THE ROLE OF GLYCATION

AND

FREE RADICALS

IN

HYPERGLYCEMIA-INDUCED MALFORMATIONS

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ABSTRACT

Although the risk of malformations in the offspring of diabetic mothers remains the leading cause of perinatal mortality, the pathogenesis has not been elucidated. The hypothesis of this study was that protein glycation and oxygen free radicals play a role in hyperglycemia-induced malformations. CD-1 mouse embryos (0-2 somites) were cultured under hyperglycemic conditions for 48 hours with the exogenous addition of anti-glycating agents and oxygen free radical scavengers. The exogenous addition of aspirin (ASA) and D-lysine reduced significantly the malformations and embryonic glycated protein levels. Salicylate, arachidonic acid (AA), and to a lesser extent, indomethacin also exerted protective effects, but with no effect on glycated protein levels. We hypothesize that ASA, salicylate and indomethacin are protective by exerting free radical scavenging action; and ASA and D-lysine are acting as potent anti-glycating agents. Moreover, we suggest that AA may have inhibited hyperglycemia-induced malformations through the protective action of prostaglandins against free radical damage. Serum media lipid peroxidation (LPO) was reduced in the ASA and indomethacin groups possibly due to either a direct free radical scavenging action and/or the inhibitory effects of these agents on cyclooxygenase activity thereby decreasing the oxygen free radicals produced by this enzyme system. On the other hand, AA was associated with an increased level of LPO in the serum media. As the evidence has shown, the cause of hyperglycemia-induced malformations appears to be multifactorial and no one agent can completely eliminate the problem, although protective action can be exerted at different levels of the glycation-free radical cascade of tissue damage.

RÉSUMÉ

Bien que le risque de malformations chez les rejetons de mères diabétiques reste la cause principale de mortalité périnatale, la pathogenèse n'a pas été élucidée. L'hypothèse de cette étude était que la glycosylation non enzymatique des protéines et les radicaux libres d'oxygène jouent un rôle dans les malformations provoquées par l'hyperglycémie. Les embryons de souris CD-1 (0-2 métamères) ont été cultivés dans des conditions d'hyperglycémie pendant 48 heures avec l'addition exogène d'agents anti-glycosylants et de phagocytes de radicaux libres d'oxygène. L'addition exogène d'aspirine (ASA) et de D-lysine a réduit de façon significative les malformations et les niveaux de protéines glycosylées de l'embryon. Aussi, le salicylate, l'acide arachidonique (AA), et dans une moindre mesure, l'indométacine, exercent des effets protecteurs, mais n'ont pas d'effets sur les niveaux de protéines glycosylées. Nous avons émis l'hypothèse que l'ASA, le salicylate et l'indométacine agissent comme protecteurs en exerçant une action de phagocytose des radicaux libres, et que l'ASA et la D-lysine jouent un rôle d'agents anti-glycosylants puissants. De plus, nous suggérons que l'AA peut avoir inhibé les malformations provoquées par l'hyperglycémie au moyen de l'action protectrice des prostaglandines contre les lésions causées par les radicaux libres. La peroxydation des lipides dans les média sériques a été réduite dans les groupes ayant reçu de l'ASA ou de l'indométacine, probablement en raison d'une action directe de phagocytose des radicaux libres et/ou des effets inhibiteurs de ces agents sur l'activité de cyclo-oxygénase causant une diminution des radicaux libres d'oxygène produits par ce système enzymatique. D'un autre côté, l'AA a été associée à un niveau accru de peroxydation dans le milieu sérique. Comme il a été démontré, la cause des malformations provoquée par l'hyperglycémie semble être multifactorielle et aucun agent ne peut éliminer complètement le problème, bien qu'une action protectrice soit exercée à différents niveaux de la cascade des radicaux libres de glycosylation des lésions tissulaires.

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ABBREVIATIONS

$HbA1_c$	Glycosylated hemoglobin
AA	Arachidonic acid
AGE	Advanced glycosylation endproducts
ASA	Aspirin
CAT	Catalase
CNS	Central nervous system
DHBA	Dihydroxybenzoic acid
GPX	Glutathione peroxidase
HETE	Hydroxyeicosatetraeonoic acid
HPLC	High performance liquid chromatography
LPO	Lipid peroxidation
NSAID	Nonsteroidal anti-inflammatory drugs
PGE_2	Prostaglandin E_2
PGI_2	Prostacyclin
PG	Prostaglandins
SOD	Superoxide dismutase
TXA_2	Thromboxane

SECTION 1: INTRODUCTION

1.1. HISTORIC BACKGROUND OF CONGENITAL MALFORMATION AND DIABETES

In diabetes mellitus, congenital malformation has remained the leading cause of perinatal mortality in the offspring of diabetic mothers (Freinkel, 1980; Mills, 1982). Autopsies of infants of diabetic mothers revealed that 17% of the deaths were due to lethal malformations (Driscoll, 1965). Karlsson and Kjellmer (1972) and Pedersen et al. (1974) have reported that anomalies were responsible for 40% of perinatal deaths. Furthermore, several studies have found that the prevalence of malformations are three times as common in these offsprings and fatal congenital abnormalities occur six times more frequently (Breidahl, 1966 and Hubbell et al., 1965).

Naeve (1967) compared the incidence of malformations in infants of diabetic mothers with similar age, race, and gravidity. Major malformations such as anencephaly, cleft lip and palate, omphalocele, and reduction deformities of the limbs were detected in 1.62% of infants of diabetic mothers but only 0.85% in controls. Kucera (1971) reviewed retrospective data on 7,101 infants of diabetic mothers and reported a 4.8% incidence of congenital malformations. Malformations affected all major organ systems, including the heart, kidneys, skeleton, and genitalia. Abnormalities of the central nervous system such as anencephaly and spina bifida are

commonly found in infants of diabetic mothers (Soler et al., 1976 and Gabbe, 1977). Caudal regression syndrome, a condition in which agenesis of both the lower femora and the lower vertebrae, also shows a strong correlation to diabetes (Eriksson, 1991).

Despite the variations in reporting and classification, the data does suggest a higher incidence of malformations in infants of diabetic mothers. Congenital malformations in diabetic pregnancy have also been reported experimentally in animal models using mice (Sadler, 1980; Piddington et al., 1983; and Baker et al., 1990), rats (Baker et al., 1981; Eriksson et al., 1983; Giavini et al., 1986; and Zusman and Omoy, 1986) and also rabbits (Chomette, 1955 and Brinsmade et al., 1956). Malformations seen in these studies primarily affected the skeletal and central nervous system. Renal and cardiac abnormalities, as well as heart hypertrophy, were found in the offspring of diabetic rats. Increased incidence of cataract and eye defects, such as microphthalmia and anophthalmia, has been reported in the offspring of severely diabetic rats (Giavini and Prati, 1990).

1.2. Potential Mechanisms of Diabetic Teratogenicity

The overall risk for malformations for diabetic patient populations was found to be approximately 7.0 - 9.0% (Soler et al., 1976; Gabbe, 1977; Simpson et al., 1983; Mills et al., 1988b; and Greene et al., 1989). The critical period for induction of malformations in diabetic pregnancy has been estimated to occur between the third and the sixth week of gestation (Mills et al., 1979). This is the period of organogenesis which also has the greatest teratogenic susceptibility (Mills et al., 1979). The pathogenesis of the congenital malformations has not yet been elucidated. Several possible underlying mechanisms have been proposed. These include: hyperglycemia

(Cockroft and Coppola, 1977 and Sadler, 1980), hypoglycemia (Smithberg and Runner, 1963), hyperinsulinemia (Hinchliffe, 1974), and hyperketonemia (Horton and Sadler, 1983 and Lewis et al., 1983). Although the above mechanisms appear to offer explanation for diabetes-induced teratogenesis, it is likely that this condition is multifactorial in origin.

There has been ongoing research in understanding the etiology of malformations in infants of diabetic mothers. One proposed concept of "fuel-mediated teratogenesis" by Freinkel (1980) looks at the alterations in the maternal glucose, amino acid and lipid levels as instrumental in the induction of dysmorphogenesis in the offspring. The intrauterine environment of most diabetics is one of constant change. Disturbances in the maternal metabolism alter the fuel mixture offered to the embryo. These effects on the offspring can manifest as malformation in early gestation (Freinkel, 1980).

In experimental diabetic pregnancy, there is a lowered blood flow to the placenta (Eriksson and Jansson, 1984 and Palacin et al., 1985), as well as alterations in the transport of glucose (Thomas et al., 1990) and lipids (Thomas, 1987). Experimental studies in rodents have shown marked morphological alterations and impaired function of the visceral yolk sac of early embryos (Zusman et al., 1987). Morphological alterations were also seen after embryos were cultured in elevated glucose concentrations (Sadler, 1980 and Pinter et al., 1986). The proposed mechanisms by which high glucose plays a role in the disturbed embryogenesis include: accumulation of sorbitol (Baker et al., 1990); deficiency of myo-inositol (Eriksson and Freden, 1988 and Baker et al., 1990); deficiency of arachidonic acid (Goldman et al., 1985 and Pinter et al., 1986): and increased oxygen free radical generation (Eriksson and Borg, 1991). Thus, the concept of "fuel-mediated teratogenesis" has obtained experimental

support to demonstrate the impairment of embryonic development by a variety of cellular processes (Eriksson, 1991).

Hyperglycemia has also been associated with a decreased number of lipid droplets and an elevation in the oleic acid of embryonic nonesterified fatty acids (Benjamin and Gellhorn, 1964). This is seen as an premature event, which occurs before the placenta and the embryonic liver are capable of performing functions of the yolk sac. This could result in the failure of organogenesis, since the yolk sac is first organ that produces essential proteins, including prealbumin, albumin, transferrin and apolipoproteins (Shi et al., 1985). In addition, vitelline circulation is the first circulation originating within the yolk sac during organogenesis (Moore, 1982). Insults to the yolk sac could lead to deleterious effects on the development of embryonic vessels and blood cells, which seems to underlie many abnormal consequences in hyperglycemia (Pinter et al., 1988). Hyperglycemia has also been hypothesized to reduce DNA synthesis and slow cell division, leading to impaired growth or abnormalities (Ely, 1981). This has been proposed to occur through competitive inhibition of cellular uptake of dehydroascorbic acid resulting in reduced hexose monophosphate shunt activity, which in turn may suppress DNA synthesis (Ely, 1981).

1.3. Role of Hyperglycemia in Malformations

In clinical studies, Leslie et al. (1978), Miller et al. (1981), and Ylinen et al. (1981) have reported a significantly higher incidence of major congenital anomalies among the infants of diabetic mothers who had elevated glycosylated hemoglobin ($HbA1_c$). $HbA1_c$ is expressed as a percentage of total hemoglobin and is used as a retrospective index of hyperglycemia.

Miller et al. (1981) examined 116 infants of diabetic mothers. $HbA1_c$ levels drawn from the mothers before the 14th week of gestation appeared to have a strong correlation with malformation rates. Leslie et al. (1978) also found correlation between high $HbA1_c$ and malformations. They found that three out of five insulin-dependent diabetic mothers with elevated $HbA1_c$ levels had malformed infants. Thus, the results have demonstrated that embryos exposed to the metabolic derangements of hyperglycemia during the period of organogenesis have an increased risk for congenital malformations. Subsequent to these studies, the levels of $HbA1_c$ have been used to determine the risk for major malformation and spontaneous abortion (Miller et al., 1981; Ylinen et al., 1984; and Greene et al., 1989). However, good periconceptual regulation of diabetes and insulin therapy helped to diminish the frequency of congenital malformations (Mills et al., 1983; Freinkel et al., 1986; and Buchanan et al., 1986). Intensive metabolic control in early pregnancy could reduce the incidence of congenital malformation in infants of diabetic mothers (Fuhrmann et al., 1983). Unfortunately, strict glycemic control during pregnancy does not always result in a concomitant reduction in congenital abnormalities. This is possibly due to mild abnormalities induced by difficulties in administrating optimal insulin doses even with well-controlled diabetes (Greene et al., 1989).

Hyperglycemia in diabetic pregnancy has been suspected to be one of the alterations in the intrauterine environment which causes congenital malformations. The susceptibility of early human embryos to elevated glucose levels is not known. The teratogenic effects of glucose seem to be specific for the increased ambient concentration of D-glucose, but not L-glucose (Cockroft and Coppola, 1977; Sadler, 1980; and Eriksson, 1988). Studies done with rats have shown that diabetes increases

the placental transfer of glucose from the mother to the fetus as compared to nondiabetic rats (Thomas et al., 1990).

Many studies have examined the relationship of hyperglycemia, duration of diabetes, and vascular complications with the occurrence of anomalies (Pedersen et al., 1974; Karlsson and Kjellmer, 1972; and Miller et al., 1981). In a 10-year study, Karlsson and Kjellmer (1972) have reported a higher rate of malformations among patients with hyperglycemia, long-standing diabetes, and diabetic vasculopathy than those without these complications. Miller et al. (1980) and Wittaker et al. (1983), in a prospective study, have also reported a 30% frequency of spontaneous abortion among insulin-dependent diabetic women compared to 15% reported in the general population of pregnant women.

In the study of the teratogenic effects of hyperglycemia on embryogenesis, the rate of unfused neural tubes in whole embryo cultures exposed to hyperglycemic conditions is similar in many studies (Cockcroft and Coppola, 1977; Sadler, 1980; and Garnham et al., 1983). Sadler (1980; and et al., 1986) had investigated the effects of hyperglycemia on early stages of embryogenesis using the whole embryo culture technique. They had compared the effects of different concentrations of glucose on younger (0-1 and 2-3 somites) and older (4-6 somites) embryos. Embryos were exposed to glucose at five times (6.2 mg/ml) and eight times (9.2 mg/ml) normal blood glucose levels for a 24 hour period. Neural tube defects were observed in both concentrations of glucose. In both of his studies, the frequency of malformations were higher in younger embryos and in embryos exposed to the higher glucose concentration (9.2 mg/ml). Thus, his results showed a dose- and age-related effect of glucose on early stages of embryogenesis (Sadler, 1980 and Sadler et al., 1986).

In a study done by Cockroft (1984), rat embryos at 9.5 days were cultured in 1.6 (control), 12 or 15 mg/ml glucose levels for 48 hours. Maximum sensitivity of these embryos to hyperglycemia appeared to be during the first and second 8-hour periods of culture. Malformations were found to be most severe in highest glucose concentration (i.e., 15 mg/ml). In addition to structural abnormalities, there was also a reduction in the rate of growth and differentiation reflected by a reduced protein content, crown-rump length, and somite number. Therefore, it was seen that younger embryos were more susceptible to damage from hyperglycemia and the observed malformations were primarily neural and heart defects (Cockroft, 1984).

Embryos exposed to a hyperglycemic culture (950 mg/dl) have abnormal embryonic development and yolk sac formation (Goldman et al., 1985 and Pinter et al., 1986). The embryos exposed throughout the culture period were more severely affected than the embryos exposed to only part of the culture period (Garnham et al., 1983). Kubow et al. (1993) have demonstrated hyperglycemia affects the growth and development of CD-1 mouse embryos at 0-2 somite stage. Fifteen of 20 embryos placed in a glucose concentration of 50 mM for 48 hour culture period exhibited gross neural abnormalities (i.e., open neural tube in the cranial and/or caudal regions of the embryo). Hyperglycemia affected all parameters of growth and development as indicated by an overall decrease in morphological ratings.

Similar neural tube defects have been observed after alloxan and streptozotocin-induced diabetes in mice and rats (Watanabe and Ingalls, 1973 and Deuchar, 1977) as have been reported in the offspring of diabetic mothers (Soler et al., 1976; Gabbe, 1977; and Fuhrmann et al., 1983). Sera from streptozotocin diabetic rats (Sadler, 1980; Horton and Sadler, 1983; and Freinkel et al., 1986) and diabetic humans (Sheehan et al., 1986) also have embryotoxic and inhibitory effects on the development of early

somite embryo cultures. Serum from streptozotocin-induced diabetic rats that received insulin therapy prior to sacrifice was less teratogenic when used as culture medium than samples received no insulin. Moreover, malformation rates were directly correlated to the severity of diabetes in the insulin-treated rats (Smith et al., 1975). Exogenous insulin added to the serum from streptozotocin-induced diabetic rats did not alleviate the teratogenic effects of this serum in culture. Therefore, insulin therapy, by itself, is not sufficient to produce normal embryogenesis in embryos cultured in diabetic serum, other factors, such as hyperglycemia are operating as teratogens.

High levels of ketone bodies in poorly controlled diabetes were suspected as teratogens when it became apparent that hyperglycemia was not the sole factor involved. It has been shown that rodent embryos cultured in elevated β-hydroxybutyrate (β-OHB) exhibited neural tube defects and other morphological abnormalities (Horton and Sadler, 1983; Freinkel et al., 1986; and Sadler et al., 1986). The frequency and severity of malformations were both dose- and age-dependent (Horton and Sadler, 1983 and Sadler et al., 1986). Furthermore, the interactions between glucose and β-OHB have synergistic effects (Freinkel et al., 1986 and Sadler et al., 1986), such that subteratogenic doses of glucose and β-OHB administrated in combination in culture have shown to produce malformations in embryos (Lewis et al., 1983).

1.4. Nonenzymatic Protein Glycosylation

Nonenzymatic glycosylation is also known as the browning reaction, glycation or the Maillard reaction. Formation of glycated proteins begins when the carbonyl groups of a reducing sugar are attached to the amino groups of a protein by

nucleophilic addition without the aid of enzymes (Fgiure 1). This results in the formation of unstable Schiff base adducts which can rearrange into more stable 1-deoxy-1-amino-2-ketose derivatives (also known as ketoamines or fructosamines). Ketoamines being the major species of glycated protein (Schleicher and Weiland, 1989) contain a ketol group, making them candidates for superoxide anion (O_2^-) formation when in contact with oxygen molecules (Gillery et al., 1988 and Azevedo et al., 1988). Ketoamines can thereby initiate oxidative degradation of dioxygen to superoxide anion. Hydrogen peroxide and hydroxyl radicals can be generated from superoxide in the presence of catalytic metal ions (Jones et al., 1988 and Gillery et al., 1988).

Following a slow chemical rearrangement, a more stable but still chemically reversible sugar-protein adduct, called the Amadori product is formed (Higgins and Dunn, 1981 and Mortensen and Christophersen, 1983). These early glycation products increase proportionally with blood glucose levels and return to normal after insulin treatment (McFarland et al., 1979). Amadori products slowly undergo a series of rearrangements and dehydrations to form chemically irreversible complexes called advanced glycosylation endproducts (AGE) (Monnier and Cerami, 1983) (Figure 1). The level of AGE products does not return to normal after the blood glucose level is normalized.

In recent years, the nonenzymatic glycosylation of proteins has gained great relevance in mammalian pathophysiology. Increased glycation of proteins has been implicated to be involved in the process of aging, and the development of cataracts, atherosclerosis and cancer (Cerami et al., 1988). Nonenzymatic glycosylation might induce tissue derangement by modifying the structure, function and biochemical characteristics of proteins via the formation of irreversible and highly reactive foreign groups (Sensi et al., 1991). The nonenzymatic glycosylation of glucose and protein has

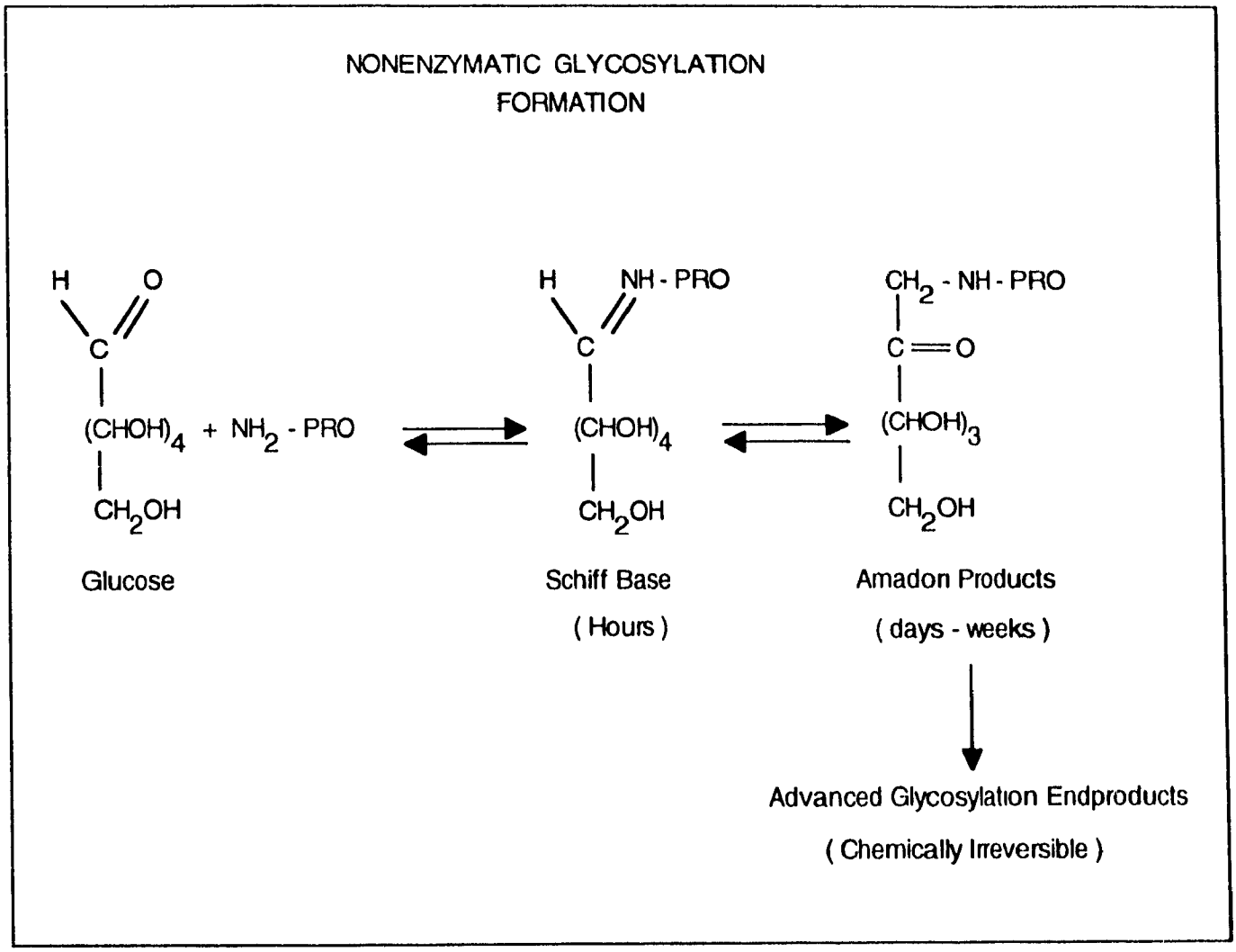

Figure 1: Pathway for nonenzymatic glycosylation formation of relatively unstable intermediate products (Schiff base and Amadori products), and eventually, AGE, which have been implicated to play a role in disorders associated with diabetes.

also been implicated as one of the mechanisms involved in the development of diabetic complications such as atherosclerosis, retinopathy, nephropathy and angiopathy (Brownlee et al., 1987).

Proteins known to undergo the nonenzymatic glycosylation reaction include hemoglobin, immunoglobulins, serum albumin, collagen and lens crystallins (Dunn et al., 1978; Stevens et al., 1977; Day et al., 1979; and Bailey and Kent, 1989). The significance of the process to human diabetes is not clear, but the extent of tissue browning has been correlated with the incidence and severity of several complications in human diabetics (Monnier et al., 1986). Glycation can affect the structure, function and biochemical characteristics of proteins such as calcium ATPase (Gonzalez-Flecha, 1990), calmodulin (Kowluru et al., 1989), hemoglobin, lens crystalline, and collagen (Lee and Cerami, 1990).

1.4.1. Measurement of Glycosylated Protein

One common measurement developed for the quantitative determination of early glycosylated protein during diabetic pregnancy is the measurement of the concentration of $HbA1_c$. Various methods are also used to separate glycosylated from non-glycosylated hemoglobin. These methods include: i) ion exchange chromatography on cation resin; ii) isoelectric focusing on thin-layer polyacrylamide gels; iii) agar electrophoresis; and iv) boronate affinity chromatography (Sensi et al., 1991). The use of glycated hemoglobin measurements is to assess long-term glycemic control in the management of diabetes (Kennedy and Lyons, 1989 and Lester, 1989).

AGE have characteristic spectral and fluorescent properties which form the basis for several assays used to quantify protein-glycation. Furoyl-furanyl-imidazole, for example, is an AGE which has spectral characteristics that have been used to estimate differences in AGE content in lens protein, collagen (Monnier and Cerami, 1983), and DNA (Bucala et al., 1984). AGE such as furoyl-furanyl-imidazole, can be measured by high performance liquid chromatography (HPLC) or radioimmunoassay.

One of the amino acids, lysine, and its interaction with sugars has been studied extensively. Lysine is involved in the glycation process mainly due to its free and highly reactive ε-amino groups. Upon hydrolysis of the sugar-lysine adduct, ε-N (2-furosylmethyl)-L-lysine, called furosine, is formed. Furosine has been used as a useful indicator to determine if lysine-glucose reactions have occurred (Schleicher and Wieland, 1980 and Furth, 1988). Quantification of glycated lysine residues is achieved by hydrolyzing the glycosylated protein in 6N HCl and measuring the amount of furosine formed in the process (Fluckiger and Gallop, 1984). Recently, HPLC analytical methods have been developed for the separation and identification of the decomposition products of Amadori compounds. Quantification of glycated proteins is also possible using a fluorescence probe (Yaylayan et al., 1992).

1.4.2. Nonenzymatic Glycosylation and Diabetic Teratogenicity

Congenital malformations in diabetic pregnancies have been associated with the extent of nonenzymatic glycosylation of umbilical cord tissue (Pollak et al., 1988). Glucitollysine, an indicator which reflects the extent of nonenzymatic glycosylation processes, was extracted from the umbilical cords of infants of diabetic mothers. Pollak et al. (1988) have observed that there was a higher glucitollysine content in the

umbilical cord extracts of infants with congenital malformations when compared to infants of control mothers or infants of diabetic mothers with no congenital malformations. This led to their hypothesis that glycated proteins play a role in alteration of normal development of embryonic structural proteins leading to a teratogenic role in diabetic pregnancies. Fetal hemoglobin in infants of diabetic mother also showed an increased level of glycation (Sosenko et al., 1979 and Peterson and Jovanovic, 1986).

The role of glycation in hyperglycemia-induced malformations has been previously assessed in rodent embryos. Sadler et al. (1986) used the boronate affinity chromatography to assay glycation products in rodent embryos which were cultured for 48 hours under hyperglycemic conditions. However, they found no increase in the percentage of glycated proteins in either the embryos or visceral yolk sacs after culturing. Although this method has been demonstrated to be sufficient to separate glycated from non-glycated hemoglobin, the polyboronate chromatographic assay has certain limitations for intracellular studies of embryonic tissues. To separate enzymatic and nonenzymatic glycosylated proteins, it is important to raise the ionic strength, temperature and pH of the eluting solution in order to enhance the differences between glycoproteins and glycated proteins in binding to the boronate resin (Furth, 1988). These factors, however, were not described in Sadler et al. (1986). Since high ambient glucose concentrations could inhibit proteoglycan accumulation in precartilage mesenchymal cells (Leonard et al., 1989), an increase in embryonic protein glycation during hyperglycemia may be accompanied by a decrease in glycoprotein synthesis. Thus, the amount of glycated protein can be underestimated if the polyboronated assay conditions are not calibrated to accurately distinguish between enzymatically and nonenzymatically glycosylated proteins. In addition, a pooled samples of four embryos were required to detect glycated protein by Sadler et al. (1986) whereas, the combined

techniques of microwave sample hydrolysis (Gilman and Woodward, 1990) and HPLC detection of furosine allow detection of glycated protein in individual embryos. The polyboronate chromatographic assay may further underestimate the extent of endogenous glycated protein due to poor retention of proteins glycated by sugar phosphates such as glucose 6-phosphate on polyboronate resins (Furth, 1988). Since the embryo relies mainly on glycolysis for its energy requirements (Tanimura and Shepard, 1969), relatively high amounts of glucose 6-phosphate would be generated and available for protein glycation. Therefore, it is important to measure phosphorylated sugar glycation since phosphorylated sugars glycate proteins much more readily than nonphosphorylated ones.

1.4.3. Anti-glycating Agents

The abundance of reducing sugars in the body and the nonspecificity of this reaction with amino groups, inevitably, will result in glycation with a number of proteins, especially those with extended half-lives. Increased glycation of lipoproteins, basement membrane might contribute to the pathogenesis of aging, cataract, and diabetic complications such as atherosclerosis, microangiopathy and thrombosis (Brownlee et al., 1987). Therefore, many studies have aimed at blocking the formation of the reactive Amadori products. Fluorescence can be generated by glycation of proteins, and it can be used to measure the degree of glycation. It was suggested that serum protein fluorescence is due to oxidative changes (Jones et al., 1988), and the process of glycation and development of fluorescence can be inhibited through the blocking of ketoamine carbonyl groups on glycated proteins (Brownlee et al., 1986).

Aspirin (ASA) has demonstrated a role as an anti-glycating agent by acetylating proteins such as albumin, immunoglobulins, and fibrinogen (Pinckard et al., 1968 and Hawkins et al., 1969). Huby and Harding (1988) have shown that pre-incubation of the lens crystallins with ASA prior to the addition of galactose (also a reducing sugar) prevented galactosylation. When ASA was incubated simultaneously with galactose, a dose-related inhibition of galactosylation was observed. ASA acetylates the protein lysine/serine residues thereby protecting them against sugar addition (Huby and Harding, 1988).

D-lysine, an amino acid, which is neither metabolized by nor incoporated into protein in mammalian systems, has been previously reported to inhibit protein glycation (Sensi et al., 1989). D-lysine has also been shown to be effective in limiting the *in vitro* formation of AGE (Sensi et al., 1990). Aminoguanidine, on the other hand, is a nucleophilic hydrazine compound which acetylates proteins. It has been shown that aminoguanidine prevents the formation of AGE fluorescence and cross links both *in vitro* (Brownlee et al., 1986 and Khatami et al., 1988) and *in vivo* (Nicholls and Mandel, 1989 and Odetti et al., 1990).

1.5. Oxygen Free Radicals and Diabetes

Toxic oxygen free radicals have been implicated as important pathological mediators in many clinical disorders, such as cancer (Totter, 1980), atherosclerosis (Harman, 1984 and Harman, 1986) and diabetes (Baynes, 1991 and Chisolm et al., 1992). There is evidence that diabetes is accompanied by enhanced lipid peroxidation or lipoprotein oxidation and that hyperglycemia and accelerated oxidation may be related (Lyons, 1991 and Baynes, 1991). Studies have found that oxygen free radicals

contribute to the destruction of β cells of the Langerhans islets (Burkart et al., 1992 and Yamada et al., 1993).

It has been suggested that transition metal-catalyzed glucose enediol oxidation (glucose autoxidation) generating oxygen-centered free radicals is responsible for the changes observed in experimental glycation models of tissue damage (Wolff and Dean, 1987 and Hunt et al., 1988). In the presence of albumin (1 mg/ml), the levels of H_2O_2 detected were approximately 6-fold lower than in the absence of the protein (Jiang et al., 1990). Albumin chelates copper ions (Halliwell, 1988) and inhibits glucose autoxidation via chelation of copper since autoxidation is dependent upon transition metals (Hunt et al., 1988). Hunt et al. (1988) have proposed that the fragmentation and conformational changes observed in experimental glycation models of tissue damage, are dependent upon hydroxyl radicals produced by glucose autoxidation. Various hydroxyl free radical scavengers, therefore, may inhibit tissue damage in diabetes.

Oxygen radicals are capable of reversibly or irreversibly damaging compounds such as nucleic acids, protein and free amino acids, lipids and lipoproteins, carbohydrates, and connective tissue macromolecules (Cross et al., 1987). Experimental work has shown that there is an alteration in free radical tissue defense mechanisms (Wohaieb and Godin, 1987), a decrease of reduced glutathione in red blood cells, and increased activity of glutathione peroxidase in diabetic animals (Gandhi and Roy-Cowdhury, 1979). The evidence has shown that oxidative transformation of plasma lipoproteins enhances their ability to induce atherosclerosis (Fogelman et al., 1980), which shows high prevalence in diabetics. Other complications of diabetes, suggested by experimental studies involving oxygen radicals, include retinal damage (Crouch et al., 1978) and renal injury (Paller et al., 1984).

Elevated levels of lipid peroxidation (LPO) products in plasma have been observed in diabetic humans and rats (Sato et al., 1979; Tsuchida et al., 1985; Uzel et al., 1987; and Jain et al., 1989). LPO is a natural event which occurs in the cells through autoxidation or enzymatic reactions (i.e., prostaglandin H synthase and lipoxygenase). Free radicals and lipid hydroperoxides, formed during LPO, can be scavenged by anti-oxidants or reduced enzymatically (superoxide dismutase (SOD), catalase (CAT) or glutathione peroxidase (GPX)).

Diabetic rats, induced by streptozotocin, have demonstrated increased oxidative stress in various tissues resulting in the alteration of antioxidant enzyme activities (SOD, CAT, and GPX) (Wohaieb and Godin, 1987). Vitamin E was found to be deficient in erythrocytes of diabetic rats (Jain et al., 1991). However, the addition of vitamin E (Hayward et al., 1992) and antioxidant (U78518F) (Rabinovitch et al., 1993) have both reduced the incidence of diabetes in experimental mice. Furthermore, the addition of oxygen free radical scavengers (i.e., desferrioxamine and nicotinamide) protected islets from immune destruction and significantly enhanced the survival of cultured islet allografts (Mendola et al., 1989).

Oxygen free radicals, which are formed in increasing amounts in diabetes may be due to electron exchange between the sugar moiety of the glycated protein and molecular oxygen (Gillery et al., 1989). The formation of glycated protein may promote the formation of oxygen free radicals at high glucose concentrations (Gillery et al., 1989 and Hunt et al., 1988). In the process of nonenzymatic glycosylation, reducing sugars such as glucose can enolize and reduce molecular oxygen to produce hydrogen peroxide and hydroxyl radicals (Wolff and Dean, 1987). The increased amount of Amadori products associated with the diabetic condition increases the probability of the formation of superoxide radicals when in contact with oxygen

molecules. Gillery et al. (1988) have demonstrated that at pH 7.4 and temperature 37° C, glycated proteins form superoxide radicals *in vitro*. In the presence of high glucose concentrations, glucose autoxidation can also generate oxygen free radicals (Hunt and Wolff, 1990).

Oxygen free radicals may exert their effects by inactivating enzymes (Kong and Davidson, 1980), breaking DNA strands (Brawn and Fridovich, 1980), cross-linking proteins (Biorkstein, 1974), and oxidizing lipids (Mead, 1976). In diabetic patients, a significant increases in membrane LPO in erythrocytes have been documented (Jain et al., 1989). Tissue peroxidases are ineffective in the presence of high levels of lipid hydroperoxides (Jain et al., 1983). Membrane LPO can result in decreased cell survival, altered membrane phospholipid asymmetry (Jain, 1984; Jain, 1985; and Wali et al., 1987), hypercoagulability (Jain, 1985), and increased adhesion to endothelial cells (Wali et al., 1987).

1.5.1. Free Radicals and Diabetic Teratogenesis

As LPO inhibits mitosis (Wilbur et al., 1957), the rapidly dividing cells of embryonic tissues could have a greater susceptibility to peroxidation, due to both the greater degree of lipid unsaturation in neonatal tissues than in adult tissues (Wolfson et al., 1956) as well as a relatively low activity of antioxidant systems (i.e., superoxide dismutase) (Kehler and Autor, 1977). Therefore, the reaction of oxygen free radicals with cellular macromolecules such as proteins and lipids could have cytotoxic effects which affect embryonic development (Halliwell, 1987).

Eriksson and Borg (1991) proposed a direct coupling between free radical generation in diabetic pregnancies and the induction of congenital malformations. Rat embryos were incubated in 50 mmol/L glucose media for 48 hour, and free oxygen radical scavenging enzymes SOD, CAT and GPX were added to the media. The addition of SOD to the media resulted in a complete normalization of both the crown rump length and the number of somites as well as an improvement of morphological scores. The addition of CAT and GPX, although they did not result in full normalization, significantly improved the embryonic development. There were also significant increases in enzyme activities in both the embryos proper, and their membranes.

Free oxygen radicals can be generated due to a decrease in radical scavenging capacity or an increased production of free oxygen radicals in the conceptus in a glucose-rich environment (Eriksson and Borg, 1991). Oberley (1988) found decreased activity of SOD or GPX and decreased concentrations of vitamin E in various organs of diabetic humans and animals. However, based on their results, Eriksson and Borg (1991) suggested an increased production of free oxygen radicals in the conceptus in high glucose environment. An increased glucose concentration causes an increased flow through electron transport chain. At a high oxygen pressure, there was an increase endogenous production of oxygen free radicals, and the mitochondria of the neuroectoderm are morphologically altered in cultured embryos (Morriss and New, 1979). Since the mitochondria of early stage embryos are not mature, younger embryos are more vulnerable to a glucose challenge due to their poorly developed defense mechanisms and immature embryonic oxidative systems compared to older embryos (Sadler, 1980 and Eriksson et al., 1989). An enhanced formation of free oxygen radicals in the embryos may, therefore, directly relate to the increased risk of congenital malformations in diabetic pregnancy.

1.6. PROSTAGLANDIN SYNTHESIS

Arachidonic acid (AA), the most abundant of the prostaglandin precursor fatty acids in almost all tissues (Oliw et al., 1983), is stored in the cell membrane phospholipids. With an increased in activity of phospholipase A_2, there is a corresponding increase in both prostaglandin synthesis and the release of AA (Halushka et al., 1977; Halushka et al., 1979; Gerrard et al., 1980; and Takeda et al., 1981). Therefore, the limiting step in prostaglandin synthesis is the release of phospholipid by the action of phospholipases when activated by substances such as peptide hormones and catecholamines (Halushka et al., 1985). Fatty acids such as AA are metabolized through two distinct enzyme activities, a cyclooxygenase, which is the first component of the bifunctional prostaglandin H synthase enzyme complex, and hydroperoxidase. This enzyme complex converts polyunsaturated long chain fatty acids to thromboxanes and two cyclic endoperoxides: prostaglandin G_2 and prostaglandin H_2. The activity of cyclooxygenase is dependent on the availability of non-esterified fatty acid (substrates) and lipid peroxides (activators) (Warso and Lands, 1983). Sufficient hydroperoxide is needed by prostaglandin H synthase so that the released AA upon stimulation can be readily oxidized to prostaglandins (PG).

In diabetes mellitus, decreased proportions of AA in serum fatty acid and platelet phospholipids have been reported (Jones et al., 1983; Tilvis and Miettinen, 1985; Jones et al., 1986; and Kanzaki et al., 1987). The synthesis of prostacyclin (PGI_2), a vasodilator and an antiaggregatory substance, was decreased in diabetes mellitus. In addition, there was an increased production of thromboxane A_2 (TXA_2) (Jeremy et al., 1983). The increased rate of TXA_2 has been suggested to contribute to

the accelerated large vessel disease (atherosclerotic or macrovascular), and abnormalities affecting the capillaries, i.e., microangiopathy, seen in diabetes (Colwell et al., 1979).

Nonsteroidal anti-inflammatory drugs (NSAID) have been identified as cyclooxygenase inhibitors (Lands and Hanel, 1983). ASA and indomethacin bind to the cyclooxygenase moiety of prostaglandin synthase in an irreversible manner (Kulmacz et al., 1991). Both of these drugs inhibit the cyclooxygenase in a concentration-dependent and time-dependent manner (Rome and Lands, 1975). However, indomethacin is a much more effective inhibitor than aspirin on an equimolar basis (Smith and Lands, 1971 and Egan et al., 1978).

1.6.1. Prostaglandin Synthesis and Embryogenesis

AA is an important structural component of membrane and a precursor for the regulatory eicosanoids in placental and fetal development (Kuhn et al., 1990). Abnormalities in fetal growth and development have been associated with alterations in placental eicosanoid production. In diabetic pregnancy, the placental eicosanoid movement is found to be predominantly in the direction of fetal-to-maternal (Glance et al., 1986 and Kuhn et al., 1990). Hydroxyeicosatetraenoic acid (HETE) (a major lipoxygenase metabolite of AA in human vasculature), was shown to inhibit PGI_2 production (Mocada et al., 1976). On the other hand, PGE_2, having an anti-fertility effects in rats, hamsters, rabbits and mice was shown to have teratogenic effects on embryonic development in mice (Persaud, 1975). PGE_2 and/or its metabolites readily cross the mouse placenta. A single dose (25 μg) showed a high incidence of developmental defects in fetuses surviving to term (Persaud, 1975).

Recently, the notion of prostaglandin imbalance affecting embryonic morphogenesis has been supported by the beneficial effect on glucose-induced malformations by the addition of various prostaglandins (i.e., PGE_2, $PG_{2\alpha}$ and PGI_2) to whole embryo culture (Goto et al., 1992). Goldman et al. (1985) have found that the addition of AA reversed the hyperglycemia-induced teratogenesis both *in vivo* and *in vitro*. There were also significant decreases in neural fusion defects, incidences of micrognathia and cleft palate in 262 mouse embryos treated with AA. The presence of 10 ug/ml arachidonic acid resulted in 67% neural tube fusion of the embryos in the presence of 8 mg/ml D-glucose. This 67% of neural tube fusion was similar to what was observed in the control group (74%) but significantly different from the embryos in the D-glucose (8 mg/dl) in the absence of AA (32%).

Similar studies have been repeated by other researchers. Pinter et al. (1986) have found that addition of AA prevented malformations induced by hyperglycemic condition (950 mg/dl of D-glucose). The addition of 20 or 80 ug/ml of AA resulted in a significant increase in both the conceptus diameter and embryonic crown-rump length. The cellular arrangement, mitotic rate in the neural tube, and neuroepithelium of these embryos were similar to controls. The yolk sacs also demonstrated a significant increase in vitelline vessel conformation. AA was found to prevent advanced neuropil formation in the neural tube. The reduction of quantity of rough endoplasmic reticulum, a decrease in number of lipid droplets, and an increased number of lysosome-like structures within the endodermal yolk sac cells were all prevented by the addition of AA.

Yolk sac is the first organ that produces essential proteins during organogenesis (Shi et al., 1985). The rough endoplasmic reticulum of the endodermal cells may be a

possible site for the production of these proteins. Hyperglycemia reduces the quantity of rough endoplasmic reticulum in the visceral endodermal cells. However, this was returned to normal with the addition of AA. This may have preserved the synthetic activity of the yolk sac (Pinter et al., 1986). Insults to the yolk sac during organogenesis could adversely affect the development of vitellin vessels, which could lead to deleterious effects on the development of embryonic vessels and blood cells and to asphyxia with secondary adverse effects on organogenesis. Therefore, Pinter et al. (1986) have suggested that a deficit in the concentration of AA is the result of the disturbances in the developing conceptus, and will result in embryopathy.

Further study done by Pinter et al. (1988) has also suggested that diabetes-related embryopathy is associated with quantitative and qualitative abnormalities in essential fatty acids. Under AA supplementation (80 ug/ml), total fatty acid content in all major lipid groups was higher in the yolk sac than in rat embryos, which is similar to what was observed in controls. AA also prevented the elevation of oleic acid/stearic acid ratio seen in hyperglycemic group. According to Pinter et al. (1988), a variety conditions could activate cell membrane lipases in many tissues. This will result in the release of free fatty acids such as oleic and AA. In hyperglycemia-exposed conceptuses, release of these fatty acids may be due to an insufficient blood supply with significant decrease in the yolk sac and neural tube vascularity (Pinter et al., 1986). The uptake process of fatty acid is also decreased in hyperglycemic condition, but the decrease is preventable by AA supplementation.

Neural tube fusion requires embryonic cell movements and apposition of the two advancing layers (Waterman et al., 1983 and Moran and Rice, 1975), with cell death at that point leading to fusion and closure (Schluter, 1973 and Goldman et al., 1981). In the malformation model of glucocorticoid- and phenytoin-induced cleft

palate, the availability of AA in the fetal palates is reduced during their differentiation (Tzortzatou et al., 1981). Glucocorticoids or phenytoin interact with a cytosolic receptor and the receptor complex induces phospholipase A_2-inhibiting proteins (Gupta et al., 1984). An increase in phospholipase A_2-inhibiting proteins sharply decreases the availability of AA (Goldman et al., 1985). Experiments demonstrated that specific blockade of the cytosolic glucocorticoid receptor or the addition of exogenous AA will significantly reverse the production of cleft palate (Tzortzatou et al., 1981; Piddington et al., 1983; and Goldman et al., 1983). On the other hand, indomethacin has been shown to inhibit the effect of AA in preventing glucocorticoid-induced cleft palate both *in vivo* and *in vitro* (Tzortzatou et al., 1981 and Piddington et al., 1983).

There seems to be conflicting mechanisms involved in the prevention of hyperglycemia-induced malformations. Both ASA (Kubow et al., 1993) and AA (Goldman et al., 1985; Pinter et al., 1986; and Pinter et al., 1988) have been shown to have beneficial effects in preventing malformations. However, mechanistically, these two substances appear to work in opposite directions. ASA, an inhibitor of cyclooxygenase, has been proposed to prevent malformations by acting as an anti-glycation agent and free radical scavenger. On the other hand, sufficient amounts of AA seem to play an important role in preventing malformations. Therefore, further studies are needed to investigate the ASA and AA paradox in preventing hyperglycemia-induced malformations.

SECTION 2: HYPOTHESES AND OBJECTIVES

2.1. HYPOTHESES

The hypothesis for the first part of the experiment is that anti-glycating agents and free radical scavengers will have a protective effect on hyperglycemia-induced congenital malformations. Existing studies have shown that hyperglycemia-induced congenital malformations during organogenesis are related to high levels of glycated protein and free radicals being generated during this period of early development. The addition of a low dose of ASA (0.005 mM) has been shown to significantly reduce the formation of glycated protein, as well as the incidence of malformations in mouse embryos (Kubow et al., 1993). Furthermore, furosine levels were lower in the ASA and glucose + ASA embryos as compared to the normal glycemic and hyperglycemic embryos. ASA may also exert protective effects on malformations via the free radical scavenging action of its salicylate metabolite (Figure 2). As well, increased amounts of free radicals and congenital malformations under hyperglycemic conditions have been shown to be reduced by adding free radical scavengers such as SOD, CAT, and GPX (Eriksson and Borg, 1991). On the other hand, lysine (either L- or D-isomer) is the most active amino acid involved in the formation of glycated protein (Schleicher and Wieland, 1980 and Furth, 1988). Furthermore, the addition of D-lysine may also provide protective effects against hyperglycemia-induced malformations via the

formation of glucose-lysine product (Sensi et al., 1989). However, because the D-isomer of lysine cannot be utilized by the embryo for its nutritive needs, the protective action of this isomer could only reside in its anti-glycating role.

The hypothesis for the second part of the experiment is that exogenous supplementation of AA will reverse the effects AA deficiency on hyperglycemia-induced congenital malformations. Although it has been shown that the addition of AA to mouse embryos significantly reverse the inhibition of neural tube closure caused by high glucose concentration (Goldman et al., 1985), the addition of polyunsaturated long chain AA could also increase the formation of LPO. ASA and indomethacin have been shown to be potent inhibitors of cyclooxygenase, through which prostaglandins are formed (Roth et al., 1975; Kaplan et al., 1978; and Dejana, et al., 1981), and oxygen free radicals can be generated (Mead, 1976 and Kehler and Autor, 1977). Therefore, the presence of ASA and indomethacin could lower the level of LPO produced. This approach could thus help to elucidate the relative importance of LPO in embryonic malformations.

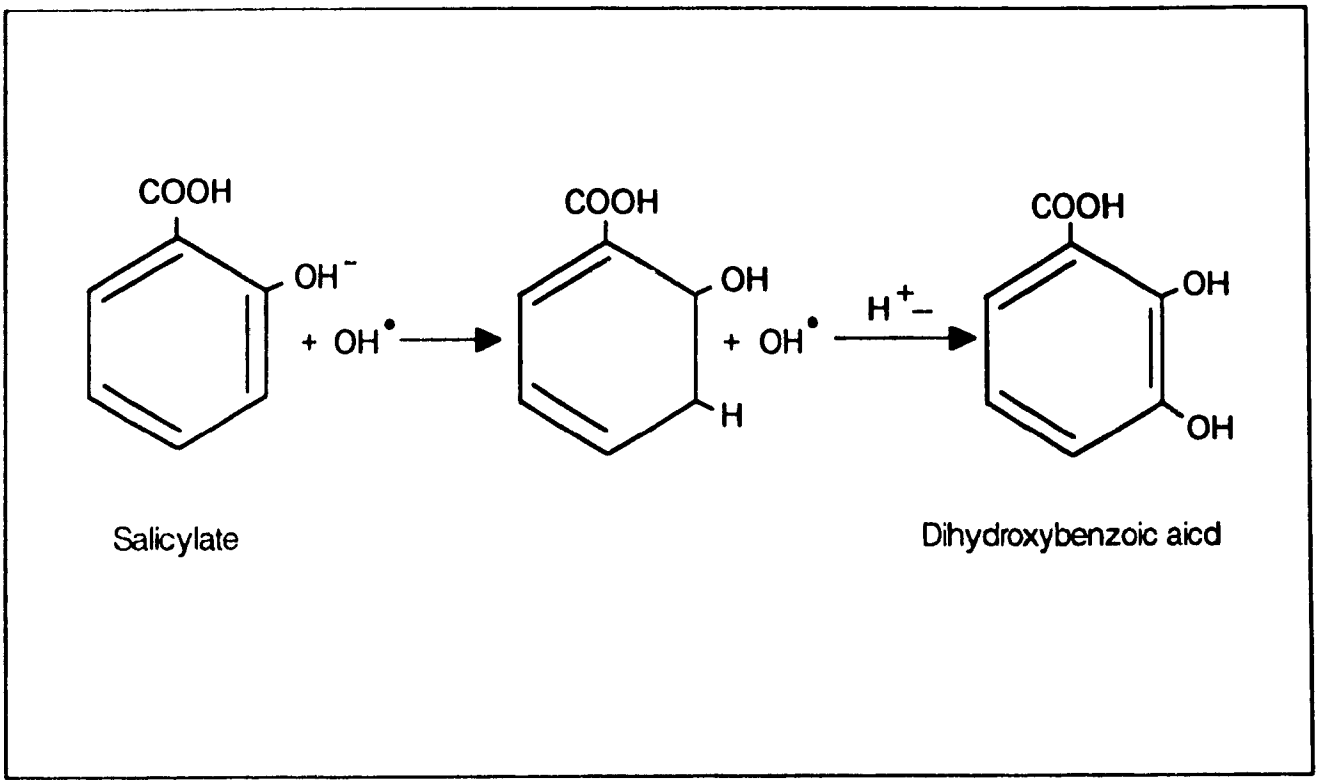

Figure 2: Free Radical Scavenging Action of Salicylate. Salicylate traps hydroxyl radicals yielding 2,3- and 2,5-dihydroxybenzoic acid (DHBA).

2.2. OBJECTIVES

The goals of these experiments are to examine the following objectives in the CD-1 mouse model of hyperglycemia-induced teratogenicity.

1) Phase 1: Teratogenicity

a) To validate the experimental model for hyperglycemia-induced teratogenicity in early stage CD-1 mouse embryos with 0-2 somites using embryo culture.

b) To examine the severity of malformations of early stage mouse embryos under hyperglycemic condition (50 mmol/L) in embryo culture.

c) To determine the formation and amount of glycated products and oxygen free radicals in early stage mouse embryos under hyperglycemic conditions using embryo culture.

d) To investigate the relationship between glycated protein and oxygen free radical concentration with malformations in early stage mouse embryos using embryo culture.

e) To investigate the role of free radicals and glycation in hyperglycemia -induced malformations in embryo culture by studying the protective roles of anti-glycating agents and free radical scavengers.

2) Phase 2: Lipid Peroxidation

a) To determine the formation and amount of LPO in early stage mouse embryos using embryo culture.

b) To investigate the relationship between the level of LPO and malformations in early stage mouse embryos using embryo culture.

c) To investigate the protective role of AA on hyperglycemia-induced teratogenicity in embryo culture.

d) To investigate the mechanism(s) of the apparent paradoxical effects of ASA and AA as protective agents against hyperglycemia-induced teratogenicity in embryo culture by studying their effects on glycation and LPO.

SECTION 3: MATERIALS AND METHODS

The experimental work described in this section is organized to reflect the order of the entire experiment. The experiment comprises: morphological assessment of the embryos, protein analysis, glycated protein and hydroxyl free radical measurements, and lipid peroxide concentrations.

The entire experiment was divided into two phases, so the effects of glycated protein (phase 1) and lipid peroxide (phase 2) on hyperglycemia-induced malformations could be determined.

3.1. EXPERIMENTAL DESIGN

CD-1 mice (Charles River Canada, St. Constant, Quebec) were kept in a temperature-controlled room (24 $\pm$ 1 °C) with a 12 hour light-dark cycle (0200h to 1400h). The mice were housed in plastic shoebox cages with Beta Chip bedding (Northwestern Products Corp., Warrensburg, NY). Purina Mouse Chow® (Ren's Feed and Supply, Oakville, Ontario, Canada) and tap water were fed *ad libitum*. Male mice were housed individually, and female mice were housed three per cage. Mice were mated by placing one male with three females, at random, between 900h to 1100h. The

presence of a vaginal plug indicated insemination and was designated as gestational day 0. Dams were kept until gestational day 8 and were sacrificed by cervical dislocation. Embryos at 0-2 somite stage were included in the experiment. Embryos were cultured for 48 hours and morphological scoring was performed at the end of the culture period.

3.2. 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 exsanguinating male Sprague-Dawley rats (Charles River Canada, St. Constant, PQ) under halothane anaesthetic. The blood was collected through cardiac puncture and was immediately centrifuged. The plasma clot was removed and the serum was re-centrifuged. The serum was pooled and heat-inactivated at 56°C for 30 min and filter sterilized using a 0.45 micron mesh filter (Millipore). The heat-inactivated, pooled serum was stored at -80°C. The serum was thawed and heat-inactivated again before use in embryo cultures. On gestational day 8, mice were killed by cervical dislocation and their uteri excised immediately. Using aseptic techniques, the decidua were dissected from the uterus in Hank's Balanced Salt Solution (HBSS) (Gibco, Burlington, Ontario) under a stereomicroscope. The embryo was exposed by removing the decidual tissue and Reichert's membrane. The ectoplacental cone, amnion, and viceral yolk sac were left intact.

Embryos with 0-2 somites were randomly assigned to the treatment groups; 1) normoglycemic (control) (glucose concentration at 8 mM); 2) control + treatment (Figure 4); 3) hyperglycemic medium (50 G) prepared by adding D-glucose (final culture concentration of 50 mM) to the rat serum; and 4) 50 G + treatment (Figure 4).

Embryos were placed into marked 60-ml culture bottles; each contained 1.6 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 male rat serum. The D-glucose (Sigma Chemical Co., St. Louise, MO) was dissolved in distilled water and included as 3% of the final volume of the culture medium. Control embryos received equivalent volumes of distilled water. Other test compounds (Figure 4) were also added as 3% of the final volume of the culture medium. The bottles were gassed for 2 min with 5% O_2-5 % CO_2-90% N_2 immediately after the addition of the embryos. The culture flasks were rotated at 30-rpm rotator wheel in a 37°C incubator. Embryos were re-gassed at 12 hour intervals on the second day of culture with 20% O_2-5% CO_2-75% N_2 and 40% O_2-5% CO_2-45% N_2. After 48 hours in culture, the embryos were transferred to Hank's saline solution for morphological scoring.

The amount of each treatment added was determined prior to the actual experiment by a dose-response curve using 0-2 somite mouse embryos. A treatment at different concentrations was added to control embryos and cultured for 48 hours. Morphological scoring was used to determine the effects of the different treatment concentrations on the growth of the embryos. Ten embryos were used for each concentration to determine the points of the dose-reponse curve. The highest concentration which gave the best morphological result, along with the absence of any visible malformations (to the naked-eye), was chosen to be the concentration used in the actual experiment. This process was repeated for lysine, salicylate and indomethacin (Figures 10 to 12, Appendix B). Literature values were chosen to be the concentrations used in the experiments for ASA (Kubow et al., 1993) and AA (Pinter et al., 1986).

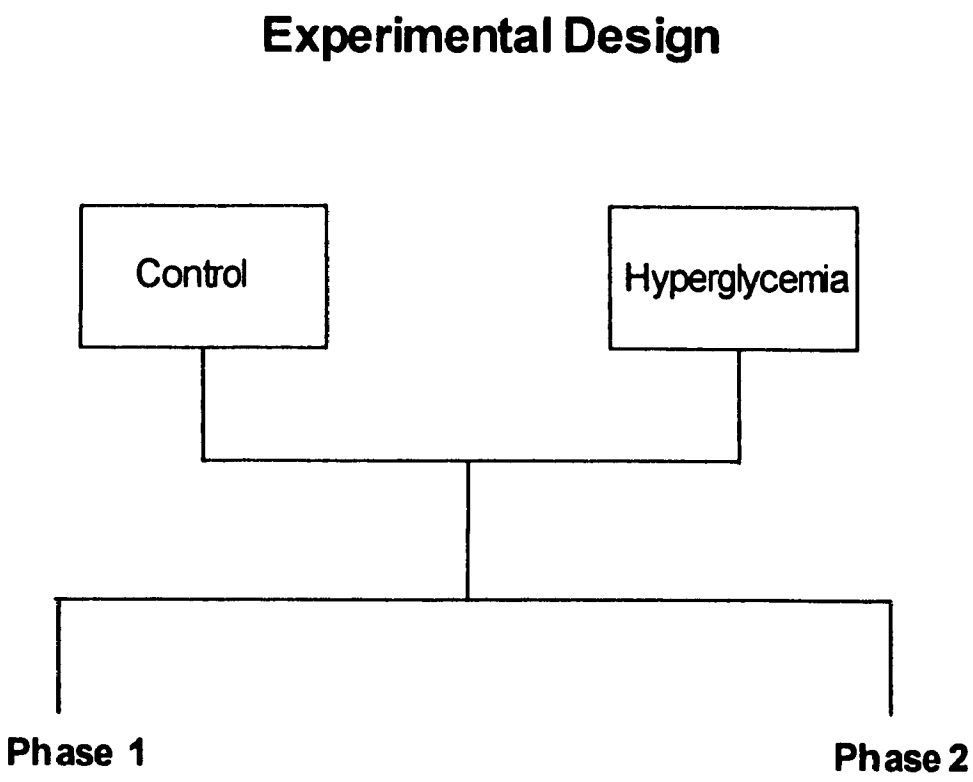

Figure 3: Experimental design. The entire experiment was divided into a control group, a hyperglycemic group, and other experimental groups which were subdivided into Phase 1 and Phase 2 of the experiment. These groups are detailed in Figure 4.

Experimental Design

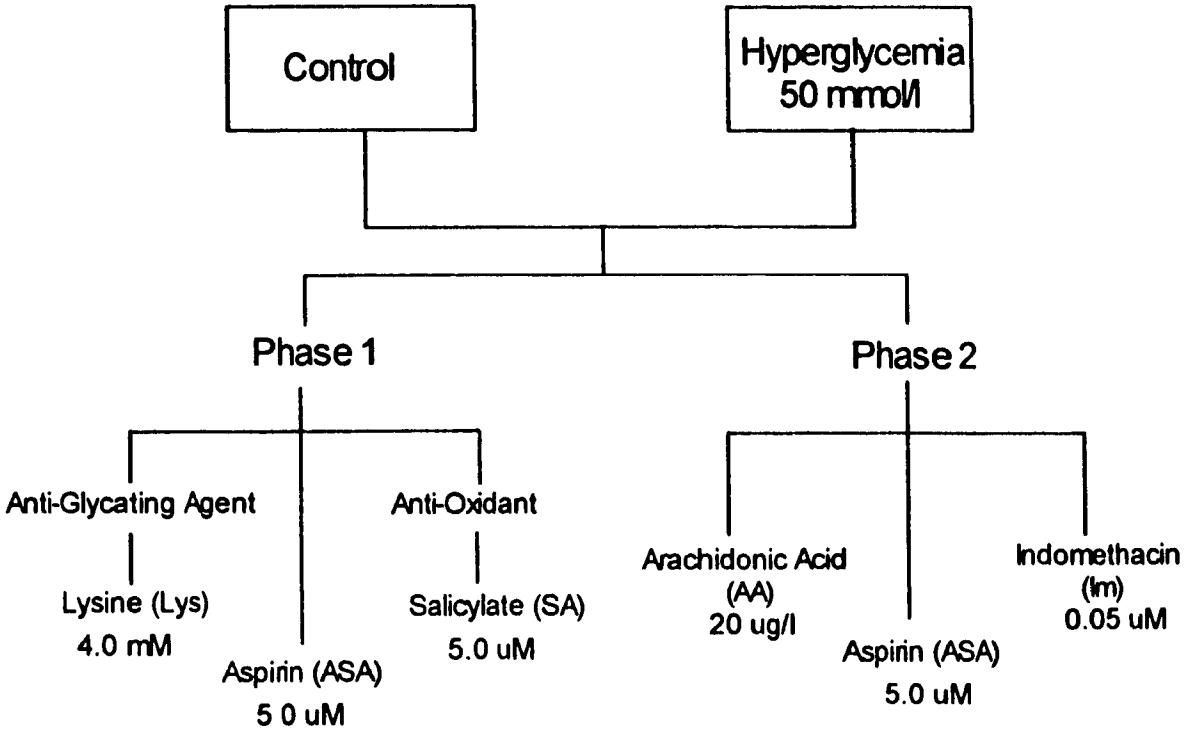

Figure 4: Details of the Experimental Design for Phase 1 and Phase 2. The groups were subdivided to meet the objectives described in Section 2: Hypotheses and Objectives. Letters in parentheses represent the abbreviations for the compounds. The amount of lysine, salicylate, and indomethacin used was determined by a dose-response curve prior to the actual experiment (Figures 10 To 12, Appendix B) The concentrations used in the experiment are presented below the test compounds. Lysine, aspirin, salicylate, arachidonic acid, and indomethacin were added as 3% of the final volume The control medium received 3% distilled water as the final volume of the culture medium.

All treatments were dissolved and added as a water based solution except for AA and indomethacin. These two compounds were first dissolved in ethanol and then diluted with distilled water. The amount of ethanol used as the final volume of the media was 0.3% for AA groups and 0.03% for indomethacin groups. These ethanol concentrations were added to the embyros (n=6/group) prior to the experiment. Results indicated no apparent effect on the growth and development of the embryos after 48 hours of culture.

3.3. Morphological Assessment

All embryos were examined under a dissecting microscope. Survival was indicated by the presence of a heartbeat and yolk sac circulation. Dead embryos were discounted from further analysis. The yolk sac diameter, crown rump length and head length of live embryos were measured using an eyepiece micrometer. Embryos were assessed following the method of Brown and Fabro (1981) (Appendix A), in which 13 morphological criteria are each given a numerical score ranging from 0 to 5. Each score corresponds to the stage of morphological development. The sum of the scores from each individual morphological feature gives a total morphological score. Somite counts were also performed on embryos as one of the criteria for the stage of development of the embryos. Where somite numbers could not be counted, the total sum of the scores was added and categorized as "Sum Less". Embryos which displayed at least one external malformation were considered malformed.

3.4. TOTAL PROTEIN ANALYSIS

Kirazov et al. (1993) have reported that upon storage (15 days) of the membrane-bound proteins at -20°C, there was a decrease in the amount of protein estimated by the Bradford protein assays. Therefore, in order to determine the shelf-life of the frozen embryo samples, the Bradford Microprotein Assay (Bradford, 1976) (Bio-Rad with 0.1 NaOH) was used to compare the protein values between samples stored at -20 and -80°C prior to the actual experiment. Fresh mouse liver was homogenized with a polytron® tissue homogenizer. The homogenate was then divided into aliquots with half the samples stored at -20°C and the other half stored at -80°C. Using the protein values (Beckman, DU-40 Spectrophotometer) from the fresh samples as the baseline, the samples stored at both temperatures were each measured at three day-intervals and compared to the fresh sample values. At day 21, samples stored at -20 and -80°C produced the same protein values as the fresh samples. Consequently, all embryo samples were stored at -80°C after morphological examination, and the protein values were obtained within 10 days after culture.

Individual frozen embryos were transferred to 12x75 mm test tubes and homogenized with a polytron® PT 3000 tissue homogenizer (Brinkman). Each embryo was homogenized with 1 ml 0.9% saline (0.009 g sodium chloride/L) distilled water. The 1.0 ml sample was stored in 1.5 µl eppendorf tubes, 200 µl of the solution was then removed for protein analysis, with the remaining sample analyzed for total furosine and hydroxyl free radical measurements. The total protein content of the embryos was measured using the Bradford Microprotein Assay (Bradford, 1976). Bovine serum albumin was used as the standard protein. Microassays were prepared by adding saline to the homogenized samples to a volume of 0.8 ml, and then mixing with

0.2 ml of Bio-Rad dye reagent concentrate. Absorbance was measured at 595 nm in 1 ml cuvettes (Beckman, DU-40 Spectrophotometer). The weight of the embryonic protein was plotted against the corresponding absorbance of a standard curve (Figure 13, Appendix B). The amount of embryonic protein was calculated and expressed as µg protein/embryo.

3.5. HYDROXYL FREE RADICAL ANALYSIS

The amount of hydroxyl free radicals was measured according to a modified procedure of Onodera and Ashraf (1991) in 8 embryos of each treatment group, in both phase 1 and 2 of the experiment (Figures 3 and Figure 4). The 800 µl embryo samples were treated with 50 µl of 2 N HCL and 20 µl of 100 µM 2,4-dihydroxybenzoic aicd (DHBA) as an internal standard. Samples were extracted with 2 ml of HPLC grade diethyl ether on a vortex mixer for 80 sec. The diethyl ether layer was pipetted into 1.0 ml conical tubes and allowed to dry on a dry bath at 52°C. This extraction was repeated six times. At the end of the last extraction, the few drops of the ether were evaporated to dryness by N_2. The residues were dissolved in 150 µl of methanol and 50 µl of 2 N HCL.

Twenty µl of sample was injected into a Beckman ODS C-15 column (4.6 x 15 cm, pore size 5 µm). The high performance liquid chromatographic (HPLC) system consisted of a solvent delivery module (Beckman, system Gold, module 110B), a programmable detector (Beckman, system Gold, module 166) and an analog interface (Beckman, system Gold, module 406). The pump, the detector and analog interface were computer controlled using Beckman Gold system software. The mobile phase was 80% 0.03 M citric acid - 0.03 M acetic acid buffer (pH 3.3) and 20% methanol.

Samples were injected at a flow rate of 0.8 ml/min, and a wavelength of 315 nm was used.

3.6. FUROSINE ANALYSIS

The remainder of all the frozen embryo samples, in all experimental groups, were analyzed for the total furosine content. A modified procedure of Gilman and Woodward (1990) was followed for the vapor phase microwave hydrolysis. The 800 µl protein samples from individual embryos were transferred into 200 µl tapered glass microvials (Hewlett Packard, Palo Alto, CA), and evaporated using Speed Vac (Savant Corporation, Farmingdale, NY). After samples were completely dried, the microvials were placed inside a Teflon ®PFA digestion vessel (CEM Corporation) together with 10.0 ml of 6 N HCL. The vessel cap was sealed using a Capping Station (CEM Corporation) and then connected to a surge trap and a manifold. The sealed Teflon ® PFA vessel (with up to 10 samples inside) was placed in a liquid nitrogen bath to freeze the 6 N HCL. This could prevent "bumping" and possible contamination of the sample tubes during degassing.

After the acid was frozen, the vessel was evacuated and purged five times with nitrogen gas. The vessel was then placed on the turntable of the microwave unit under positive nitrogen pressure (15 psig.). The pressure controller was set to begin controlling the microwave power when the pressure in the vessel reached 132 psig. Full microwave power (660 W) was initially applied to the vessel. When the pressure reached 132 psig., the magnetron on/off cycle was controlled to maintain a constant pressure in the system.

All embryo protein samples were hydrolyzed for 8 min. Longer hydrolysis times showed increased degradation of the furosine product. After hydrolysis, samples were placed in the Speed Vac (Savant Corporation, Farmingdale, NY) to remove any condensed acid. Samples were then dissolved in 200 µl EGTA (0.1 mg/ml) for HPLC analysis. Samples were filtered using microfilters (0.45µm, Nihon Millipore, Japan) before being injected into the HPLC. The HPLC system, for this part of the analysis, was the same as the system described above used to measure hydroxyl free radicals. Elution was performed using 5.6 mM phosphoric acid solution at a linear gradient with a flow rate of 1.0 ml/min for 20 min (Fluckiger and Gallop, 1984). The furosine peak was monitored at a wavelength of 280 nm. Each sample was injected three times and the average amount of glycated protein per individual embryo was expressed as pmol hexose per µg protein.

3.7. Lipid Peroxidation Concentrations

Lipid peroxide concentrations were also measured in phase 2 of the experiment, in addition to all of the analyses described above.

The spectrophotometric kit (Kamiya Biochemical Co., Thousand Oaks, CA) was used to measured the lipid peroxide concentrations in individual embryos. The principle of the assay is based on the equimolar conversion of a methylene blue derivative to methylene blue by a haemoglobin catalyzed reaction with lipid hydroperoxides. This assay has been validated by a standard iodometric assay for the determination of lipid peroxide concentrations (Oshishi et al., 1985; Kanazawa et al., 1989). Embryos were put individually into 16x100 mm test tubes. Two ml of a chloroform:methanol mixture (2:1 ratio) was added into the test tubes and vortexed.

Saline was added at 250 µl to the test tubes. The test tubes were centrifuged at 5 rpm x 1000 for 5 min. The methanol/saline layer was siphoned off into a vacuum flask whereas the chloroform layer was transferred into a 16x100 mm test tube. The choloroform was dried completely with N_2 while being incubated in a waterbath at 37° C. Fifteen µl of isopropanol was added into each test tube and vortexed, of which 10 µl was transferred into a microplate (0.35 ml well capacity, Titertek® Flow Laboratories, Inc. Mclean, Virginia) containing a reagent, and incubated at 30°C for 2 to 5 min. The second reagent was then added and incubated at the same temperature for 10 min. Absorbance was read by a spectrophotometer at 670 nm (Titertek Multiskan Plus MK II spectrophotometer) using cumene hydroperoxide as a reference standard.

Unfortunately, the lipid peroxide concentrations in individual embryos were found to be undetectable. Subsequently, embryos were pooled together in groups of four and seven. However, the lipid peroxide concentration level remained undetectable. Therefore, it was decided that the serum from the embryo culture should be analyzed using a serum lipid peroxide spectrophotometric kit with the method described below (Kamiya Biochemical Co., Thousand Oaks, CA).

The sample preparation protocol outlined follows the instructions of the kit manufacturer for serum samples. Ten µl of the serum samples were added to a reagent containing lipoprotein lipase and incubated at 30°C for 2 to 5 min. Methylene blue derivative was then added to the reaction mixture. Samples were incubated again at 30° C for 10 min, and the absorbance was read spectrophotometrically at 670 nm (Titertek Multiskan Plus MK II spectrophotometer). The serum lipid peroxide concentrations were measured both prior to the addition of any gas to the media at the beginning of the culture and at the end of the 48 hour culture.

3.8. STATISTICAL ANALYSES

The morphological criteria, total protein, furosine concentrations, and LPO concentrations of each group were compared by one-way analysis of variance (ANOVA) to determine the main effect of treatments on these parameters. When significances occurred, Bonferroni's multiple comparisons test was used to identify specific treament mean differences. The frequency of neural tube abnormalities, blebs, and specific types of malformations per groups were compared with chi-square test. Pearson chi-aquare was calculated to test the independence between rows and columns. Continuity adjusted chi-square was used to correct for the fact that chi-square statistics calculated with small numbers will overestimate the true chi-square statistic (Bruning and Kintz, 1977). Pearson's correlation was also done for many parameters and the p values are given in Tables 9 through 11. All analyses were performed using the SPSS for Windows (SPSS Institute, Chicago, Illinois; Version 5.0, 1992). Results from analyses are listed in figures and tables in Section 4 and Appendices B and C. A probability of $p < 0.05$ was accepted as the minimal level of significance for all analyses.

SECTION 4: RESULTS

4.1. MORPHOLOGICAL ASSESSMENT OF MOUSE EMBRYOS AFTER 48 HOUR CULTURE

4.1.1. Embryonic age

Embryonic age of the embryos after the 48 hour culture was computed and the data is presented in Table 1.

The apparent embryonic age was computed based on the total morphological scores of the embryos. Brown and Fabro (1981) had found that the embryonic age and the total morphological score were highly linear ($R^2 = 0.991$). Therefore, by using the following formula, the apparent embryonic age of the embryos could be computed.

Morphological Score = -202.28 + 20.932 Embryonic Age

Since the compution of embryonic age is based on the total morphological score, the data presented includes only those embryos with countable somite numbers. Embryos with tail malformation and/or ventrally convex tails were excluded. The mean

scores for embryonic age, except hyperglycemic group, are similar between all the treatment groups.

4.1.2. Embryonic growth

The effects of treatment on specific embryological growth parameters were compared, and the data is presented in Table 1.

After a 48 hour culture, all embryos cultured in the hyperglycemic groups, with the exception of hyperglycemic+lysine (hyper+lys) group, had significantly lower somite numbers than those in the control group. Embryos in all groups, except hyperglycemic+salicylate (hyper+SA) and hyperglycemic+indomethacin (hyper+Im), have significantly higher somite numbers than the hyperglycemic group. The highest somite numbers are found in embryos in the control group, as well as those with the addition of lysine and ASA. Hyper+lysine is the only hyperglycemic group with somite numbers not significantly different from the control and the control+ASA embryos (Table 1).

Hyperglycemic treatment resulted in the shortest yolk sac diameter (Table 1). The addition of indomethacin to a hyperglycemic medium, resulted in the production of embryos with similar yolk sac diameters as those in the hyperglycemic group. All other embryos (control or treatment) displayed a significantly greater crown rump length than those in the hyperglycemic group. Embryos cultured under hyperglycemic conditions had the smallest head diameter when compared to those in all other treatment groups except for those treated with indomethacin. The addition of indomethacin to the control

Table 1: Effects of Treatments on Specific Embryological Growth Parameters [1,2]

Treatment Groups	Embryonic Age	Somite Number	Yolk Sac Diameter (mm)	Crown Rump Length (mm)	Head Diameter (mm)
Control	11.6 ± 0 08	28.5 ± 0 32 [a]	(54) [3] 3 44 ± 0.06 [a,c]	2 79 ± 0 05 [a]	1.39 ± 0.03 [a]
Hyperglycemic	8.7 ± 0.04	20 8 ± 0.85 [b]	(54) 2.78 ± 0 06 [b]	2 05 ± 0 07 [b]	0.86 ± 0.04 [b]
Control + ASA	11.62 ± 0 09	29 3 ± 0.41 [a]	(25) 3.63 ± 0 09 [a,c]	2 93 ± 0 09 [a]	1.44 ± 0.07 [a]
Hyper + ASA	11.5 ± 0 10	24 6 ± 1.08 [c]	(25) 3.20 ± 0.06 [c,d]	2.66 ± 0 10 [a]	1.19 ± 0.06 [a,c,d]
Control + SA	11.17 ± 0 16	27.7 ± 0.4 [a,d,e]	(16) 3.52 ± 0 12 [a,c]	2.97 ± 0 09 [a]	1.43 ± 0.06 [a,c]
Hyper + SA	11.43 ± 0.11	23.3 ± 0.8 [b,c]	(16) 3.24 ± 0.15 [a,c,d]	2.65 ± 0.11 [a]	1.17 ± 0.07 [a,c,d]
Control + Lys	11.53 ± 0.06	29.5 ± 0.56 [a]	(16) 3.70 ± 0.10 [a,c]	2.92 ± 0.08 [a]	1 45 ± 0.08 [a]
Hyper + Lys	11 41 ± 0 07	26 1 ± 0.77 [a,d,e]	(16) 3 19 ± 0 10 [a,c,d]	2.57 ± 0 09 [a]	1 22 ± 0.11 [a,c,d]
Control + AA	11 38 ± 0.12	27 7 ± 2.2 [a,d]	(18) 3 44 ± 0.08 [a,c,d]	2 75 ± 0 10 [a]	1 41 ± 0.07 [a,c]
Hyper + AA	11.31 ± 0 16	24 4 ± 0 89 [c,d]	(18) 3.32 ± 0 13 [a,c,d]	2.60 ± 0 11 [a]	1 21 ± 0.00 [a,c,d]
Control + Im	11.57 ± 0.08	26.5 ± 0 93 [a]	(21) 3 23 ± 0 09 [a,c,d]	2 53 ± 0 11 [a]	1 09 ± 0.07 [b,c,d]
Hyper + Im	11.43 ± 0 07	23 9 ± 1.04 [b,c,e]	(21) 3 00 ± 0 10 [b,d]	2 47 ± 0 15 [a]	1.06 ± 0.08 [b,d]

Table 1 (continued)

[1] Growth parameters were measured according to Brown and Fabro (1981), Appendix A.

[2] Values are means ± SEM. Means not sharing a common superscript, within a column, are different at the $p < 0.05$ level by Bonferroni's multiple comparison test.

[3] Number in parentheses equals the total number of embryos.

group resulted in a significantly smaller head diameter than the control and control groups with treatments (i.e., control, control+ASA, and control+Lys).

Embryonic age was directly and positively correlated to the total morphological score ($r=1.000$, $p<0.001$) and somite numbers ($r=0.800$, $p<0.001$) (Table 11). The number of somites was found to be positively correlated with the size of the yolk sacs ($r=0.586$, $p<0.001$), the crown-rump lengths ($r=0.646$, $p<0.001$), and the head diameters ($r=0.611$, $p<0.001$) (Table 9).

4.1.3. Tail abnormality rate

The various types of tail abnormalities and the percentage of occurrence amount embryos of different groups are shown in Table 2.

Embryos having tails rotated to the left side after the 48 hour growth period were considered as having tail malrotation, and the frequencies were relatively low amoung all of the treatment groups. On the other hand, it was observed that 44% of the embryos cultured in the hyperglycemic groups had kinked tail. Embryos with unaccountable/unrecognizable somites were included in the "tail malformation" category. Those with ventrally convex tails (i.e., unturned tails) were included in the "ventrally convex" category. Hyperglycemic group had tail malformation significantly higher (31%) than all other treatment groups. In addition, 20% of the embryos in this group had ventrally convex tails. Kinked tail was found in all treatment groups but the relative frequencies of occurrence were found higher in the hyperglycemic groups. Hyperglycemic+AA (hyper+AA) and hyper+Im had higher frequencies of kinked tailed compared to other groups, and the percentages are 22% and 24%, respectively.

Table 2: Types and Percentages of Tail Abnormalities of Embryos After 48 Hour Culture [1,2]

Treatment Groups	Tail Malrotation[3] N	%	Kinked Tail N	%	Ventrally Convex N	%	Tail Malformation[5] N	%
Control	2(54)[4]	4	4(54)	7[a]	0(54)	0[a]	0(54)	0[a]
Hyperglycemic	4(54)	7	24(54)	44[b]	11(54)	20[b]	17(54)	31[b]
Control + ASA	1(25)	4	1(25)	4[a]	0(25)	0[a]	0(25)	0[a]
Hyper + ASA	1(25)	4	3(25)	12[a]	3(25)	12[b]	1(25)	4[a]
Control + SA	1(16)	6	2(16)	12[a]	0(16)	0[a]	0(16)	0[a]
Hyper + SA	0(16)	0	3(16)	19[a,b]	2(16)	12[a]	0(16)	0[a]
Control + Lys	0(16)	0	1(16)	6[a]	1(16)	6[a]	0(16)	0[a]
Hyper + Lys	0(16)	0	2(16)	12[a]	3(16)	19[b]	0(16)	0[a]
Control + AA	0(18)	0	1(18)	5[a]	0(18)	0[a]	0(18)	0[a]
Hyper + AA	1(18)	5	4(18)	22[a,b]	3(18)	17[b]	0(18)	0[a]
Control + Im	1(21)	5	2(21)	9[a]	2(21)	9[a]	0(21)	0[a]
Hyper + Im	0(21)	0	5(21)	24[a,b]	3(21)	14[b]	0(21)	0[a]

Table 2 (continued)

[1] See Tables 12-13 in Appendix C for specific Chi-square comparisons and p values
[2] Numbers not sharing a common superscript are different at the $p < 0.05$ level.
[3] Tail rotated to the left side.
[4] Number of embryos (total number of embryos).
[5] Somite numbers could not be counted due to malformation.

The hyperglycemic groups had higher frquencies of embryos having ventrally convex tails. No hyperglycemic groups had frequencies significantly different from the respective control groups in all categories of abnormal tail growth. Furthermore, kinked tail and ventrally convex tail were significantly correlated to the amount of furosine in the embryos, ($r=0.183$, $p=0.045$) and ($r=0.188$, $p=0.039$), respectively.

4.1.4. Enlarged pericardium

The frequencies of having an enlarged pericardium in the mouse embryos were calculated and the data is presented in Table 3.

Embryos in the hyperglycemic group exhibited a significantly higher frequency of enlarged pericardiums. The hyperglycemic group, with the addition of salicylate, is the only hyperglycemic group to exhibit a frequency significantly lower than that of the hyperglycemic group. The frequency of enlarged pericardiums was negatively related to the size of the yolk sacs ($r=-0.216$, $p<0.001$), the crown-rump lengths ($r=-0.271$, $p<0.001$), the head diameters ($r=-0.232$, $p<0.001$) (Table 9), number of somites ($r=-0.368$, $p<0.001$), total morphological scores ($r=-0.444$, $p<0.001$), and the total protein concentrations ($r=-0.343$, $p<0.001$). On the other hand, the higher the frequency of enlarged pericardiums, the higher the amount of furosine found. ($r=0.340$, $p<0.001$) (Table 10).

4.1.5. Embryonic blebs

Both chorion and pericardium blebs were grouped and counted as one category: embryonic blebs. The frequencies of embryonic blebs are calculated and presented in Table 3.

Blebs were commonly found in all hyperglycemic groups. Embryos cultured under hyperglycemic conditions exhibited a significantly higher frequency of embryonic blebs when compared to controls (except hyper+lys). The highest number of embryos having blebs were found in the hyperglycemic group. Within the same treatment, hyper+ASA and hyper+SA had a significantly higher frequency of blebs compare to their controls. The higher the frequency of blebs in a group of embryos, the less the growth of the embryos as indicated by the growth parameters. The frequency of having blebs was negatively correlated to the size of yolk sacs ($r=-0.158$, $p=0.006$), crown-rump lengths ($r=-0.162$, $p=0.005$), head diameters ($r=-0.2544$, $p<0.001$) (Table 9), somite numbers ($r=-0.357$, $p<0.001$), and the total morphological scores ($r=-0.385$, $p<0.001$). Similar to the enlarged pericardium, the frequency of blebs was positively correlated with the amount of furosine in the embryos ($r=0.273$, $p<0.003$) (Table 10).

4.1.6. Mortality rate

The number and percentage of embryos which died after the 48 hour culture are presented in Table 3.

Table 3: Effects of Treatment on the Frequency of Enlarged Pericardiums, Blebs, Mortality and Total Malformation Rate [1,2]

Treatment Groups	Enlarged Pericardiums	Blebs [3]	Mortality Rate N	Mortality Rate % [4]	Total Malformation Rate [5] N	Total Malformation Rate [5] %
Control	2 (54) [6 a]	3 (54) [a]	0	0 [a]	4(54)	7 [a]
Hyperglycemic	18 (54) [b]	27 (54) [b]	8	15 [b]	54(54)	100 [b]
Control+ASA	2 (25) [a]	0 (25) [a]	0	0 [a]	2(25)	8 [a,c]
Hyper+ASA	4 (25) [a,b]	8 (25) [b]	2	8 [a]	5(25)	20 [c]
Control+SA	0 (16) [a]	0 (16) [a]	0	0 [a]	1(16)	6 [a]
Hyper+SA	0 (16) [a]	6 (16) [b]	2	12 [a]	4(16)	25 [a]
Control+Lys	0 (16) [a]	0 (16) [a,b]	0	0 [a]	1(16)	6 [a]
Hyper+Lys	2 (16) [a,b]	3 (16) [a,b]	1	6 [a]	3(16)	19 [a]
Control+AA	0 (18) [a]	1 (18) [a]	0	0 [a]	1(18)	5 [a,d]
Hyper+AA	2 (18) [a,b]	7 (18) [b]	2	11 [a]	6(18)	33 [d]
Control+Im	1 (21) [a]	4 (21) [a]	1	5 [a]	3(21)	9 [a,b,c]
Hyper+Im	3 (21) [a,b]	9 (21) [b]	3	14 [b]	7(21)	33 [e]

Table 3 (continued)

[1] See Tables 14-16 in Appendix C for specific Chi-square comparisons and p values

[2] Numbers not sharing a common superscript are different at the $p < 0\ 05$ level.

[3] Includes chorion and pericardium blebs.

[4] Percent of embryos which died after the 48 hr culture.

[5] Total malformation includes all tail abnormalities, CNS malformations, and enlarged pericardium

[6] Number of embryos (total number of embryos).

All control embryos, except those in the control+indomethacin (control+Im) group, survived in the media throughout and after the culture period. The hyperglycemic and hyper+Im groups developed significantly higher rates of mortality (15% and 14%, respectively) compared to the control group.

4.1.7. Total Malformation

The frequency of total malformation includes all types of tail abnormalities, malformation in the central nervous system, and enlarged pericardium. The frequencies and percentages are presented in Table 3.

Embryos cultured under hyperglycemic condition exhibited a total malformation rate of 100%. It is significantly higher than the frequencies found in all other treatment groups. Both hyper+AA and hyper+Im have significantly higher malformation rate than the control group. Hyperglycemic+treatments did not differ with the respective control groups in the total malformation rate.

4.1.8. Frequency of malformation in the central nervous system

The frequency of having malformation in the central nervous system (CNS) was defined as having scored all of the four criteria (E to H, Appendix A) for neural and brain development below the predetermined cutoff points (Figure 5). The cutoff points were determined by using control embryos as the standard reference. After 48 hour culture, all control embryos have closed posterior (score 4) and anterior (score 3) neuropores. In addition, mesencephalic (score 2) and prosencephalic folds (score 2)

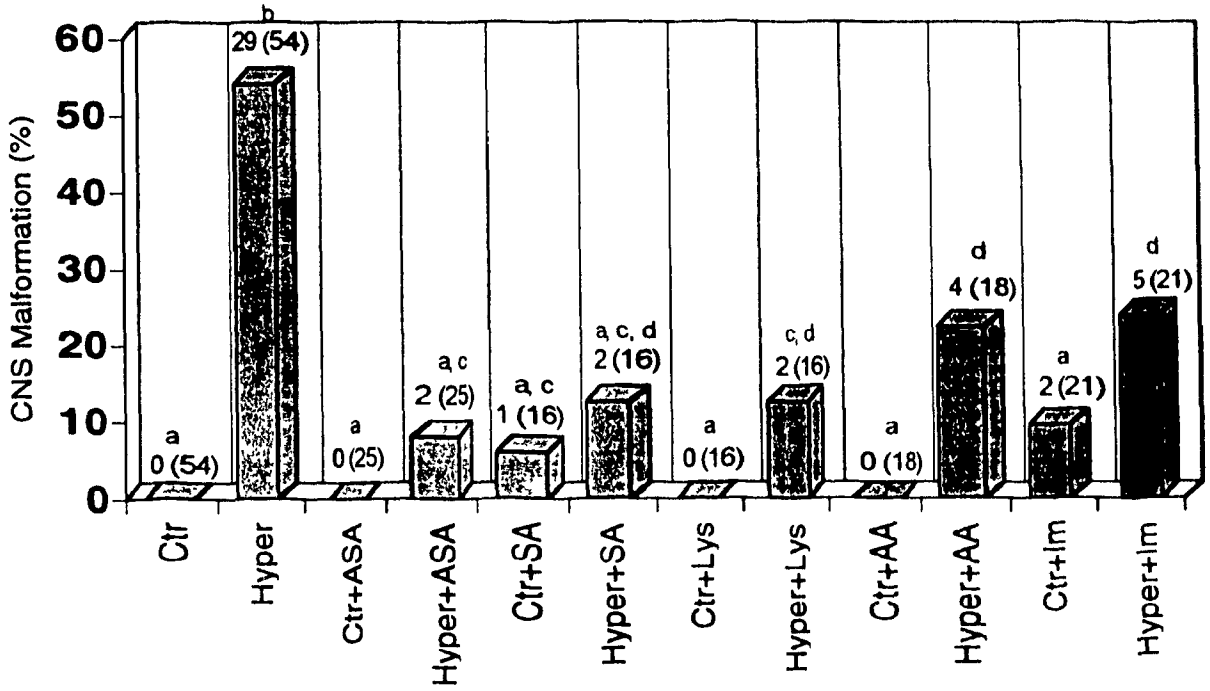

Figure 5: Frequency of Malformation in the Central Nervous Sytem. Malformation is defined by the observed scores in the following criteria: caudal neural tube ≤ 3; hindbrain ≤ 2; midbrain ≤ 1; and hindbrain ≤ 1 (Brown and Fabro, 1981; Appendix A). N (n) refers to the number of embryos with CNS malformation (total number of embryos). Groups not sharing a common letter are significantly different at the $p < 0.05$ level. See Table 17, Appendix C for Chi-square and p values.

were completely fused. Therefore, embryos having scores lower than these points for all of the four criteria were considered as having CNS malformations.

The embryos cultured in the hyperglycemic medium exhibited the highest frequency of CNS malformations, and are significantly higher than all the other treatment groups. The hyper+Im resulted in the second highest frequency of CNS malformations in comparison to all other treatment groups (23.8%), which however, was still significantly lower than the hyperglycemic group (Table 17, Appendix C). Furthermore, all the hyperglycemic groups exhibited some type of CNS malformations. Hyper+Lys, hyper+AA and hyper+Im all have significantly higher frequency of CNS malformations compared to the controls of the same treatment. It should also be noted, that the addition of ASA and salicylate to the hyperglycemic medium resulted in CNS malformations not significantly different from their respective control groups. Furthermore, the somite numbers ($r=-0.416$, $p<0.001$), total morphological scores ($r=-0.487$, $p<0.001$), and total protein ($r=-0.410$, $p<0.001$) have a negative correlation with the frequencies of CNS malformation, which on the other hand, increased with the increase in furosine concentration ($r=0.270$, $p<0.001$) (Table 10).

4.1.9. Morphological scores

The mean scores for all morphological parameters were compared and the data is shown in Tables 4 to 7. Total morphological score of the embryos, excluded embryos with somite numbers considered not countable (tail malformation and ventrally convex). The mean total morphological score for each group is presented in Figure 6.

Embryos in hyperglycemic group consistently received lower morphological scores for all parameters. Hyper+Im also scored the second lowest in all the parameters (Tables 4 to 7). The addition of ASA and salicylate to hyperglycemic groups have significantly improved the mean scores for caudal neural tube and hind brain. The mid brain and fore brain scores were significantly improved with the addition of ASA and salicylate, but the mean scores remain significantly lower than the control group (Table 5). The addition of lysine to hyperglycemic group had significantly improved the mean scores for hind, mid, and fore brain, but remain significantly lower than the control group. Although the addition of AA also significantly increased the mean scores for hind, mid, and fore brain, the mean score for mid brain remain significantly lower than all the control groups (except for control+AA) (Table 5).

Other morphological scores in Tables 6 and 7 also show that hyperglycemic group received the lowest scores for all the parameters. Although hyper+Im had significantly higher mean scores (except otic and optic system) than hyperglycemic group, the mean scores were significantly lower compared to the control group. Furthermore, the addition of all other treatments had significantly improved the mean scores of all the morphological parameters (Tables 6 and 7).

The total morphological scores (Figure 6) showed that all embryos which were cultured under hyperglycemic conditions exhibited a mean score significantly lower than the control embryos. In addition, the embryos receiving the hyperglycemic treatment had the lowest mean morphological scores, which were significantly lower than the mean scores in the other treatment groups. The multiple comparison test also showed that hyper+SA was the only hyperglycemic group which did not differ in total morphological score when compared to the respective control group. The total

Table 4: Mean Scores for the Morphological Assessment [1,2]

Treatment Groups	Yolk Sac Circulatory System	Allantois	Flexion	Heart
Control	3.24 ± 0.06[a]	2.42 ± 0.07[a,c,e]	2.93 ± 0.04[a]	2.50 ± 0.07[a,c]
Hyperglycemia	1.59 ± 0.10[b]	1.28 ± 0.07[b]	0.80 ± 0 10[b]	1.17 ± 0.09[b]
Control + ASA	3.32 ± 0.12[a]	2.52 ± 0.10[a,c]	2.86 ± 0.07[a,c,e]	2.52 ± 0.10[a,c]
Hyper + ASA	2 68 ± 0 14[c]	2 00 ± 0.11[c,d]	2 32 ± 0 14[a,c,d]	2.14 ± 0.13[a,c,d]
Control + SA	3.44 ± 0 20[a]	2.69 ± 0 11[a]	2.87 ± 0.08[a,c,e]	2.75 ± 0.14[a]
Hyper + SA	2 62 ± 0.20[a,c]	1.94 ± 0 14[c,d]	2.37 ± 0.13[a,c,d]	2.31± 0.12[a,c,d]
Control + Lys	3.44 ± 0.12[a]	2.37 ± 0.12[a,c,d]	2.94 ± 0.06[a,e]	2 69 ± 0.12[a,c]
Hyper + Lys	2.56 ± 0 20[c]	1.87 ± 0.15[d]	1.84 ± 0.24[c,d]	1.94 ± 0.11[c,d]
Control + AA	3 05 ± 0 10[a,c]	2.33 ± 0.14[a,d]	2.78 ± 0.10[a,c,d]	2.44 ± 0.12[a,c,d]
Hyper + AA	2 37 ± 0 17[c]	1 95 ± 0.14[d,e]	2.10 ± 0.18[d]	1.84 ± 0 08[b,d]
Control + Im	3 00 ± 0 17[a,c]	2.09 ± 0.12[a,d]	2 33 ± 0.19[d,e]	2.09 ± 0.17[a,d]
Hyper + Im	2.48 ± 0.19[c]	1.86 ± 0.14[d]	1.76 ± 0.25[d]	1.62 ± 0.17[b,d]

[1] Values are means ± SEM.

[2] Means not sharing a common superscript, within a column, are different at the $p < 0.05$ level by Bonferroni's multiple comparison test.

Table 5: Mean Scores for the Morphological Assessment [1,2]

Treatment Groups	Caudal Neural Tube	Hind Brain	Mid Brain	Fore Brain
Control	4.00 ± 0.00 [a]	3 05 ± 0 03 [a]	3.19 ± 0 06 [a]	2.91 ± 0.07 [a]
Hyperglycemia	1.50 ± 0 17 [b]	1.14 ± 0.11 [b]	1 35 ± 0.12 [b,c]	1 21 ± 0.09 [b]
Control + ASA	3 96 ± 0.04 [a]	3.00 ± 1.10 [a]	3.20 ± 0.14 [a]	2.96 ± 0 13 [a]
Hyper + ASA	3 16 ± 0.18 [a,c]	2 48 ± 0.14 [a]	2 72 ± 0 13 [c,d]	2.20 ± 0.11 [b,c,d]
Control + SA	3.37 ± 0 27 [a]	2.69 ± 0 18 [a]	3.03 ± 0 24 [a,c]	2.81 ± 0 16 [a,c]
Hyper + SA	3.00 ± 0 24 [a,c]	2 25 ± 0 17 [a]	2 31 ± 0.12 [b,c,d]	2.12 ± 0 12 [c,d]
Control + Lys	3 75 ± 0.14 [a]	3 00 ± 0.09 [a]	3 00 ± 0 09 [a,c]	2 75 ± 0 14 [a,c]
Hyper + Lys	3.12 ± 0.24 [a,c]	2.31 ± 0 19 [a]	2.25 ± 0 17 [d,e]	1 87 ± 0.19 [d]
Control + AA	3 94 ± 0.05 [a,c]	3.00 ± 0.00 [a]	3 17 ± 0.09 [a,c]	2 89 ± 0.16 [a,c]
Hyper + AA	3.42 ± 0.19 [a,c]	2 74 ± 0 10 [a]	2.42 ± 0.18 [d,e]	1 92 ± 0.14 [d]
Control + Im	3 52 ± 0 21 [a,c]	2 67 ± 0 16 [a]	2 71 ± 0 24 [c,d,e]	2.26 ± 0.20 [d]
Hyper + Im	2.81 ± 0 31 [b,c]	2.09 ± 0.24 [b]	2.33 ± 0 28 [e]	1 36 ± 0.15 [b,d]

[1] Values are means ± SEM.

[2] Means not sharing a common superscript, within a column, are different at the $p < 0.05$ level by Bonferroni's multiple comparison test

Table 6: Mean Scores for the Morphological Assessment [1,2]

Treatment Groups	Otic System	Optic System	Olfactory System	Branchial Bars
Control	3 22 ± 0.07 [a]	3 07 ± 0.06 [a]	2.68 ± 0.06 [a,c]	2.94 ± 0.05 [a]
Hyperglycemia	1.05 ± 0 12 [b]	1.09 ± 1.10 [b]	1.13 ± 0.10 [b]	1.44 ± 0.11 [b]
Control + ASA	3 32 ± 0 12 [a]	3.08 ± 0 11 [a]	2.60 ± 0.12 [a,c]	2.96 ± 0.09 [a]
Hyper + ASA	2 56 ± 0 16 [a,c]	2 40 ± 0 18 [a]	2 16 ± 0.15 [a,c,d]	2.52 ± 0.14 [a,c]
Control + SA	3 25 ± 0 14 [a,c]	3 19 ± 0.18 [a]	2.78 ± 0.12 [a]	2 87 ± 0.08 [a]
Hyper + SA	2 75 ± 0 17 [a,c]	2 62 ± 0.20 [a]	2 19 ± 0.14 [a,c,d]	2.72 ± 0.13 [a,c]
Control + Lys	3 34 ± 0 15 [a,c]	3 19 ± 0 19 [a]	2.50 ± 0.13 [a,c,e]	3.00 ± 0.14 [a]
Hyper + Lys	2 34 ± 0 26 [c,d]	2 12 ± 0.18 [c]	1.81 ± 0.10 [c,d]	2.44 ± 0.18 [a,c]
Control + AA	3 17 ± 0.17 [a,c,d]	3.00 ± 0 14 [a]	2 61 ± 0 12 [a,c,e]	2.89 ± 0.07 [a]
Hyper + AA	2 37 ± 0.19 [c,d]	2 21 ± 0.16 [c]	1 87 ± 0 12 [d]	2.47 ± 0.16 [a,c]
Control + Im	2 67 ± 0 20 [a,c,d]	2.43 ± 0 21 [a]	2.24 ± 0 19 [a,c,d]	2.38 ± 0.18 [a,c]
Hyper + Im	1 48 ± 0 29 [b,d]	1 71 ± 0 27 [b,c]	1 90 ± 0.24 [d,e]	2.09 ± 0 26 [c]

[1] Values are means ± SEM

[2] Means not sharing a common superscript, within a column, are different at the $p < 0.05$ level by Bonferroni's multiple comparison test

Table 4: Mean Scores for the Morphological Assessment [1,2]

Treatment Groups	Yolk Sac Circulatory System	Allantois	Flexion	Heart
Control	3 24 ± 0.06 [a]	2.42 ± 0.07 [a,c,e]	2.93 ± 0.04 [a]	2 50 ± 0.07 [a,c]
Hyperglycemia	1.59 ± 0.10 [b]	1.28 ± 0.07 [b]	0.80 ± 0 10 [b]	1.17 ± 0.09 [b]
Control + ASA	3.32 ± 0.12 [a]	2.52 ± 0.10 [a,c]	2.86 ± 0 07 [a,c,e]	2.52 ± 0.10 [a,c]
Hyper + ASA	2.68 ± 0.14 [c]	2 00 ± 0.11 [c,d]	2.32 ± 0.14 [a,c,d]	2.14 ± 0 13 [a,c,d]
Control + SA	3.44 ± 0.20 [a]	2.69 ± 0.11 [a]	2 87 ± 0.08 [a,c,e]	2.75 ± 0.14 [a]
Hyper + SA	2 62 ± 0 20 [a,c]	1.94 ± 0 14 [c,d]	2.37 ± 0 13 [a,c,d]	2 31± 0 12 [a,c,d]
Control + Lys	3.44 ± 0 12 [a]	2 37 ± 0.12 [a,c,d]	2.94 ± 0.06 [a,e]	2 69 ± 0.12 [a,c]
Hyper + Lys	2.56 ± 0 20 [c]	1.87 ± 0.15 [d]	1.84 ± 0.24 [c,d]	1.94 ± 0.11 [c,d]
Control + AA	3.05 ± 0.10 [a,c]	2.33 ± 0.14 [a,d]	2.78 ± 0.10 [a,c,d]	2 44 ± 0.12 [a,c,d]
Hyper + AA	2.37 ± 0.17 [c]	1.95 ± 0.14 [d,e]	2 10 ± 0 18 [d]	1.84 ± 0.08 [b,d]
Control + Im	3 00 ± 0.17 [a,c]	2.09 ± 0.12 [a,d]	2.33 ± 0.19 [d,e]	2.09 ± 0.17 [a,d]
Hyper + Im	2 48 ± 0.19 [c]	1 86 ± 0.14 [d]	1.76 ± 0 25 [d]	1 62 ± 0.17 [b,d]

[1] Values are means ± SEM.

[2] Means not sharing a common superscript, within a column, are different at the $p < 0.05$ level by Bonferroni's multiple comparison test.

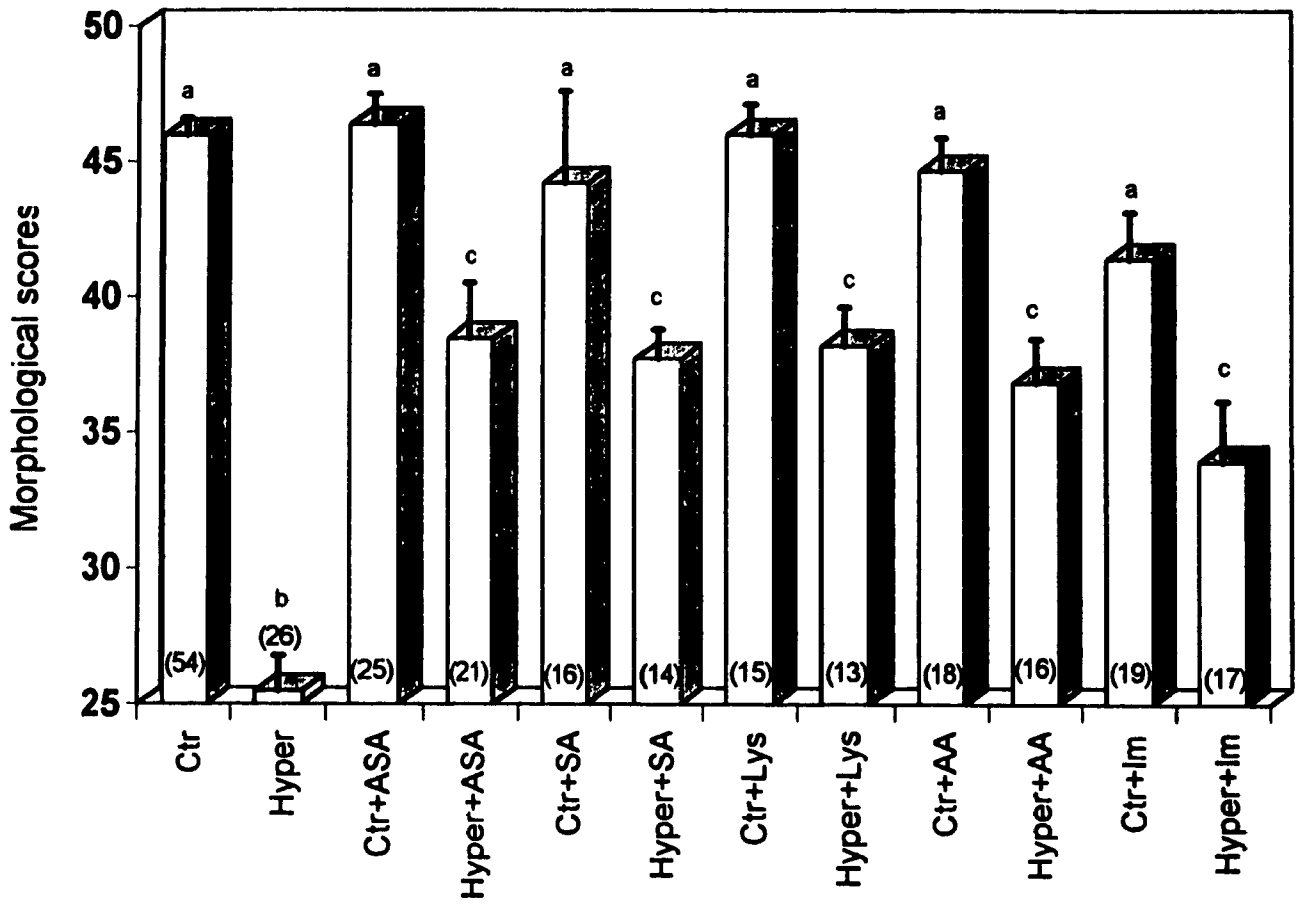

Figure 6: Mean Morphological Score. DC-1 mouse embryos were cultured for 48 hours as described in Section 3: Materials and Methods. Embryos were assessed following the Brown and Fabro (1981) morphological scoring system (Appendix A). Embryos with "Sum Less" scores are excluded (N) refers to the total number of embryos Means not sharing the same letter are significantly different at the $p < 0.05$ level as determined by the Bonferroni multiple comparison test See table 18, Appendix C for ANOVA and p values.

morphological score was positively correlated with the size of yolk sacs ($r=0.605$, $p<0.001$), the crown-rump lengths ($r=0.637$, $p<0.001$), head diameters ($r=0.590$, $p<0.001$) (Table 9), number of somites ($r=0.7986$, $p<0.001$) and the amount of protein ($r=0.2430$, $p<0.001$) (Table 11). On the other hand, it displayed a negative relationship with abnormal CNS ($r=-0.487$, $p<0.001$), blebs ($r=-0.385$, $p<0.001$), enlarged pericardiums ($r=-0.444$, $p<0.001$) (Table 10), and furosine content ($r=-0.341$, $p<0.001$) (Table 11).

4.2. Hydroxyl Free Radical Analysis

The attempt to measure embryonic free radical production, unfortunately, has been unsuccessful. Embryos were treated and the level of hydroxyl free radicals was measured following the methods described in Section 3: Materials and Methods. However, the HPLC chromatogram showed no detection of hydroxyl free radicals. Furthermore, the hydroxyl free radicals were not detected even after embryos were pooled in groups of two, four, and eight. As a final attempt, samples were pooled and concentrated to ten µl prior to injection into the HPLC. Unfortunately, the hydroxyl free radical was again undetectable.

4.3. Embryonic Protein and Glycated Protein

4.3.1. Embryonic protein

Embryonic protein is presented as the mean protein concentration per treatment group. Protein concentrations are expressed as µg protein per embryo (Table 8).

The mean protein concentrations were similar in almost all of the treatment groups. The embryos in the hyperglycemic medium with and without indomethacin had the lowest protein concentrations, and are significantly lower than the control embryos. Furthermore, embryos cultured in hyper+Im medium had mean protein concentrations even lower than those in the hyperglycemic medium alone. The embryonic protein was found to increase with the size of yolk sacs ($r=0.205$, $p<0.001$), crown-rump lengths ($r=0.250$, $p<0.001$), head diameters ($r=0.207$, $p<0.001$) (Table 9), somite numbers ($r=0.2263$, $p<0.001$), and total morphological scores ($r=0.2430$, $p<0.001$).

4.3.2. Glycated protein

The mean glycated protein concentration for each treatment group was calculated and expressed as furosine in pmol hexose/μg protein (Table 8).

The furosine content was found to be the highest in embryos cultured in the hyperglycemic group. The embryos cultured under hyperglycemic conditions with salicylate, AA and indomethacin have furosine contents very close to those of the hyperglycemic group. On the other hand, hyperglycemia with the addition of ASA had a furosine content significantly lower than that of the hyperglycemic group. It is also the only hyperglycemic group with a furosine content lower than the control and treatment groups (Figure 14, Appendix B). Hyper+ASA and hyper+lys were the two hyperglycemic groups with furosine not significantly different from the control groups of the same treatment. Control+lys has also resulted in the lowest amount of furosine produced in all the treatment groups. The amount of furosine found in the embryos was

Table 8: The Effects of Treatment on the Embryonic Growth as Measured by Protein and Glycated Protein [1,2]

Treatment Groups	Protein (μg/embryo)	Furosine (ρmol hexose/μg protein)
Control	(51)[3] 293 29 ± 12 41 [a]	121.88 ± 24.51 [b]
Hyperglycemic	(50) 211.62 ± 12.78 [b]	304 69 ± 85.51 [a]
Control + ASA	(25) 281 65 ± 15.33 [a]	92.70 ± 23.19 [b]
Hyper + ASA	(25) 267.77 ± 17 39 [a]	69.72 ± 19.02 [b,c]
Control + SA	(16) 276.97 ± 33.74 [a]	96.66 ± 18.07 [b]
Hyper + SA	(16) 259.74 ± 24.15 [a]	272.74 ± 65.34 [a]
Control + Lys	(16) 280.69 ± 10.97 [a]	48.04 ± 13.04 [c]
Hyper + Lys	(16) 260.68 ± 25.07 [a]	97.75 ± 23.31 [b]
Control + AA	(15) 283.62 ± 19 14 [a]	73.86 ± 14 43 [b,c]
Hyper + AA	(15) 266 31 ± 12 34 [a]	260.28 ± 74.56 [a]
Control + Im	(21) 255.12 ± 23.02 [a]	91 03 ± 11.97 [b]
Hyper + Im	(21) 208 69 ± 13.31 [b]	247.99 ± 34 60 [a]

[1] Values are means ± SEM Means not sharing a common superscript, within a column, are different at the $p < 0.05$ level by Bonferroni's multiple comparison test.

[2] See Table 18 in Appendix C for ANOVA F and p values

[3] Number in parentheses equals the total number of embryos.

higher with increased frequency of CNS malformation ($r=0.270$, $p<0.001$), blebs ($r=0.273$, $p=0.003$) and enlarged pericardiums ($r=0.340$, $p<0.001$) (Table 10).

4.4. SERUM MEDIA LIPID OXIDATION PRODUCTS (LPO)

4.4.1. Lipid peroxide concentrations in serum media

Serum oxidation products (LPO) were analyzed to examine the lipid oxidation. LPO was measured in phase 2 of the experiment, before (Figure 15) and after the 48 hour culture. The results are presented in Figure 7 and Table 19 of Appendix C.

Serum with the addition of AA exhibited a high LPO concentration, with the hyperglycemic group being the highest. In fact, these two groups had significantly higher LPO concentrations when compared to all other treatment groups. Control+Im produced the lowest level of LPO during the 48 hour culture, which was significantly lower than control and hyperglycemic group. All the hyperglycemic groups have produced relatively higher (but not significant) LPO concentrations than the respective control groups. LPO was found to be negatively correlated to size of yolk sacs ($r=-0.273$, $p=0.002$), crown-rump lengths ($r=-0.332$, $p<0.001$), head diameters ($r=-0.301$, $p=0.001$) (Table 9), and total protein ($r=-0.180$, $p=0.045$) (Table 11).

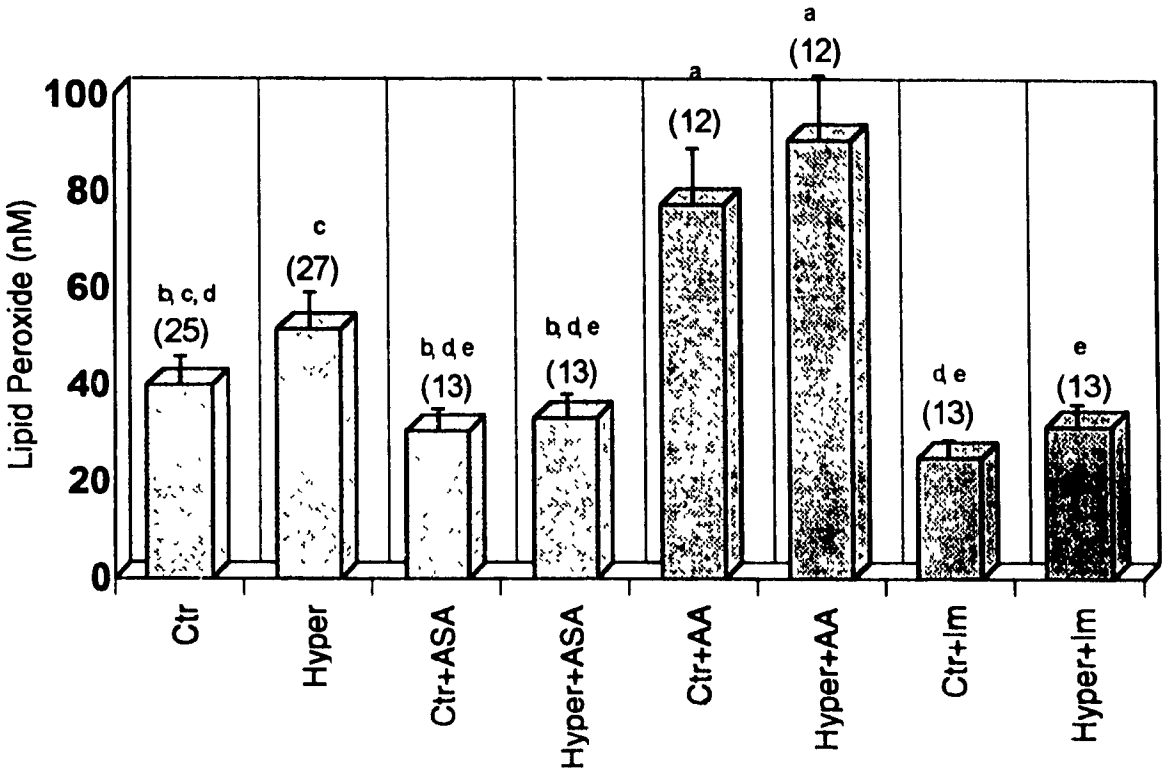

Figure 7: Lipid Peroxide Concentrations of Serum Media After 48 Hour Culture. Lipid peroxide concentrations were measured with the spectrophotometric kit as described in Section 3: Materials and Methods. Serum lipid peroxide concentrations were pooled and the values are expressed as nMoles per (n) of embryos. (N) refers to the total number of embryos per group. Means not sharing a common letter are significantly different at the $p < 0.05$ level as determined by Bonferroni's multiple comparison test.See Table 19, Appendix C for ANOVA F and p values.

Table 9: Correlation Results for Growth Parameters [1]

	Variables					
	Yolk Sac		Crown-rump Length		Head Diameter	
	Corr. coef.	$p > F$	Corr. coef.	$p > F$	Corr. coef.	$p > F$
Somite #	0.586	<0.001	0.646	<0.001	0.611	<0.001
Embryonic Age	0.605	<0.001	0.638	<0.001	0.590	<0.001
Sum Score	0.605	<0.001	0.637	<0.001	0.590	<0.001
Total Protein	0.205	<0.001	0.250	<0.001	0.207	<0.001
Furosine	-0.081	0.189	-0.215	0.009	-0.194	0.017
LPO	-0.273	0.002	-0.332	<0.001	-0.301	0.001
Blebs	-0.158	0.006	-0.162	0.005	-0.2544	<0.001
Enlarged Pericardium	-0.216	<0.001	-0.271	<0.001	-0.232	<0.001

[1] Analyses were performed utilizing the Pearson Correlation in SPSS for Windows, version 5.0.

Table 10: Correlation Results for Growth Parameters [1]

	Variables					
	Abnormal CNS [2]		Blebs		Enlarged Pericardium	
	Corr. coef.	$p > F$	Corr. coef.	$p > F$	Corr. coef.	$p > F$
Somite #	-0.416	<0.001	-.357	<0.001	-0.368	<0.001
Sum Score	-0.487	<0.001	-0.385	<0.001	-0.444	<0.001
Total Protein	-0.410	<0.001	-0.060	0.307	-0.343	<0.001
Furosine	0.270	0.001	0.273	0.003	0.340	<0.001
Total Malformation	0.059	0.312	0.105	0.700	0.003	0.963

[1] Analyses were performeded utilizing the Pearson Correlation in SPSS for Windows, version 5.0.

[2] Abnormal CNS includes morphological scores for the following criteria (Brown and Fabro, 1981; Appendix): caudal neural tube ≤ 3
hind brain ≤ 2
mid brain ≤ 1
fore brain ≤ 1

Table 11: Correlation Results for Growth Parameters [1]

	Variables							
	Total Morphological Score		Total Protein		Embryonic Age		Furosine	
	Corr. coef.	$p > F$	Corr. coef.	$p > F$	Corr. coef.	$p > F$	Corr. coef.	$p > F$
Somite #	0.799	<0.001	0.226	<0.001	0.800	<0.001	-0.319	0.001
Morphological Score			0.243	<0.001	1.000	<0.001	-0.341	<0.001
Total Protein	0.243	<0.001	0.637	<0.001			-0.162	0.770
LPO	-0.132	0.1780	-0.180	0.045	-0.132	0.178	0.226	0.094
Caudal Neural Tube	0.606	<0.001	0.271	<0.001			-0.275	0.002
Hind Brain	0.656	<0.001	0.338	<0.001			-0.323	<0.001
Mid Brain	0.747	<0.001	0.271	<0.001			-0.301	0.001
Fore Brain	0.782	<0.001	0.301	<0.001			-0.270	0.003

[1] Analyses were performed utilizing the Pearson Correlation in SPSS for Windows, version 5.0.

SECTION 5: DISCUSSION

5.1. PHASE 1: TERATOGENICITY

The morphological growth and development of the embryos in the present study clearly demonstrates the teratogenic effects of hyperglycemia on early stage (0-2 somites) embryos. The morphological assessment of all 17 criteria (Brown and Fabro, 1981; Appendix A) indicates that hyperglycemia had an inhibitory effect on the growth of the embryos. Hyperglycemia also caused a reduction in the rates of growth and differentiation, as evidenced by reduced protein contents, somite numbers, yolk sac sizes, crown-rump lengths, head diameters and the overall morphological scores. These results are in agreement with previous studies which looked at the teratogenic effects of hyperglycemia in rodent embryos *in vitro* (Sadler, 1980; Cockroft, 1984; Pinter et al., 1986; and Styrud and Eriksson, 1992). Hyperglycemia produced abnormalities that were both mild (e.g., kinked tail), or severe enough to be fatal (e.g., CNS malformation). The mortality rate was also found to be high (15% of the embryos) in the hyperglycemic group. The significantly high incidence of neural-tube defects in this group also confirmed previous studies which showed the inhibitory effect of hyperglycemia on neural-tube closure (Sadler, 1980; Goldman et al., 1985; Sadler et al., 1989; Kubow et al., 1993; and Styrud and Eriksson, 1992). The frequency of malformations in the CNS was over 50% for the embryos cultured in the

hyperglycemic media. This is in agreement with the frequencies observed by Sadler (1980), Garnham et al. (1983) and Goldman et al. (1985). Thus, the present experiment also validates that CD-1 mouse embryos represent a good experimental model for hyperglycemia-induced teratogenicity in early stage embryos of 0-2 somites, *in vitro*.

5.1.1. Glycation and Teratogenicity

The formation of glycated protein in the embryos was confirmed by the presence of furosine which was detected by the HPLC analysis. Hyperglycemia resulted in the production of higher amounts of glycated protein in the cultured embryos. Malformations seen in the embryos under hyperglycemic conditions could be due to the increased production of glycated proteins in the embryos since glycation increases proportionally with the levels of glucose (McFarland et al., 1979). Increased glycation of proteins might induce tissue derangement by altering the structure and function of the proteins (Sensi et al., 1991), and thereby induced malformations in the embryos. This is supported by the results obtained from the morphological assessments. Glycated protein had a direct positive correlation with the frequency of CNS malformations, blebs, and enlarged pericardiums. On the other hand, the higher the furosine level, the less the growth and development of an embryo (e.g., somite number and total morphological scores). Therefore, a high amount of glycated protein could be one of the mechanisms involved in the hyperglycemia-induced malformations observed in these early somite stage embryos.

The addition of ASA, salicylate, and lysine reduced the teratogenic effects of hyperglycemia. The reversal of inhibition of neural tube fusion was also observed along

with the significant reduction of CNS malformations. Although the reduction of the glycated embryonic protein suggests that the primary effect of hyperglycemia can be mediated via glycation of embryonic protein, the lack of effect of salicylate on furosine concentrations indicated that other mechanisms may also be involved. The mechanism(s) by which the compounds protect against hyperglycemia-induced malformations is/are possibly due to the anti-glycating effects of ASA and lysine.

5.1.2. Effects of ASA on Teratogenicity

The protective effect of ASA in hyperglycemia-induced malformations was also seen in a previous study (Kubow et al., 1993). This protective effect can be attributed to a direct ASA-protein interaction as a result of the acetylation of protein by ASA. The inhibitory action of ASA on glucose-mediated glycation, via the acetylation of protein, was observed (Yaylayan, 1993) when ASA was incubated with albumin. ASA acetylates proteins and forms acetylated protein and salicylic acid (Rainsford, 1984). Thus the protective effect of ASA on hyperglycemia-induced malformations in early somite embryos may also be due to the acetylation of protein by ASA, thereby preventing the critical biological event(s) which lead to the formation of malformations under hyperglycemic conditions.

Another mechanism by which ASA can prevent the hyperglycemia-induced malformations may involve the free radical scavenging action of the salicylate metabolite of ASA (Onodera and Ashraf, 1991 and Udassin et al., 1991). In the process of glycation, reducing sugars such as glucose can produce hydrogen peroxide and hydroxyl radicals via autoxidation (Wolff and Dean, 1987). The reduction of hyperglycemia-induced defects was reported to be associated with the addition of free

radical scavenging enzymes to rat embryo cultures (Eriksson and Borg, 1991). Part of the objectives of this experiment were to measure the amount of hydroxyl free radicals produced by embryos under hyperglycemic conditions, and to correlate the formation of free radicals with malformations in the embryos. Unfortunately, the detection of hydroxyl free radicals in the embryos was unsuccessful. This can be due to several reasons: 1) the level of production of hydroxyl radicals could be too low for detection, due to the small size of the embryos; 2) the production of radicals by glucose in the presence of protein has a steady-state levels in the submicromolar range (Jiang et al., 1990), thus it could be too low for detection using the current method; and 3) the method used in the experiment was initially developed for the measurement of hydroxyl radicals in the post-ischemic reperfused heart using 1 mM salicylic acid (Onodera and Ashraf, 1991), and thus, the relatively low amount of salicylate used (5 μM) was not effective in trapping the highly reactive free radicals.

Higher doses of ASA (e.g., 1.0 mM) were shown to have teratogenic effects in various rodent species, including mice (Larsson and Eriksson, 1966). ASA was rapidly cleared from the body as salicylate and its conjugates following both intravenous and intraperitoneal injection (Flower et al., 1985). In the present study, the addition of ASA at a relatively low dose (5.0 mM) to the control embryos exhibited no teratogenic effects. However, it is not clear if ASA has similar potential protective effect *in vivo*.

5.1.3. Effects of Lysine on Teratogenicity

In addition to ASA, D-lysine has also demonstrated a role as an anti-glycating agent in the present study. Similar to ASA, the addition of lysine has reversed the teratogenic effects of hyperglycemia, and reduced the formation of glycated protein.

The amino acid D-lysine, appears to inhibit protein glycation. D-lysine is not incorporated into mammalian protein, but its chemical characteristics are the same as its physiological isomer (Sensi et al., 1989). Therefore, the protective role of D-lysine is not likely to be due to improved growth as a result of increased essential amino acid availability. Lysine contains a highly reactive ε-amino group, making it a candidate to form an unavailable lysine-sugar complex. During the glycation process, protein D-lysine competes with protein L-lysine for the same glucose molecule. This hypothesis was demonstrated by Sensi et al. (1989 and 1990) who observed that D-lysine was effective in limiting the *in vitro* formation of AGE. The present study also supports this hypothesis by showing that D-lysine has a protective effective in relation to hyperglycemia-induced malformations. The addition of lysine improved the growth and development of the embryos under hyperglycemic conditions, i.e., increased morphological scores, protein contents, somite numbers and embryonic sizes. The use of D-lysine as an anti-glycating agent in reducing hyperglycemia-induced malformations can be advantageous since, unlike ASA, it would not interfere with either the protein structure, or the function of the protein. However, additional work *in vivo* is needed to determine any possible side-effects and to define physiological parameters such as, intestinal absorption, circulatory retention, and catabolism.

5.1.4. Effects of Salicylate on Teratogenicity

In the present study, the inhibitory effects of salicylate have resulted in the same inhibitory effects on hyperglycemia-induced malformations as ASA and lysine. Its presence in the medium significantly improved the growth and development of the embryos with regards to all parameters. The frequency of malformation, mortality, and CNS malformation were very low when compared to other hyperglycemic groups.

However, it is important to note that the amount of furosine produced was not reduced by salicylate. Therefore, the inhibitory effects of salicylate on hyperglycemia-induced malformations must be through mechanism(s) other than the anti-glycating effect. It could possibly be due to the hydroxyl radicals trapping ability of salicylate, by preventing the reaction of toxic oxygen radicals with cellular macromolecules such as proteins and lipids. This could reduce the damaging effects of oxygen radicals on cellular compounds such as nucleic acids, amino acids, and lipids (Cross et al., 1987). Salicylate has been demonstrated to trap hydroxyl radicals in *in vivo* (Onodera and Ashraf, 1991 and Udassin et al., 1991) and *in vitro* (Yaylayan, 1993). Although the action of salicylate as a free radical scavenger could not be verified, this study demonstrated that part of the protective action of ASA could be attributed to its salicylate metabolite.

5.2. PHASE 2: LIPID PEROXIDATION

LPO occurs naturally in cells through autoxidation or enzymatic reactions. Elevated levels of LPO have been observed in diabetic humans and rats (Sato et al., 1979; Tsuchida et al., 1985; Uzel et al., 1987; and Jain et al., 1989). In the present study, baseline serum LPO was not significantly different between the groups. After the 48 hour culture, the serum LPO had significantly risen in all groups. Therefore, it is reasonable to conclude that the LPO in the serum was produced by either the embryos during the culture period, by glucose autoxidation within the serum, or as a result of peroxidation of the lipids present in the serum. The total morphological scores, protein concentrations and somite numbers, and embryonic age decreased with the increase in serum LPO concentrations. In addition, serum LPO concentrations were correlated with the furosine concentrations found in the embryos.

5.2.1. Aspirin and Lipid Peroxidation

Polyunsaturated fatty acids are essential for growth and cell differentiation, and they are especially important in early fetal development (Crawford et al., 1981). The rapidly dividing cells of embryonic tissues are highly susceptible to peroxidation, due to the greater degree of lipid unsaturation (Wolfson et al., 1956), and a relatively low activity of antioxidant systems (Kehler and Autor, 1977). Hyperglycemia, as discussed above, produces significant deleterious effects on the growth and development of the embryos. However, ASA significantly improved the morphological development of the embryos in the hyperglycemic condition. In the presence of ASA, the serum LPO levels after the culture were significantly lower than the hyperglycemic group.

One potential mechanism by which ASA could prevent the hyperglycemia-induced malformations was via inhibition of glycation by acetylating embryonic protein and thereby reducing the formation of free radicals generated through glycation. ASA could also reduce the LPO concentrations through the free radical scavenging action of its salicylate metabolite. Another possible mechanism of the protective action of ASA was via its inhibitory action on prostaglandin H synthase activity (Flower et al., 1985). The formation of prostaglandins G_2 (PGG_2) involves the addition of two molecules of dioxygen to AA. Abstraction of protons concomitant with the rearrangement of the double bonds produces a PGG_2 with a peroxy group present at the 15-position (Miyamoto et al., 1976 and Lands, 1979). Subsequent peroxidase activity at the 15-hydroperoxy group of PGG_2 liberates an oxygen radical and forms PGH_2, which is the central precursor for the production of prostaglandins and thromboxanes (Rainsford, 1984) (Figure 9). The oxygen radicals, if not scavenged, could inactivate enzymes (Kong and Davidson, 1980), break DNA strands (Brawn and Fridovich, 1981), cross-

link proteins (Biorkstein, 1974), peroxidize lipids (Mead, 1976), and lead to cell death (Rainsford, 1984). The addition of ASA (an NSAID) inhibits the PGG_2 peroxidase activity and, therefore, reduces the production of oxygen radicals. Furthermore, the phenol structure of ASA can act as a free radical scavenger via the salicylate metabolite and, again, reduce the toxic effects of oxygen radicals produced from glycated protein, glucose autoxidation, or from the metabolism of AA through the cyclooxygenase pathway (Figure 8). Although LPO concentrations showed a significant negative correlation with size of yolk sacs, crown-rump lengths, and head diameters, other negative effects of LPO concentrations on growth parameters were weakly associated. It is possible, however, that the elevated LPO levels were simply associative with the deleterious elevated glucose levels rather than exerting damaging effects per se. It is also important to recognize that the prostaglandin synthase activity was not measured in the present study. Subsequently, the significance of the inhibition of ASA on prostaglandin synthase activity in hyperglycemia-induced malformations can not be elucidated.

5.2.2. Indomethacin and Lipid Peroxidation

Another NSAID, indomethacin, was shown to be more effective in the inhibition of cyclooxygenase than ASA on a equimolar basis (Smith and Lands, 1971; Egan et al., 1978). The concentration of indomethacin used in this experiment was much lower than that of the ASA (Im = 0.05 μM, i.e., 100-fold lower). However, indomethacin was shown to be active in inhibiting prostaglandin synthase at concentrations 50- to 500-fold lower than aspirin (Smith and Lands, 1971).

Unlike other treatments, the addition of indomethacin to a hyperglycemic medium produced embryos with somite numbers, yolk sac sizes, head diameters and protein contents similar to those seen in the hyperglycemic group. Although the addition of indomethacin had improved the growth parameters compared to the hyperglycemic group, hyper+Im produced the second highest mortality rate and CNS malformation of the embryos. On the other hand, indomethacin significantly reduced the production of serum media LPO. This could be the result of indomethacin of a direct free radical scavenging action (Sagar et al., 1992) or as a cyclooxygenase inhibitor in the AA cascade. The inhibition of the prostaglandin H synthase by indomethacin could result in a reduction of oxygen radicals produced by the embryos. The lack of a more pronounced protective effect on hyperglycemia-induced malformations, could be due to the absence of an anti-glycation effect of indomethacin. Another reason could be contributed to the deficiencies of PG as a result of the inhibitory action of indomethacin on prostaglandin H synthase. Therefore, the amount of AA converting to PG could be reduced and, subsequently, lead to suboptimal growth of the embryos (Figure 8).

It should also be considered that, the ameliorative effect of indomethacin on growth parameters could indicate that this hypothesis is faulty or that the dose of indomethacin was insufficient to reduce prostaglandin synthase activity. The latter is less likely due to the reduced LPO observed in the serum following indomethacin treatment, which is probably the result of a reduced production of oxygen free radicals as generated through prostaglandin synthase activity. The fact that indomethacin was observed to reverse the protective effects of exogenous arachidonic acid (Tzotzatou et al., 1981 and Piddington et al., 1983) could be the result of the teratogenic doses administrated in these studies. Contrary to these studies, indomethacin was added in a

dose which was determined through a dose-response curve on the embryos. Thus the dose added to the culture medium did not substantially reduce embryonic growth.

5.2.3. Arachidonic Acid and Lipid Peroxidation

Similar to other studies (Goldman et al., 1985 and Pinter et al., 1986), the addition of exogenous AA has significantly improved the growth and development of the embryos (i.e., yolk sacs, crown-rump lengths, head diameters, total morphological scores, and protein contents) compared to those in the hyperglycemic group. Goldman et al. (1985) observed the frequency of neural-tube defects in the hyperglycemic group and the hyperglycemic with AA (10 μg/ml) groups to be 67% and 32%, respectively. Malformation rates were found to be 20% by Pinter et al. (1986) with 20 μg/ml of exogenous AA. In the present study, CNS malformations for the hyperglycemic and the hyper+AA (μg/ml) groups were 54% and 22%, respectively. The frequency of malformation was 17% compared to 52% in the hyperglycemic group.

The above mentioned results show that exogenous supplementation of AA could be beneficial for the development of early stage embryos. However, contrary to this, were the significantly higher levels of LPO produced in the treatment groups with the addition of AA. This could explain the fact that the frequency of CNS malformations with the addition of AA to hyperglycemic group, was relatively high compared to the other treatment groups. In the control medium, the antioxidant enzymes in the embryos probably protected the embryos from the deleterious effects of LPO and oxygen radicals. On the other hand, the antioxidant enzyme activities can be suppressed and/or altered, with a concomitant increase in the production of oxygen radicals under

hyperglycemic conditions. This increase in oxygen radicals may therefore play a role in the hyperglycemia-induced malformations via its toxic effects. This hypothesis is based on the fact that the addition of free radical scavenging enzymes to a serum media resulted in the improvement of morphological scores of the embryos (Eriksson and Borg, 1991). Since the measurement of embryonic LPO concentrations was not possible, it is not sure if the serum LPO is representative of that in the embryos.

The major products of LPO, the hydroperoxides, are known to stimulate the biosynthesis of prostaglandin, but inhibit the production of PGI_2 (Warso and Lands, 1983). This imbalance may have harmful effects during the critical period of development of the embryos. The exogenous addition of AA was, perhaps, beneficial in restoring the balance of prostaglandin metabolism. Another protective action of AA could be exhibited through the increased PG action against free radicals, as PGE_1 and PGE_2 have been indicated to inhibit O_2^- generation (Das and Devi, 1988). In contrast to this beneficial effect, the addition of AA can also result in the increased production of LPO which, again, can inhibit the production of prostaglandin. However, due to the lack of detrimental effects on embryonic growth as a result of LPO production, as well as the lack of association between LPO and malformations (i.e., CNS malformations and enlarged pericardiums), may indicate that the amount of LPO produced was not physiologically significant.

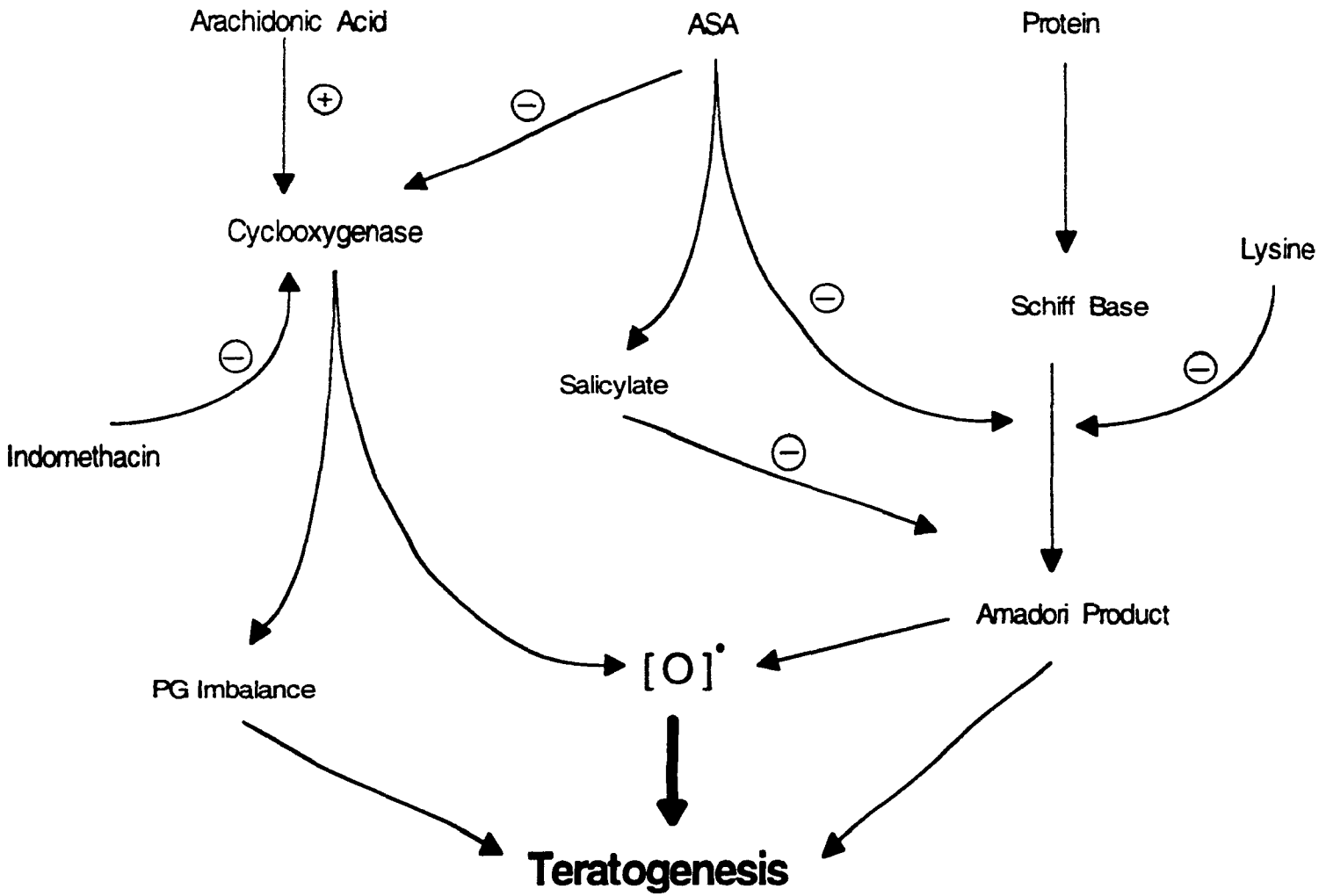

Figure 8: Sites of Inhibition of Teratogenesis by ASA, Salicylate, Lysine, Indomethacin and AA.

SECTION 6: CONCLUSIONS

This experiment was designed to study the role of glycated protein and free radicals in the hyperglycemia-induced malformations in early stage embryos. Hyperglycemia has resulted in a significant reduction in the size of the embryos, alterations in morphological features, as well as significant changes in metabolic pathways. Phase 1 of the experiment showed that the formation of glycated protein, mediated by hyperglycemia may be one of the causes for embryopathy. The amount of glycated protein produced was directly related to the severity of the malformations. The protective effects of lysine confirmed the role of glycation in hyperglycemia-induced malformations, since lysine was acting solely as an anti-glycating agent by competitive acetylation for the same protein with its physiological isomer. Supplementation of ASA also confirmed previous reports on the anti-glycating properties of this compound (Kubow et al., 1993) and thus, resulted in a reduction of hyperglycemia-induced malformations. ASA has played a protective role by acetylating embryonic protein, and demonstrated its role as a free radical scavenger by reducing the amount of free radicals generated through protein glycation and/or glucose autoxidation. Although the hydroxyl radical scavenging action of salicylate could not be confirmed, it is possible that salicylate reduced hyperglycemia-induced malformations by playing a role as a free radical scavenger. These results warrant further research on the suitability of

administrating these compounds *in vivo*, the optimal dosage, as well as possible physiological side effects.

Lipid peroxides were produced in the serum of cultured embryos in phase 2 of the experiment. The level of LPO was higher in embryos cultured under hyperglycemic conditions. ASA reduced hyperglycemia-induced malformations, and the relevant mechanisms could include a reduction of glycated protein, and a reduction in the amount of lipid peroxides produced via scavenging the oxygen radicals through its salicylate metabolite. Both ASA and indomethacin exerted a mild protective effect by reducing the production of lipid peroxides via the inhibitory action on the cyclooxygenase. However, the reduction of hyperglycemia-induced malformations by indomethacin was limited. This may be due to: 1) its inability to scavenge the oxygen radicals and inhibit glycation produced under hyperglycemic condition; and 2) its inhibitory action vis-a-vis the release of phospholipids, and the resulting deficiency of AA available for the developing embryos. The exogenous supplementation of AA has confirmed the results of previous studies in this area. AA supplementation resulted in lower neural-tube defects and an overall improvement in morphological development. However, there was no evidence that the addition of AA was fully effective. This is possibly due to the high levels of LPO as a result of supplementation of AA, and/or the lack of anti-glycating action of AA. As mentioned, it is not positive whether the serum LPO is representative of the LPO in the embryos. In addition, reduced levels of antioxidant enzyme activities may also have resulted in adverse effects in the development of the embryos. This could indicate that oxygen radicals/glycation may play a relatively more important role than AA in the hyperglycemia-induced malformations in these early stage mouse embryos.

This experiment further demonstrated that hyperglycemia-induced malformations are multifactorial in origin. Malformations are the result of a combination of factors including glycation, oxygen free radicals, AA deficiency, etc. This is supported by the fact that no single treatment was completely effective. However, a possible unified theory is put forth and presented in Figure 9. The formation of Amadori products, as a result of hyperglycemia-mediated protein glycation, can be teratogenic to the growing embryos. ASA and lysine can effectively prevent the teratogenic effects by inhibiting the glycation of embryonic protein. On the other hand, free radicals generated via glycation, glucose autoxidation, and LPO cause alterations in protein and DNA. This leads to further cell membrane and tissue damage which results in the teratogenesis of the embryo. Free radical scavengers such as ASA and salicylate protect against free radical damage. In addition, increasing the availability of AA increases the PG synthesis which, perhaps, also scavenges free radicals and prevents teratogenesis.

The results of this study provide an interesting basis for further research. It is necessary to investigate the application of these compounds in *in vivo* studies. A more sensitive technique is required to study the role of salicylate as a free radical scavenger in rodent embryos. Further studies are needed to evaluate the relative importance of ASA and AA in their roles as protective agents in hyperglycemia-induced malformations. Although ASA has been demonstrated in our study to have more protective effects than AA under the present experimental conditions, AA is an essential fatty acid required for the metabolism of eicosanoids, whereas ASA is a drug that could have potential side effects at a higher dosage. Therefore, the objective of future studies should be to establish the nontoxic levels of ASA or other relevant anti-glycating agents, free radical scavenging compounds, and AA required to achieve

preventive effects against hyperglycemia-induced malformations *in vivo*, and eventually, in humans.

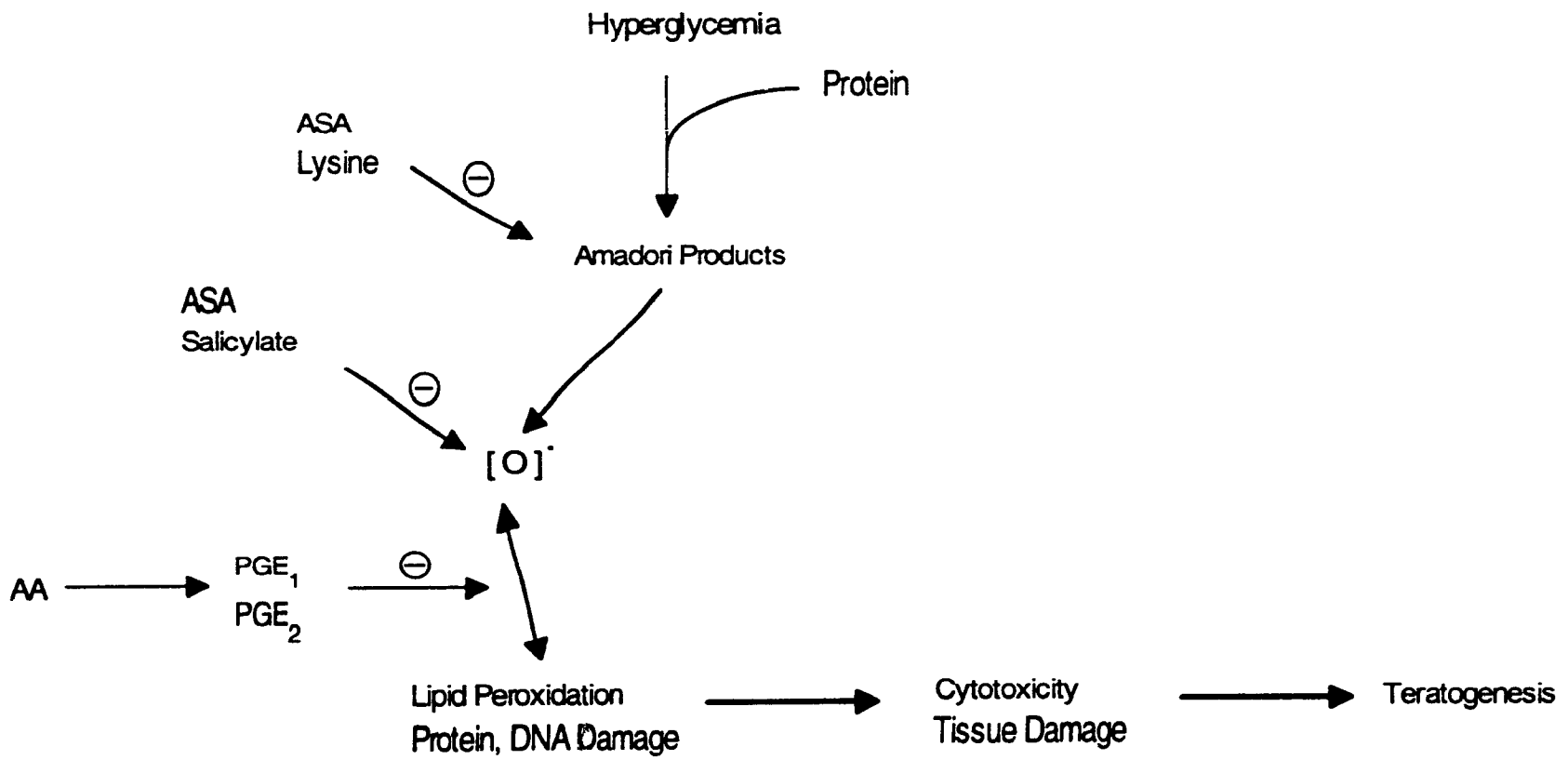

Figure 9: The proposed theory of hyperglycemia-induced teratogenesis and the role of various protective agents in preventing irreversible damage which results in teratogenesis.

SECTION 7: NOVEL FINDINGS

1. A large portion of the protective effects of ASA could be attributed to the salicylate portion.
2. Lysine is a highly specific anti-glycating agent which reduces hyperglycemia-induced malformations by competitively acetylating protein.
3. Free radical scavenging agents can protect against hyperglycemia-induced malformations despite relatively high levels of glycated embryonic protein.
4. Indomethacin exerts limited protective effects on hyperglycemia-induced malformations in early stage mouse embryos (0-2 somites) at a low dosage (0.05 μM) *in vitro*.

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APPENDIX A

Sample of the Morphological Assessement Sheet

Species Mice media Used Rat Serum

Stage of Gestation (Day) 8.5 (no. somites) 0-2

Time in _____ Time out _____

Scoring Date June 10

Treatment CTR

		0	1	2	3	4	5	A	B	C	D	E	F
A	YOLK SAC CIRCULATORY SYSTEM	no visible or scattered blood islands	Corona of blood islands w or w/o anastamoses	w/ or w/o anastamoses w/ few yolk sac vessels	Full yolk sac plexus of vessels	Yolk stem obliterated vitelline artery & vein well separated		3	3	3			
B	ALLANTOIS	Allantois trap in ectoderm	Allantois fused with chorion	Umbilical vessels	Separate aortic origins of umbilical and vitelline vessels			2	2	2			
C	FLEXION	Ventrally convex	Turning	Dorsally convex	Dorsally convex with spiral torsion			3	3	3			
D	HEART	Endocardial rudiment not visible or visible but not beating	Beating S shaped cardiac tube	Convoluted cardiac tube	Bulbus cordis, atrium common and ventricles common	Dividing atrium common		2	3	2			
E	CAUDAL NEURAL TUBE	Neural plate or neural folds	Closing but unfused neural folds (gaping)	Neural folds fused at level of somites 4-5	Posterior neuropore formed but open	Posterior neuropore closed		4	4	4			
F	HIND BRAIN	Neural plate	Rhombomeres A and B	Anterior neuropore formed but open	Anterior neuropore closed rhombencephalon formed	Pronounced dorsal flexure with transparent roof of 4th ventricle		3	3	3			
G	MID BRAIN	Neural plate	Mesencephalic brain folds	Closing or fusing mesencephalic folds	Completely fused mesencephalon	Visible division between mesencephalon & diencephalon		3	3	3			
H	FORE BRAIN	Neural plate or no visible prosencephalon	Prosencephalic brain folds	Completely fused prosencephalon	Visible telencephalic evaginations	Well elevated telencephalic hemispheres		3	3	2			
J	OTIC SYSTEM	No sign of otic development	Flattened or indented otic primordium	Otic pit	Otocyst	Otocyst with dorsal recess	Otocyst with endolymphatic duct	3	3	3			
K	OPTIC SYSTEM	No sign of optic development	Sulcus opticus	Elongated optic primordium	Primary optic vesicle with open optic stalk	Indented lens plate	Lens pocket or lens vesicle	3	3	2			
L	OLFACTORY SYSTEM	No sign of olfactory development	Olfactory plate	Olfactory plate with rim	Distinct olfactory ridges	Lateral nasal process and medial rim		2	3	2			
M	BRANCHIAL BARS	None visible	I visible	I and II visible	I, II and III visible	II overlapping and obscuring III		2	3	2			
N	MAXILLARY PROCESS	No sign of maxillary development	Maxillary process evaginated visible cleft anterior to bar I	Maxillary process fused with nasal process				1	1	1			
P	MANDIBULAR PROCESS	No sign of mandibular development from bar I	Distal ends of bars fused and forming mandibular process					1	1	1			
Q	FORE LIMB	No sign of fore limb development	Distinct evagination of apical crest at level of somites 9-13	Fore limb bud	Paddle shaped fore limb bud	Distinct Apical ridge on fore limb bud		2	3	2			
R	HIND LIMB	No sign of hind limb development	Distinct evagination of apical crest at level of somites 20-29	Hind limb bud	Paddle shaped hind limb bud			1	1	1			
S	SOMITES	0-8	7-13	16-20	21-27	28-34	35-41	3	4	3			
	SUM							27	29	26			
	SOMITES							30	33	30			
	YS							2.5	2.7	2.3			
	C-R							1.5	1.6	1.5			
	Hd												

Comments _____

APPENDIX B

Figures 10 To 15

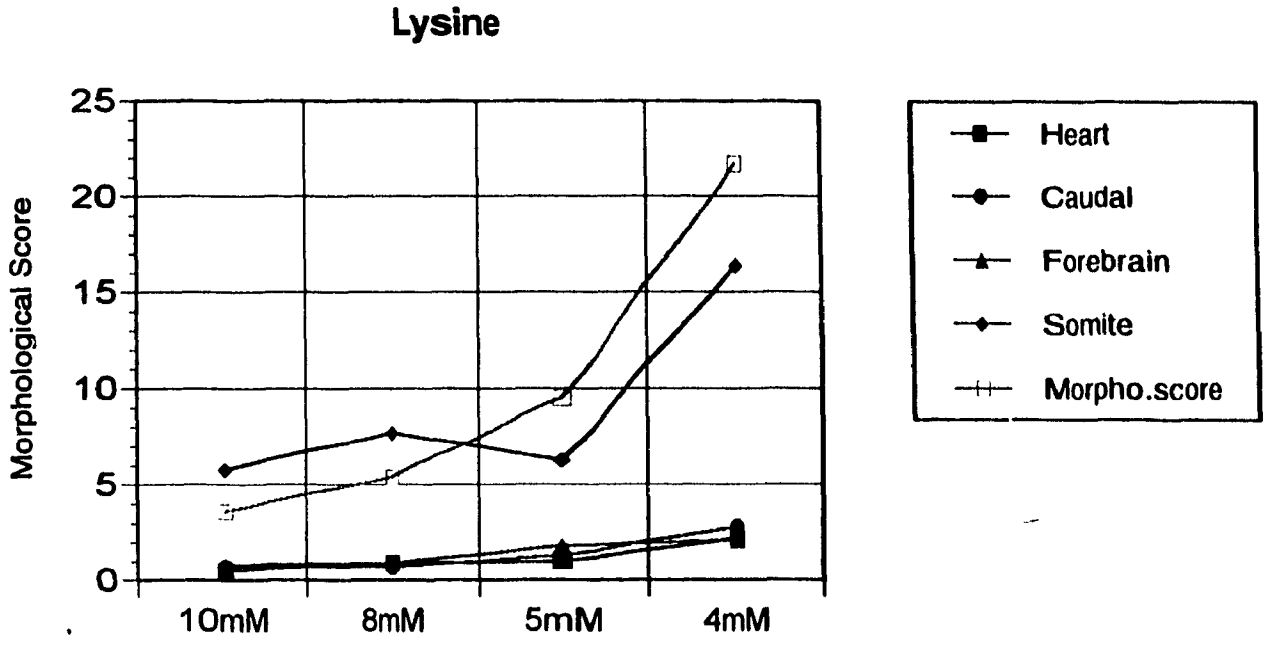

Figure 10: Dose Response Curve for Lysine. Ten embryos were used for each of the four doses. Embryos were rated on the development of the heart, caudal neural tube, forebrain, somite number and the total morphological score. The concentration which gave the best morphological result, along with the absence of visible malformations, was chosen to be the dose used in the experiment.

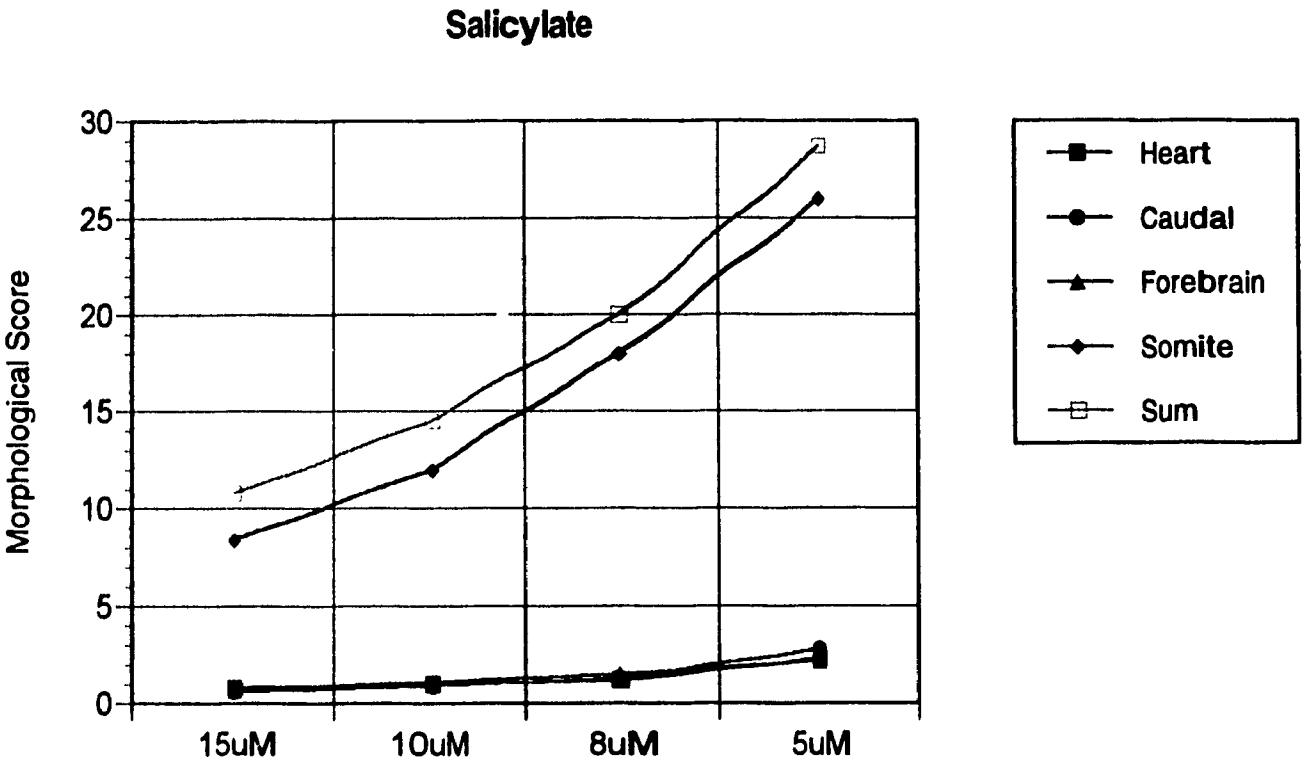

Figure 11: Dose Response Curve for Salicylate. Ten embryos were used for each of the four doses. Embryos were rated on the development of the heart, caudal neural tube, forebrain, somite number and the total morphological score. The concentration which gave the best morphological result, along with the absence of visible malformations, was chosen to be the dose used in the experiment.

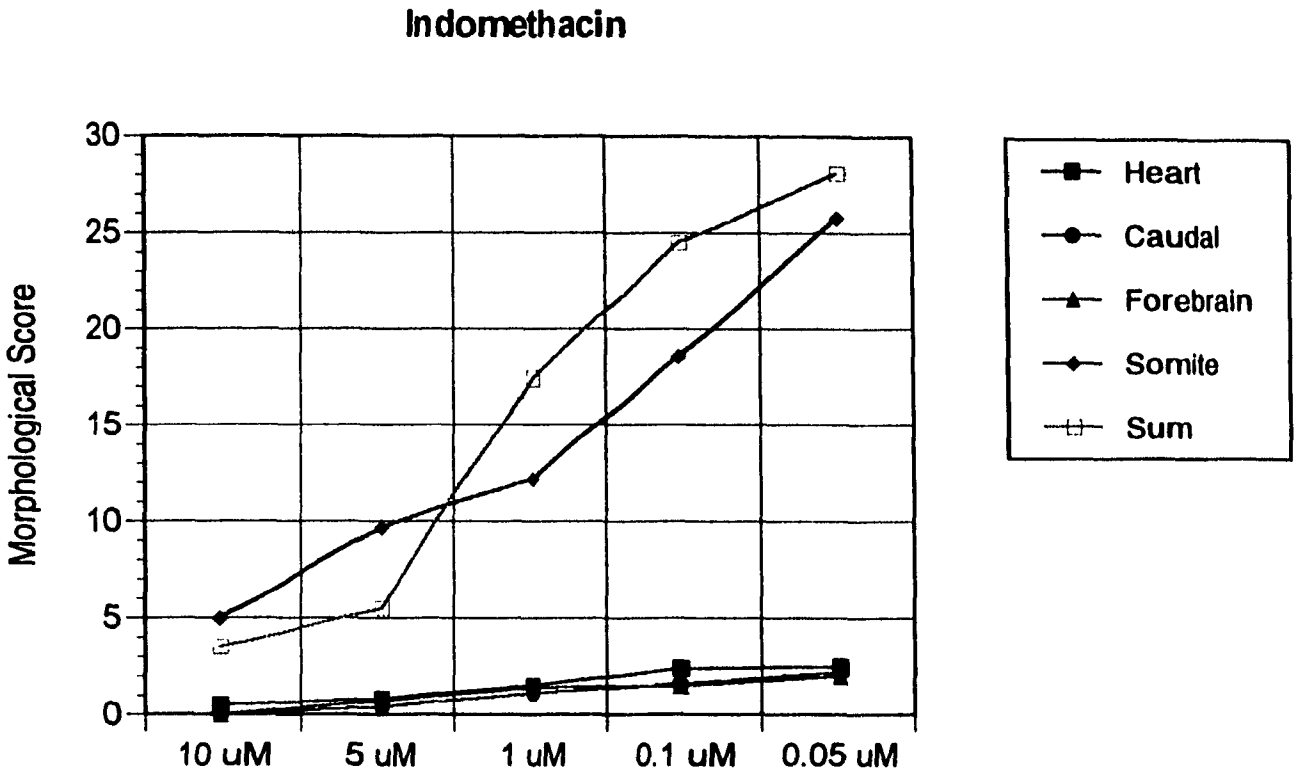

Figure 12: Dose Response Curve for Indomethacin. Ten embryos were used for each of the doses. Embryos were rated on the development of the heart, caudal neural tube, forebrain, somite number and the total morphological score. The concentration which gave the best morphological result, along with the absence of visible malformations, was chosen to be the dose used in the experiment.

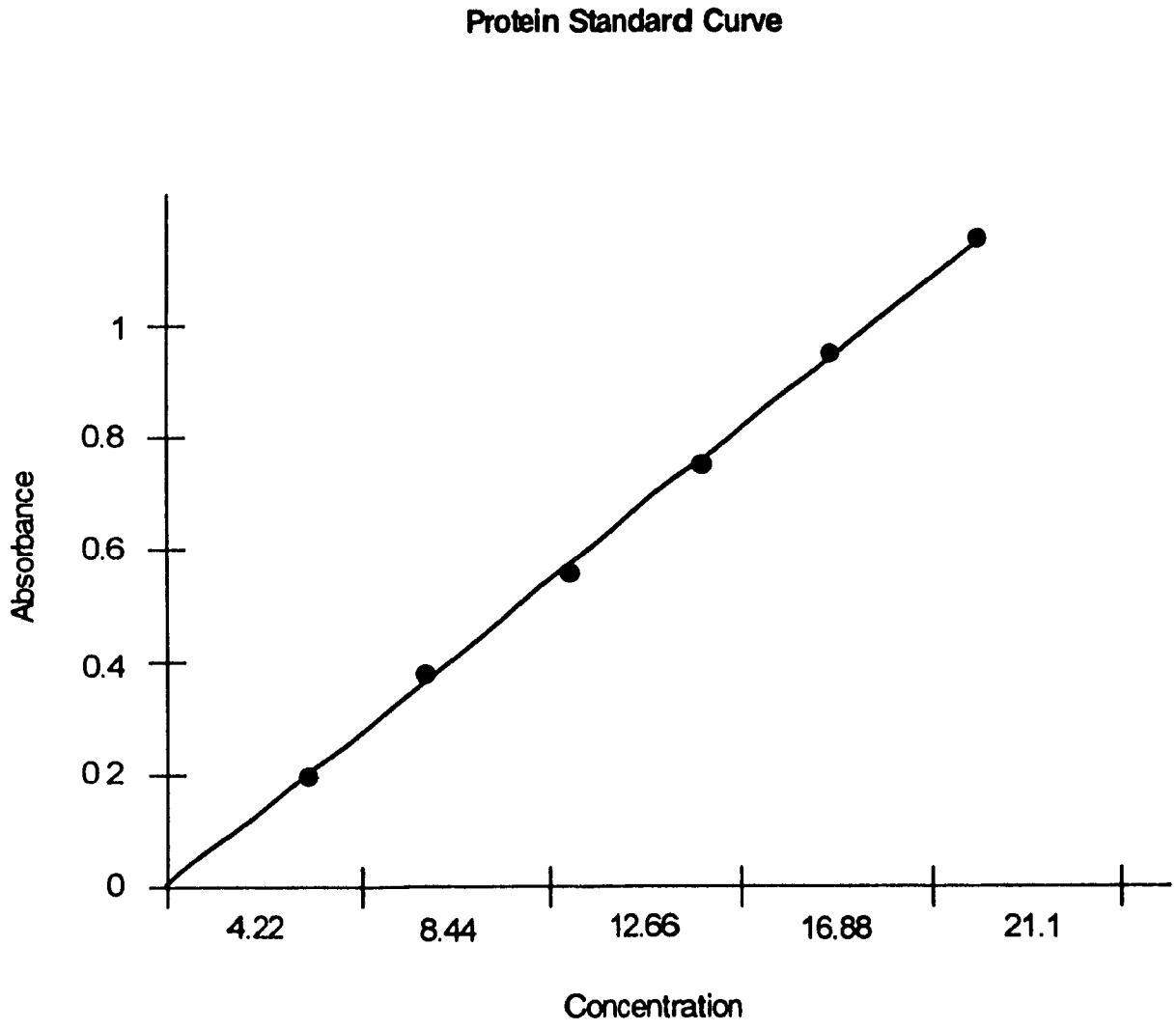

Figure 13: Sample of a Standard Curve of Bradford Microprotein Assay. Bovine serum albumin was used as the standard protein.

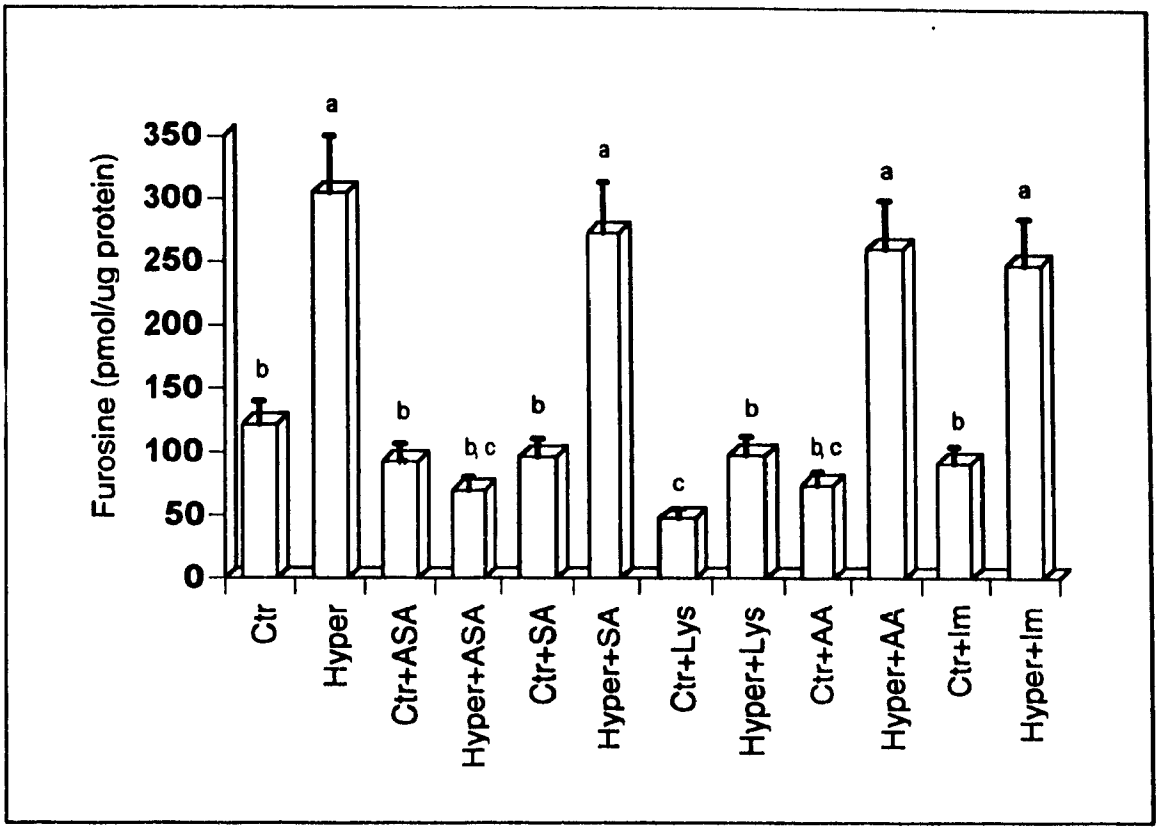

Figure 14: Mean Furosine Concentration. Furosine was prepared from mouse embryos as described in Section 3: Materials and Methods. Furosine was monitored at 280 nm wavelength. The amount of furosine was expressed as *p*mol hexose/ug protein. Means not sharing a common letter are significantly different at the $p < 0.05$ level as determined by the Bonferroni's multiple comparison test. See Table 18, Appendix C for ANOVA and *p* values.

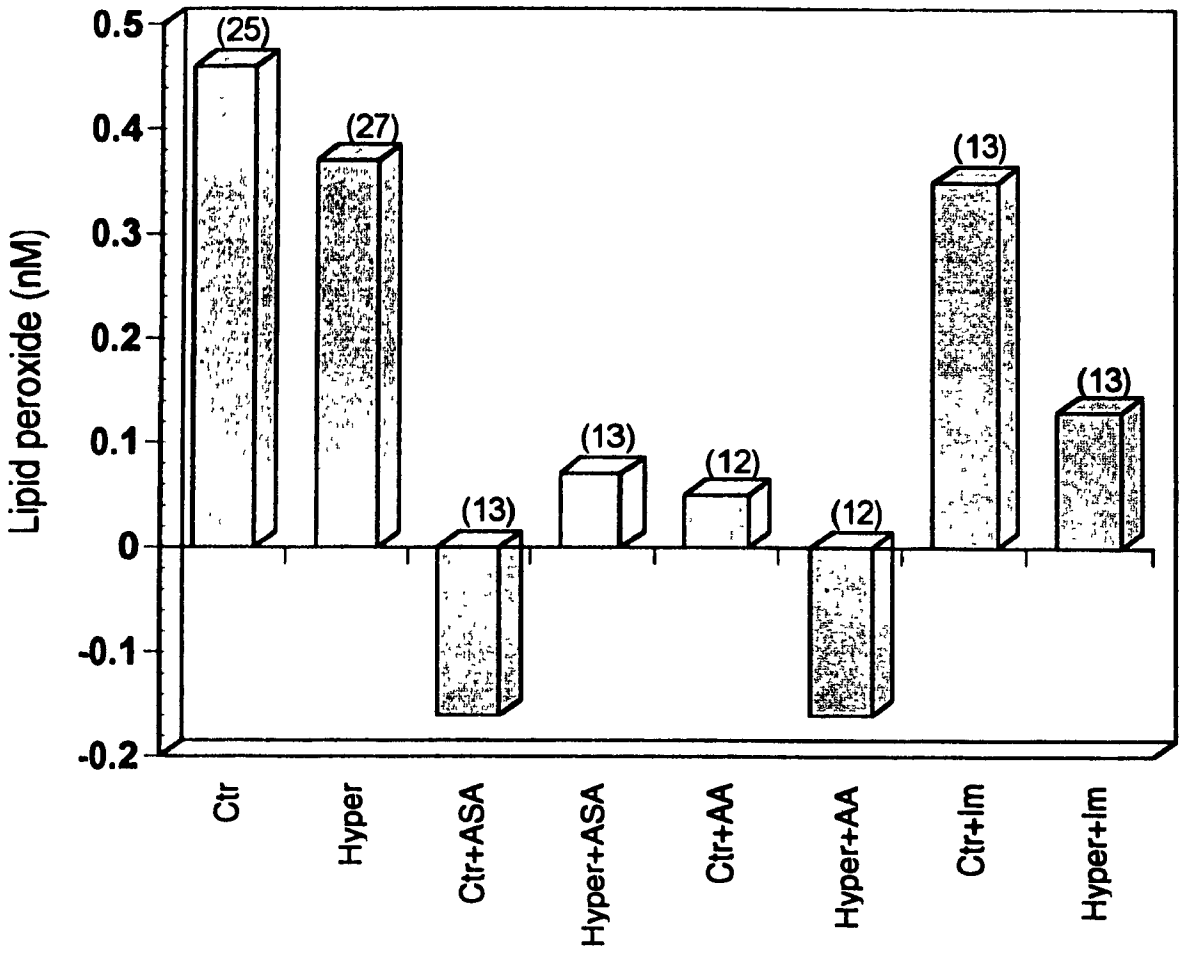

Figure 15: Lipid Peroxide Concentrations of Serum Media Before Culture. Lipid peroxide concentrations were measured with the spectrophotometric kit as described in Section 3: Materials and Methods. (n) refers to the total number of embryos per group. Serum lipid peroxide concentrations were pooled and the values are expressed as nMoles per (n) of embryos. No two groups are significantly different as determined by Bonferroni's multiple comparison test.

APPENDIX C

Tables 12 To 19

Table 12: Chi-square with Continuity Adjustment Comparisons [1,2]

	Kinked Tail		Ventrally Convex		Total Malformation	
Comparison	**F value**	**p>F**	**F value**	**p>F**	**F value**	**p>F**
Control vs Hyperglycemic	17.405	0.000	11.344	0.000	89.416	0.000
Control vs Hyper+ASA	0.058	0.808	3.852	0.050	4.450	0.035
Control vs Hyper+SA	0.729	0.393	3.175	0.075	2.236	0.135
Control vs Hyper+Lys	0.017	0.896	6.501	0.011	0.729	0.393
Control vs Hyper+AA	1.687	0.194	5.336	0.021	5.570	0.018
Control vs Hyper+Im	3.429	0.064	4.492	0.034	6.181	0.013
Ctr+ASA vs Hyper+ASA					1.339	0.593
Ctr+SA vs Hyper+SA					0.948	0.33

[1] Analyses were performed by SPSS for Windows, version 5.0.
[2] Blank spaces indicate a lack of positive outcome for a particular variable.

Table 13: Chi-square with Continuity Adjustment Comparisons [1,2]

	Kinked Tail		Ventrally Convex		Total Malformation	
Comparison	**F value**	**p>F**	**F value**	**p>F**	**F value**	**p>F**
Hyper vs Ctr+ASA	11.640	0.000			65.697	0.000
Hyper vs Hyper+ASA	7.091	0.008	0.707	0.400	49.955	0.000
Hyper vs Hyper+SA	2.885	0.089	2.869	0.090	43.742	0.000
Hyper vs Hyper+Lys	4.625	0.031	0.248	0.618	48.643	0.000
Hyper vs Ctr+AA	9.582	0.005	3.333	0.068	61.63	0.000
Hyper vs Hyper+AA	2.338	0.126	0.028	0.867	38.533	0.000
Hyper vs Hyper+Im	2.272	0.132			39.980	0.000
Ctr+Lys vs Hyper+Lys					0.286	0.593
Ctr+AA vs Hyper+AA					2.837	0.092
Ctr+Im vs Hyper+Im					1.180	0.277

[1] Analyses were performed by SPSS for Windows, version 5.0.

[2] Blank spaces indicate a lack of positive outcome for a particular variable.

Table 14: Chi-square with Continuity Adjustment Comparisons [1,2]

	Enlarged Pericardium		Blebs		Malformation Rate		Mortality Rate	
Comparison	**F value**	**p>F**	**F value**	**p>F**	**F value**	**p>F**	**F value**	**p>F**
Control vs Control+ASA	0.067	0.796	0.323	0.569				
Control vs Hyper+ASA	2.138	0.144	7.886	0.005	6.077	0.014	1.783	0.182
Control vs Control+SA	0.000	1.000	0.068	0.794				
Control vs Hyper+SA	0.000	1.000	8.571	0.003	3.174	0.075	3.175	0.075
Control vs Control+Lys	0.000	1.000			0.424	0.515		
Control vs Hyper+Lys	0.516	0.473	1.317	0.251	6.501	0.011	0.424	0.515
Control vs Control+AA	0.000	1.000	0.000	1.000				
Control vs Hyper+AA	0.289	0.591	9.141	0.002	5.336	0.021	2.561	0.109
Control vs Control+Im	0.000	1.000	1.853	0.173	2.251	0.133	0.243	0.622
Control vs Hyper+Im	1.286	0.257	13.001	0.000	7.420	0.006	4.746	0.029

[1] Analyses were performed by SPSS for Windows, version 5.0.
[2] Blank spaces indicate a lack of positive outcome for a particular variable.

Table 15: Chi-square with Continuity Adjustment Comparisons [1,2]

	Enlarged Pericardium		Blebs		Malformation Rate		Mortality Rate	
Comparison	**F value**	**p>F**	**F value**	**p>F**	**F value**	**p>F**	**F value**	**p>F**
Hyper vs Control+ASA	4.538	0.033	16.833	0.000			2.654	0.103
Hyper vs Hyper+ASA	1.765	0.184	1.574	0.210	7.687	0.005	0.234	0.629
Hyper vs Control+SA	5.540	0.018	18.990	0.000			1.413	0.235
Hyper vs Hyper+SA			0.354	0.552	6.281	0.012	0.000	1.000
Hyper vs Control+Lys					8.782	0.003	1.413	0.235
Hyper vs Hyper+Lys	1.703	0.192	3.728	0.054	4.222	0.04	0.224	0.636
Hyper vs Control+AA	6.321	0.012	9.429	0.002	13.169	0.000	1.687	0.194
Hyper vs Hyper+AA	2.618	0.105	0.521	0.471	6.078	0.014	0.006	0.936
Hyper vs Control+Im	5.102	0.024	4.765	0.029	9.593	0.002	0.652	0.419
Hyper vs Hyper+Im	1.859	0.173	0.089	0.765	5.378	0.020	0.000	1.000

[1] Analyses were performed by SPSS for Windows, version 5.0.
[2] Blank spaces indicate a lack of positive outcome for a particular variable.

Table 16: Chi-square with Continuity Adjustment Comparisons [1,2]

	Enlarged Pericardium		Blebs		Malformation Rate		Mortality Rate	
Comparison	**F value**	**p>F**	**F value**	**p>F**	**F value**	**p>F**	**F value**	**p>F**
Control vs Hyperglycemic	19.267	0.000	24.415	0.000	35.148	0.000	6.615	0.010
Ctr+ASA vs Hyper+ASA	0.189	0.663	7.292	0.007	2.446	0.118		
Ctr+SA vs Hyper+SA			5.128	0.023	0.533	0.465	1.783	0.182
Ctr+Lys vs Hyper+Lys	0.533	0.465	1.471	0.225	0.286	0.593		
Ctr+AA vs Hyper+AA	0.473	0.491	3.652	0.056	1.334	0.248	3.175	0.075
Ctr+Im vs Hyper+Im	0.276	0.599	1.782	0.182	0.194	0.659		

[1] Analyses were performed by SPSS for Windows, version 5.0.

[2] Blank spaces indicate a lack of positive outcome for a particular variable.

Table 17: Chi-square with Continuity Adjustment Comparison [1]

Comparison	Malformations of Central Nervous System F value	p > F
Control vs Hyperglycemic	36.958	0.000
Control vs Hyper+ASA	1.783	0.182
Control vs Control+SA	0.424	0.515
Control vs Control+Lys	3.175	0.075
Control vs Control+Im	10.215	0.001
Hyperglycemic vs Hyper+ASA	13.116	0.000
Hyperglycemic vs Hyper+AA	9.494	0.002
Hyper vs Control+Im	12.541	0.000
Hyper vs Hyper+Im	6.905	0.009
Ctr+Lys vs Hyper+Lys	4.803	0.028
Ctr+AA vs Hyper+AA	10.417	0.001
Ctr+Im vs Hyper+Im	4.313	0.038

[1] Analyses were performed by SPSS for Windows, version 5.0.

Table 18: ANOVA Results for Mean Total Morphological Score and Furosine [1].

Dependent Variable	Total Morphological Score		Furosine (pmol/μg protein)	
	F value	Prob > F	F value	Prob > F
Control vs Hyperglycemic	255.643	< 0.0001	4.223	0.052
Control vs Hyper+ASA	21.403	< 0.0001	2.920	0.099
Control vs Hyper+Lys	28.196	< 0.0001	0.459	0.507
Controlvs Hyper+AA	38.806	< 0.0001	4.237	0.054
Control vs Hyper+Im	51.447	< 0.0001	9.389	0.007
Hyper vs Hyper+ASA	30.700	< 0.0001	9.356	0.005
Hyper vs Hyper+SA	39.412	< 0.0001	0.073	0.790
Hyper vs Hyper+Lys	36.721	< 0.0001	3.716	0.069
Hyper vs Hyper+AA	28.845	< 0.0001	0.133	0.719
Hyper vs Hyper+Im	12.122	0.001	0.269	0.610
Control+ASA vs Hyper+ASA	12.172	0.001	0.586	0.450
Control+SA vs Hyper+SA	2.886	0.100	6.745	0.021
Control+Lys vs Hyper+Lys	18.804	0.0002	3.462	0.084
Control+AA vs Hyper+AA	14.547	0.0006	6.026	0.028
Control+Im vs Hyper+Im	6.927	0.013	18.373	0.0008

[1] Analyses were performed by SPSS for Windows, version 5.0.

Table 19: ANOVA Results for Serum Lipid Peroxide Concentrations After 48 Hour Culture [1].

	Lipid Peroxide Concentrations	
Dependent Variable	F value	Prob > F
Control vs Hyperlgycemic	2.30	0.136
Control vs Control+AA	10.369	0.003
Control vs Control+Im	5.000	0.032
Control vs Hyper+Im	1.590	0.215
Hyperglycemic vs Control+ASA	5.057	0.030
Hyperglycemic vs Hyper+ASA	4.093	0.050
Hyperglycemic vs Control+AA	4.10	0.050
Hyperglycemic vs Control+Im	8.977	0.005
Hyperglycemic vs Hyper+Im	5.061	0.030
Control+ASA vs Hyper+ASA	0.144	0.708
Control+AA vs Hyper+AA	0.377	0.545
Control+Im vs Hyper+Im	1.785	0.194

[1] Analyses were performed by SPSS for Windows, version 5.0.