statistics for the Frequency of Abnormal Embryos Between Groups

dependent variable	comparison	F-value	p>F
Frequency of abnormal embryos	(Zn injection + culture with Saline) + (Saline injection + culture with Saline)	0.117	0.732
	(Zn injection + culture with Saline) + (Zn injection + culture with ITR)	44.97	0.000
	(Zn injection + culture with Saline) + (Saline injection + culture with ITR)	34.24	0.000
	(Saline injection + culture with Saline) + (Zn injection + culture with ITR)	33.09	0.000
	(Saline injection + culture with Saline) + (Saline injection + culture with ITR)	23.99	0.000
	(Zn injection + culture with ITR) + (Saline injection + culture with ITR)	0.363	0.547

Appendix C.7

Table C.7:
One Way ANOVA for the Effect of Saline or Zn Treatments on GD 10.5 Embryonic MT Concentrations

dependent variable	source of effect	F-value	p>F
Embryonic MT	saline-treatment groups	6.100	0.0040
	Zn-treatment groups	5.964	0.0012