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Glycine decreases developmental damage induced by hyperglycaemia in mouse embryos

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Abstract

Hyperglycaemia induces neural tube defects and growth retardation in cultured mouse and rat embryos. In this study the possibility that glycine could prevent hyperglycaemia-induced embryopathy was researched. Early somite mouse embryos were cultured in normal medium, hyperglycaemic medium (50 mmol L^{-1} glucose), or with glycine (1 mmol L^{-1}) supplementation of normal and hyperglycaemic rat serum for 48 h. The embryo growth and differentiation were determined to estimate developmental and congenital malformations as well as lipid peroxidation levels. Adding glycine to the control culture medium did not affect embryonic development. Whereas the amino acid protected against telencephalon dysmorphogenesis, the decreased DNA content and number of somites, and the morphological score affectation induced by the hyperglycaemic medium, it had no preventive effect on the retarded differentiation of the otic system. Moreover, it prevented the high hyperglycaemia-induced lipoperoxidation levels of embryonic tissues. Embryos were partially protected from the hyperglycaemia-induced teratogenesis due to the antioxidative effect of glycine. As no other mechanisms related to the antiglycation or other protective effects of glycine were examined, the mechanism whereby it acted as an antiteratogenic agent needs further study.

Introduction

The incidence of congenital malformations in children of diabetic mothers is higher than that in the general population (The Diabetes Control and Complications Trial Research Group 1996), ranging from 4 to 13% worldwide according to most studies (Reece et al 1996). Although the exact cause of malformations is unknown, an association has been clearly demonstrated between spontaneous abortions or malformations and maternal hyperglycaemia in early pregnancy, detected as an excessive increase of glycated haemoglobin (HbA1c) serum levels (Rossen et al 1994). Several studies have provided evidence that hyperglycaemia may mediate dysmorphogenetic effects by disrupting myo-inositol (Khandelwal et al 1998) or arachidonic acid (Reece et al 2006), altering the trace metal status (Jankowski et al 1995), and increasing free oxygen radical formation (Gäreskog & Wentzel 2007) or non-enzymatic glycation of proteins (Kubow et al 1993).

It has been found that glycine protects kidneys (Weinberg et al 1990; Mangino et al 1991; Li et al 1995), liver (Yin et al 1998), lungs (Wheeler & Thurman 1999), and endothelial cells of the umbilical vein (Weinberg et al 1992) from damage caused by hypoxic, ischaemic and cytotoxic substances. Furthermore, this amino acid reduced cholinomimetic-induced malformations in chicken embryos (Landauer 1976) and cadmium-induced neural tube defects in mice embryos (Paniagua-Castro et al 2007). Through an anti-glycation effect it also reduced embryolethality of streptozotocin-induced diabetes (Martínez et al 2003).

This study focused on the antiteratogenic and antioxidant effects of glycine, since increased oxygen free radical generation is one of the proposed mechanisms of hyperglycaemia-induced teratogenesis. To avoid the possible maternal and streptozotocin-mediated toxic effects on offspring, post-implanted mouse embryos were cultured in a glucose-supplemented media.

Materials and Methods

Animals

Experimental procedures were reviewed and approved by our Institutional Committee in accordance with the ethical principles and regulations as specified by the standards of the National Institutes of Health of Mexico.

Swiss Webster male and female mice (25–30 g) from the National Hygiene Institute (Mexico City, Mexico) were used. The animals were kept at room temperature (24 ± 2°C) in a 12-h light/dark cycle (light at 0900 h) with free access to tap water and a commercial pellet diet. Mating was carried out by placing three females with one male from 0600 to 0800 h. Gestational day (GD) 0 was designed with the confirmation of pregnancy by the detection of a vaginal sperm plug.

Whole embryo culture

On GD 8 at 0900 h, pregnant mice were killed by cervical dislocation and the whole uteri were removed. Conceptuses were prepared according to the Cockroft technique (Cockroft 1990). Briefly, each conceptus was dissected from the uterus and decidual tissue in Hank's buffered saline solution (HBSS). Reichert's membrane and parietal yolk sac were removed, leaving the visceral yolk sac and ectoplacental cone intact. Only four to five somite embryos were chosen for culture and randomized to one of four groups: group 1, rat serum (control, 7.22 mM glucose concn); group 2, a culture media with glycine added (1 mM glycine solution); group 3, a hyperglycaemic medium to a final glucose culture concentration of 50 mM; and group 4, a hyperglycaemic medium with glycine. As many as four embryos were transferred to 60-mL sterile glass bottles containing: 3.9 mL sterile heat-inactivated rat serum at 37°C (prepared following the Cockroft (1990) indications), 100 μL sterile twice-distilled and deionized water with the proper additions of D-glucose (Sigma Chemical Co.), and glycine (Sigma Chemical Co.) to a final volume of 4 mL. The rat serum used as a culture medium was pooled immediately after the bleeding session and then preserved at –20°C until use. Serum glucose concentration was adjusted and determined by using a Wiener lab Selectra II automatic analyser before the preparation of each culture. Culture bottles were placed in a roller incubator (BTC Engineering Milton, Cambridge) at 37°C and 60 rev min^{-1}. They were gassed with 5% O_2, 5% CO_2 and 90% N_2 30 min before and immediately after the embryo transfer. The mixture was changed to 20% O_2, 5% CO_2 and 75% N_2 at 15 h, to 40% O_2, 5% CO_2 and 55% N_2 at 24 h, and to 95% O_2 and 5% CO_2 at 36 h of culture.

At 48 h of culture, embryos were transferred to Petri dishes containing HBSS and examined under a stereo microscope. Viability was assessed by the presence of a heartbeat and yolk sac circulation. Only live embryos were further analysed. The yolk sac diameter, crown–rump and head lengths were measured. Embryo morphological scoring was conducted as described by Brown & Fabro (1981) and embryonic examination for any external malformation was then carried out. Subsequently, each embryo was individually frozen at –70°C immersed in 900 μL DNA assay buffer (10 mmol L^{-1} $NaH_2PO_4.5H_2O$, 40 mmol L^{-1} Na_2HPO_4 anhydrous, 2 mmol L^{-1} NaCl, 2 mmol L^{-1} disodium EDTA (J.T. Baker)) for later analysis.

DNA quantitation

In accordance with Labarca & Paigen (1980), embryos were thawed and diluted with DNA assay buffer to a final volume of 1.9 mL and then sonicated (15 s, 8 μm) by using a Soniprep (Gallenkamp). Each sample was poured into a glass cuvette and 100 μL of 300 μL Hoechst 33258 (Sigma Chemical Co.) solution was added immediately. The mixture was stirred and the relative fluorescence (RF) at 460 nm (excitation wave length 365 nm) was read on a SLM AMINCO Spectrofluorometer (Mod. DMX-1000, SLM Instruments). The samples were read five times, being remixed between each reading. DNA content was determined by reference to a standard line of calf thymus DNA (Sigma Chemical Co.) and expressed as μg DNA per embryo.

Lipid peroxidation

Embryonic homogenates, prepared by pooling the tissue of three control or three treated embryos, were mixed thoroughly with trichloroacetic acid (TCA; J.T. Baker)–thiobarbituric acid (TBA; Sigma Chemical Co.)–HCl (J.T. Baker) (15% (w/v) TCA–0.375% (w/v) TBA–0.25 M HCl). The solution was heated for 60 min in a boiling water bath. After cooling, the flocculent was removed by centrifugation at 1000 *g* for 10 min. The absorbance of the sample was determined at 535 nm against a blank containing reagent only using a Shimadzu spectrofluorometer. The sample malondialdehyde (MDA) content was calculated at an extinction coefficient of 1.56×10^{-5} M^{-1}cm^{-1} (Buege & Aust 1978). A total of nine embryos were used for the analysis of each group. The protein content of these embryos was determined by the Coomassie blue method (Bradford 1976), using bovine serum albumin (BSA; Sigma Chemical Co.) as a standard.

Statistical analysis

Differences between means were evaluated by one-way analysis of variance followed by Newman–Keuls multiple comparison test. Differences between medians were evaluated by Kruskall–Wallis one-way analysis of variance on Ranks followed by Dunn's multiple comparison test. Intergroup comparison of the malformation incidence was performed with Fisher's exact test at the 95% significance level or chi square (with Yates' correction), whichever was applicable. Data were analysed using the Sigma Stat software Ver. 2.07, and presented as means ± s.e. The probability values of $P < 0.05$ were considered as significant.

Results

Compared with the control group, the hyperglycaemic medium resulted in a higher incidence of malformed embryos (Table 1), related mainly to the development of the central nervous and vesicular systems. When 1 mmol L^{-1} glycine was added to the hyperglycaemic medium, a decreased incidence

Table 1 Effect of glycine on types and incidence of malformations and growth of hyperglycaemia-cultured mouse embryos

	Group			
	Control (n)	Glycine 1 mM (n)	Glucose 50 mM (n)	Glucose 50 mM + glycine 1 mM (n)
Cultured embryos	28	27	30	26
Dead embryos	1 (4)	0	4 (13)	0
Viable embryos	27 (96)	27 (100)	26 (87)	26 (100)
Malformations				
Abnormal embryos	0	0	16 (62)[a]	10 (38)[a]
Central nervous system	0	0	15 (58)[a]	6 (23)[ab]
Telencephalon	0	0	11 (42)[a]	3 (12)[b]
Asymmetric	0	0	5 (19)[a]	0
Hypoplasia	0	0	6 (23)[a]	3 (12)
Open mesencephalon	0	0	4 (15)	3(12)
Vesicular systems	0	0	9 (35)[a]	5 (19)[a]
Retarded differentiation of otic system	0	0	5 (19)[a]	5 (19)[a]
Optic vesicle opacity	0	0	4 (15)	0
Growth				
Yolk sac diameter (mm)	4.46 ± 0.04	4.50 ± 0.04	4.15 ± 0.06[a]	4.27 ± 0.05[a]
Crown–rump length (mm)	4.01 ± 0.04	4.04 ± 0.06	3.77 ± 0.05[a]	3.86 ± 0.05[a]
Head length (mm)	1.91 ± 0.03	1.91 ± 0.04	1.74 ± 0.04[a]	1.81 ± 0.04
DNA content (μg)	29.72 ± 1.03	33.34 ± 1.31	22.33 ± 2.29[a]	30.63 ± 2.45[b]

Values inside parentheses represent percentage. [a]$P < 0.05$ compared with control, [b]$P < 0.05$ compared with glucose. χ^2 (with Yates' correction), Fisher's Exact Test or analysis of variance, Newman–Keuls, when applicable.

of central nervous system malformations was observed, impeding hyperglycaemia-induced telencephalic region injury. The 1 mmol L^{-1} glycine concentration used was selected based on a preliminary study (data not shown).

With regard to growth, a decreased yolk sac diameter, crown–rump and head lengths, as well as DNA content were observed in embryos from the hyperglycaemic medium (Table 1). On the other hand, adding glycine to the hyperglycaemic medium prevented DNA depletion, reflecting a protective effect of the amino acid against growth retardation.

With respect to the degree of differentiation, there was a statistically significant decrease in the morphological score and the number of somites (Table 2) in embryos from the hyperglycaemic medium compared with control embryos. In contrast, embryos cultured in hyperglycaemic medium plus glycine were protected against irregular differentiation. Finally, hyperglycaemic medium caused a significant increase in MDA levels (Figure 1), whereas the addition of amino acid to the culture medium impeded the lipid peroxidation of embryonic tissues.

Other malformations, which included right hind limb hypoplasia, retarded closure of the caudal neuropore, slight caudal neural tube oedema, and "S-shaped" closure of the neural tube, were found in all groups of embryos. However, there were no significant differences between the groups (data not shown).

Table 2 Effect of glycine on parameters of differentiation of hyperglycaemia-cultured mouse embryos

Treatment	Somites (n)	Morphological score
Control		
3rd quartile	30	51.7
median	29	47.4
1st quartile	28	45.7
Glycine 1 mM		
3rd quartile	31	51
median	29	48.8
1st quartile	28	44.5
Glucose 50 mM (hyperglycaemia)		
3rd quartile	26.7	47.1
median	26[a]	42.5[a]
1st quartile	26	39.5
Glucose 50 mM + glycine 1 mM		
3rd quartile	29	50.22
median	28[b]	47[b]
1st quartile	27	45.82

[a]$P < 0.05$ compared with control, [b]$P < 0.05$ compared with glucose 50 mM. Kruskal–Wallis, Dunn's.

Discussion

Martínez et al (2003) reported that streptozotocin-induced diabetes in pregnant mice caused a general embryolethality, but without sufficient evidence of diabetes-induced congenital malformations. However, since glycine administration protected against resorption by improving fetal viability, congenital malformations were then observed. In this study, mouse embryos were directly cultured in a hyperglycaemic medium to avoid the possible toxic effects mediated by the dam or streptozotocin, as well as other proposed mechanisms of diabetic teratogenesis such as hyperketonaemia (Eriksson &

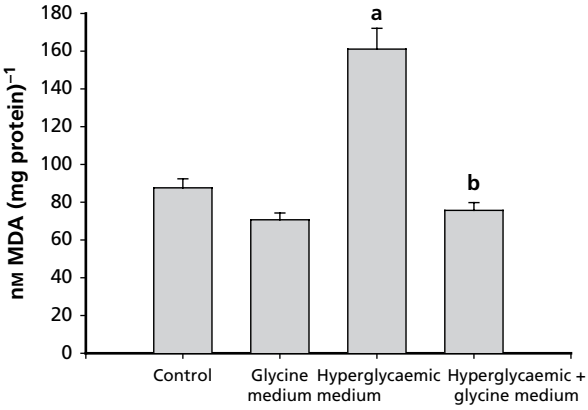

Figure 1 MDA content in embryos cultivated in glycine medium, hyperglycaemic medium, and hyperglycaemic plus glycine medium. [a]$P < 0.05$ compared with control; [b]$P < 0.05$ compared with hyperglycaemic medium (analysis of variance, Newman–Keuls).

Borg 1993) and inhibition of somatomedin (Hunter et al 1991).

Hyperglycaemia, the primary clinical manifestation of diabetes, has been suggested as an aetiological factor for diabetic dysmorphogenesis, which has been clearly related to retarded growth and neural tube defects (NTD) in rodent embryo cultures (Kubow et al 1993; Wentzel et al 1997). In this study, a hyperglycaemic medium led not only to retarded growth and NTD, but also to retarded differentiation of the otic system. On the other hand, glycine provided protection from hyperglycaemia-induced embryonic injury, preventing growth and differentiation retardation, and partially protecting from dysmorphogenesis, specifically from NTD affecting the telencephalic region. It has been proposed that the shape of the rostral head region (Klug 1991) and other NTD associated with hyperglycaemia (Pinter et al 1986) resulted from the neuroepithelium alteration. Although glycine and GABA receptors are ligand-dependent channels that mediate the synaptic inhibition in adult mammals, they are excitatory in the immature nervous cells of the embryonic stages (McDearmid et al 2006). The excitatory effect has been suggested to be mediated by an increase of Ca^{2+} influx through voltage-gated calcium channels (Belachew et al 2000). This neuronal signalling has been related with different processes of embryonic development, including differentiation, migration, neurite outgrowth, axonal growth, synaptogenesis and survival of nascent neurons (McDearmid et al 2006), which indicates that the presence of glycine is decisive for the development of the immature nervous system. On the other hand, since the amino acid had no preventive effect on retarded differentiation of the otic system, it was probable that this damage was produced by a different route.

Growth retardation may be generally defined as a delay of embryonic development. In this work high glucose exposure produced embryonic growth and differentiation reduction, which according to Klug (1991) was proportional growth retardation. Since glycine supplementation protected embryos from these effects, it could be interpreted that the amino acid protected against growth retardation caused by an excess of glucose. Whereas compounds preventing the hyperglycaemia-induced teratogenic effect such as superoxide dismutase, N-acetylcysteine (Wentzel et al 1997) and vitamins E and C (Cederberg et al 2001) only provide partial protection, glycine normalized the DNA content and the degree of differentiation of the hyperglycaemia exposed embryos. In another study, acetylsalicylic acid was partially protective against hyperglycaemia-induced growth retardation, NTD and deleterious effects on the morphological development of specific tissues in mouse embryo culture, apparently via its antiglycation properties (Kubow et al 1993). Nonetheless, in spite of the low concentration of acetylsalicylic acid used in that study, it did provide some embryotoxic effects.

Glycine has been found to react with glucose at physiological pH and temperature, and undergoes non-enzymatic glycation (Ramakrishnan & Sulochana 1993), acting as a glucose scavenger. This is the reason for studying the possible benefit of its use in preventing glycation (Carvajal et al 1999) and glycation-induced alterations, such as cataractogenesis (Ramakrishnan & Sulochana 1993), dyslipidaemia and the deleterious effects of increased glucose concentration in glomeruli (Alvarado-Vásquez et al 2003), as well as diabetes-induced embryolethality (Martínez et al 2003). Given the correlation between the extent of glycation of fetal tissue and a higher incidence of major congenital anomalies (Pollak et al 1988), as well as the positive relationship between glycated embryonic proteins and hyperglycaemia-induced dysmorphogenesis (Kubow et al 1993), the positive results obtained with glycine in this study might also have been due to its antiglycation effect. Indeed, it has been proposed that DNA glycation is associated with dysmorphogenesis in rodent models exposed to diabetic pregnancy or high-glucose cultures (Lee et al 1999).

Another hyperglycaemia-mediated mechanism involved in diabetic dysmorphogenesis is the excessive generation of oxygen free radicals resulting in the oxidation of lipids, DNA and other molecules (Wentzel et al 1997). Increased lipid peroxidation levels were measured in this study, in embryos in the hyperglycaemic medium, as the former condition probably leads to the higher incidence of defects in those embryos. In this work glycine supplementation to the hyperglycaemic medium prevented lipid peroxidation of embryonic tissues. It has been proposed that protein modification is diminished by lipid peroxidation, both by the reaction of glycine with reactive aldehydes such as malondialdehyde, and by the strong scavenging actions of active oxygen species carried out by the products of the glycine glucose reaction (Ogasawara et al 1994).

Further evidence had suggested that glycine acted as an iron chelator (Singh et al 1994) and protected umbilical cells from lethal damage caused by calcium and hydrogen peroxide (Weinberg et al 1992). Indeed, the copper–glycine complex has been described as having the ability to scavenge free radicals produced by gamma radiation (Jagetia et al 1993). Glycine also prevented nephrotoxicity induced by ischaemia–reperfusion (Mangino et al 1991) and superoxide production caused by alveolar macrophages (Wheeler & Thurman 1999). This evidence of the antioxidant and chelating effects of glycine provides a possible explanation for the mechanism of action of this amino acid in decreasing lipoperoxidation

induced by hyperglycaemia. However, since it has been suggested that the increase in the concentration of extracellular glycine caused by pre-existing hyperglycaemia on global cerebral ischaemia may have contributed to the exacerbation of the neurologic injury related to hyperglycaemia (Choi 1994; Choi et al 1994), it is necessary to proceed cautiously with the use of glycine in high glucose environments that coexist with ischaemia–reperfusion conditions.

In conclusion, glycine protected against growth retardation and partially protected embryos from NTD caused by hyperglycaemia in mouse cultured embryos. The protective mechanism of action of the amino acid may have been related to its properties of preventing oxidative damage. However, glycine might also have prevented hyperglycaemia-induced dysmorphogenesis via its antiglycation or other protective effects. Thus, further research is needed in this context.

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