

The Effects of Glycine Therapy on the Fetal Outcome of Diabetic Mice

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Abstract

The effect of glycine in preventing diabetes teratogenicity was studied in mice. Pregnant female animals were given an IP injection of 200 mg/kg of streptozotocin (STZ) on gestation day (GD) 7. Glycine was administered *ad libitum* from GD9 through GD19 (day of sacrifice) as a 1 and 2% drinking water solution. Nine whole-litter resorptions, a decreased number of live fetuses and fetal parameters, and an increase in HbA_{1c} levels in diabetic dams were found. Malformations were limited. Glycine had a protective effect from hemoglobin glycation and embryotoxicity due to STZ-induced diabetes. Also, resorptions and malformed fetuses were positively correlated to HbA_{1c} levels with a negative correlation to the number of live fetuses. The data suggest that glycine reduces the embryolethality of STZ-induced diabetes due to its anti-glycation effect.

Keywords: Diabetes, embryotoxicity, glycation, glycine, teratogenesis.

Introduction

Women with insulin-dependent (type 1) diabetes are at an increased risk of having spontaneous abortions or babies with major congenital malformations (Rosenn et al., 1994). The exact cause of these events is not known. Experimental (Eriksson et al., 1982) and clinical data (Rosenn et al., 1994; Suhonen et al., 2000), however, show an association between spontaneous abortions or malformations and maternal hyperglycemia in early pregnancy.

The mechanism whereby hyperglycemia induces dysmorphogenesis has not been completely clarified. However, several hypotheses have been proposed including sorbitol hyperaccumulation (Eriksson et al., 1987), deficient levels of

myo-inositol (Khandelwal et al., 1998) and arachidonic acid (Engström et al., 1991), altered trace metal status of fetuses (Uriu-Hare et al., 1989), increased oxygen free radical generation (Eriksson & Borg, 1991; Wentzel & Eriksson, 1998), and, recently, nonenzymatic glycation of proteins (Kubow et al., 1993).

On the other hand, glycine occurs in a relatively high proportion in proteins belonging to the collagen and elastin categories. The highest glycine content of any protein occurs in silk fibroin, i.e., close to 43% (Greenstein & Winitz, 1961). Several studies have shown that this amino acid, decreases protein glycation by scavenging glucose (Ramakrishnan & Sulochana, 1993; Carvajal-Sandoval et al., 1995). The purpose of this study was to explore the possibility of glycine preventing the teratogenic effect of diabetes by inhibiting protein glycation.

Materials and methods

Sixty virgin female CF-1 mice, weighing 25–30 g, were mated after placing them in cages with males between 6.00 and 9.00 am. The day when a positive spermatic plug was found was defined as gestational day (GD) 1. The maternal body weight was recorded on gestation days 1, 7, 10, 15 and 19. Mice were randomized to one of six groups: 1) non-diabetic controls (N), 2) non-diabetic controls receiving 1% glycine treatment (NG1), 3) non-diabetic controls receiving 2% glycine (NG2), 4) diabetic controls (D), and 5) and 6) diabetics receiving 1 and 2% glycine treatment, respectively (DG1 and DG2).

Diabetes was induced in 30 mice (experimental groups) with an IP injection of streptozotocin (STZ), 200 mg/kg of body weight (Sigma Chemical Co., St. Louis, MO), on GD

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7, and confirmed two days later by glucose in the urine, while control animals were injected with an equal volume of vehicle (citrate buffer, pH 4.6). Glycine (Sigma Chemical Co., St. Louis, MO) was administered *ad libitum*, from GD 9, as a solution added to the drinking water. Blood was obtained from the tail on GD 9 and 19 for glycosylated hemoglobin (HbA_{1c}) levels, using a DCA 2000 Analyzer (Bayer Co., Elkhart, IN). Blood glucose concentrations were measured on GD 19 with an Ames Glucometer (Bayer Co., Elkhart, IN). Animals were immediately killed by cervical dislocation and the whole uterus was removed. The number of implantation sites, resorptions, dead and live fetuses were recorded. Fetuses and placentas were weighed and measured, and the fetuses were inspected for external congenital malformations. Two thirds of offspring were subject to visceral examination for other anomalies by fixing them in Bouin's solution and sections were prepared as described by Wilson (1965a). The remaining fetuses were stained with Alcian blue and Alizarin red S. This allowed us to differentiate between cartilage and ossified skeleton (Peters, 1977).

Statistical comparisons of maternal weight during the pregnancy were made with ANOVA Two-Way Repeated Measures (one factor repetition). One-Way ANOVA or Ranked ANOVA were performed for the remaining parametric data. Fischer's Exact Test was used for statistical differences between groups in terms of malformations, and the Pearson Product Moment Correlation for a possible association between HbA_{1c} and the fetal outcome. Statistical analyses were made using the Sigma Stat software. Data are shown as the mean \pm SD.

Results

Figure 1 shows the diabetic groups having gained less weight than the normal groups ($p < 0.05$) during pregnancy. The glycine treatment had a positive effect on the weight gain of DG1 and DG2 animals. It became evident between GD15 and the day when the animals were killed before (GD19) or after removing the uterus (GD19w) when compared to group D, although there was still a difference regarding N mice.

The reproductive outcome in the different groups of pregnant mice is shown on Table 1. Diabetes had strong embryo lethal and toxic effects. Diabetic dams had more resorptions, fewer litters and live births, and smaller fetuses and placentas than those in group N. Glycine treatment protected from these toxic effects of the disease.

Streptozotocin induced a hyperglycemic state in the D groups. HbA_{1c} concentrations did not differ among groups at the beginning of diabetes (data not shown). By contrast, on GD 19, the D groups showed an increase in those levels, which glycine treatment tended to restore with the most pronounced preventive effect observed in group DG2 (Fig. 2).

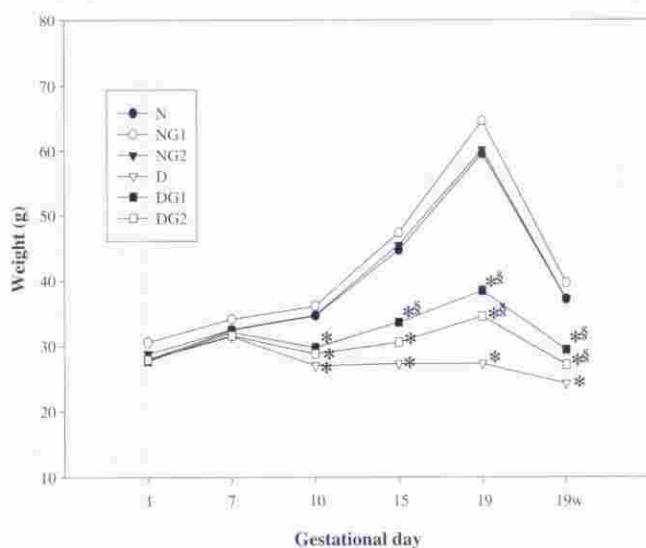


Figure 1. Maternal weight during pregnancy. Normal (N) and diabetic (D) mice were treated with 1 or 2% of glycine (NG1 and DG1, or NG2 and DG2, respectively). * $p < 0.05$ vs N, † $p < 0.05$ vs D.

The malformation rate in the fetuses of normal groups was low (Table 2). Upon visceral examination of the three fetuses from the single diabetic dam which had an offspring, one case of cleft palate and one case of hydronephrosis and hydrocephalus were found in just one of them. The incidence of external and visceral abnormalities in offspring of glycine treated diabetic dams was increased when compared to the Normal group. On the other hand, the only fetus which was examined for skeletal anomalies showed a widespread lack of osteogenesis as well as an additional rib. Even if the fetuses in groups DG1 and DG2 also showed additional ribs and lack of osteogenesis, the latter was just localized in sternbrae and occipital bones.

Finally, the correlation coefficients between HbA_{1c} levels and resorptions, the percentage of malformed and live fetuses were 0.685 ($P = 1.5 \times 10^{-9}$), 0.66 ($P = 2.7 \times 10^{-6}$), and -0.701 ($P = 4.56 \times 10^{-10}$), respectively.

Discussion

Several studies have found an association between the glycemic control in early diabetic pregnancy and the risk of congenital malformations and spontaneous abortions (Miodovnik et al., 1990; Rosenn et al., 1994; Suhonen et al., 2000) by using the glycohemoglobin level as an index of glycemic control, reflecting the metabolic control for roughly 6 weeks before measurement (Schleicher & Wieland, 1989). In this study, as in other reports, (Carvajal-Sandoval et al., 1995; Carvajal et al., 1999a,b), glycated hemoglobin is used to consider the rate of general glycation.

Torchinsky et al. (1997a) mated ICR mice 10 days after an IP injection of 240mg/kg STZ finding a resorption rate twice as high as that of their control group and a significant

Table 1. Influence of maternal diabetes and glycine treatment on reproductive outcome.

	Group					
	N	NG1	NG2	D	DG1	DG2
Pregnant mice	10	10	10	10	10	10
Litters	10	10	10	1*	5*	5*
Implantations	12.4 ± 1.7	13.9 ± 2.2	12.3 ± 1.7	10.7 ± 1.8	11.6 ± 2.6	13.3 ± 1.6
Resorptions	1.4 ± 1.6	1.1 ± 1.1	0.7 ± 0.8	10.3 ± 2.2*	6.5 ± 3.9* [§]	8.6 ± 4.0*
Live fetuses	11.0 ± 1.7	12.8 ± 2.3	11.6 ± 1.9	0.4 ± 1.3*	5.1 ± 5.4* [§]	4.6 ± 4.9* [§]
Placental weight (g)	0.11 ± 0.01	0.10 ± 0.01	0.19 ± 0.3	0.07 ± 0.005*	0.08 ± 0.01*	0.08 ± 0.04*
Placental diameter (cm)	0.79 ± 0.05	0.80 ± 0.03	0.78 ± 0.04	0.65 ± 0.06*	0.7 ± 0.05*	0.68 ± 0.04*
Fetal weight (g)	1.33 ± 0.07	1.34 ± 0.09	1.33 ± 0.08	0.58 ± 0.07*	0.88 ± 0.17* [§]	0.75 ± 0.12* [§]
Fetal crown-rump length (cm)	2.26 ± 0.10	2.28 ± 0.14	2.22 ± 0.09	1.62 ± 0.09*	1.93 ± 0.15* [§]	1.8 ± 0.09* [§]

Normal (N) and diabetic (D) mice were treated with 1% or 2% glycine (NG1 and DG1, or NG2 and DG2, respectively). **p* < 0.05 vs N, [§]*p* < 0.05 vs D.

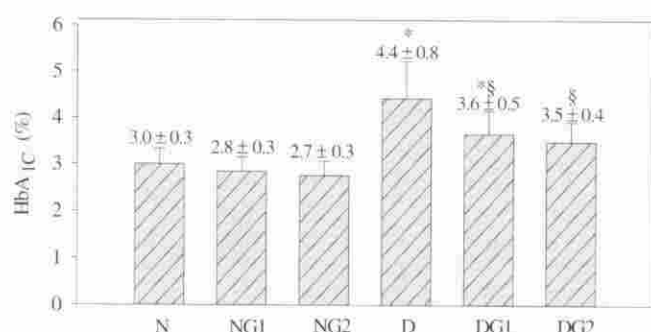


Figure 2. Effect of glycine on glycated hemoglobin concentration of diabetic pregnant mice on GD 19. Normal (N) and diabetic (D) mice were treated with 1 or 2% of glycine (NG1 and DG1, or NG2 and DG2, respectively). **p* < 0.05 vs N, [§]*p* < 0.05 vs D.

decrease in calcified ossification centers in the sternum and caudal vertebrae. Moreover, they considered glucose levels >27.8 mmol/l as the threshold for glucose-mediated teratogenesis in ICR mice. In our research, hyperglycemia (195.7 ± 31.8 mg/dl, vs 108 ± 7.3 mg/dl of N mice) induced by the STZ injection was much lower as compared to the rates reported by Torchinsky et al. Even so, a toxic effect on dams and fetuses was observed. Insufficient evidence of diabetes-induced congenital malformations was determined. However, a pronounced embryoletality was found by nine whole-litter resorptions, a rate even higher than the one found in other studies (Torchinsky et al., 1997b; Fein et al., 2002). Resorptions, according to Wilson (1965b), may provide a correlation on the extent of teratogenesis. Regarding the skeletal examination, a widespread lack of osteogenesis on the fetus of group D was seen. Again, a toxic effect of diabetes on offspring of CFI mice was determined, stronger than that reported by Torchinsky et al. (1997a). These differences between our results and those of Torchinsky could be explained at least in part by the susceptibility to STZ-induced diabetes of the strain used or by the toxicity of STZ itself on

the recently implanted embryos. Notice that we administered STZ on gestation day 7. When the injection was given before mating, mice remained in dioestrus, so we decided to induce diabetes after implantation had occurred.

On the other hand, there is evidence that non-enzymatic glycation of fetal tissue does occur under physiological conditions, and plays a role in altering the normal development of embryonic structural proteins (Pollak et al., 1988; Kubow et al., 1993). Furthermore, HbA_{1c} levels over 6 standard deviations have been suggested to produce malformed fetuses (Torchinsky et al., 1997a,b). In spite of those levels, our values for group D resulted mostly in resorptions.

Glycine, a nonessential amino acid, has been found to react with glucose at physiological pH and temperature and undergo non-enzymatic glycation (Ramakrishnan & Sulochana, 1993). It has been used as an anti-glycation agent (Ramakrishnan & Sulochana, 1993; Carvajal-Sandoval et al., 1995; Carvajal et al., 1999a,b). However, there are no reports on its use to prevent the teratogenic effects of diabetes. In our results, glycine treatment decreased the percentage of glycohemoglobin of diabetic mice, protecting them from maternal toxicity during pregnancy. In addition, the amino acid reduced embryoletality thus improving not only the viability of diabetic offspring, but also the fetal weight and crown-rump length. This improved fetal viability using glycine as an anti-glycation agent is supported by the positive and negative correlations of HbA_{1c} percentages of dams to the number of resorptions and viable fetuses, respectively. The increase in visceral malformations observed in offspring of glycine treated diabetic mice when compared to N mice could be explained by the improvement in fetal viability produced by glycine and by remembering the multifactorial etiology of dysmorphogenesis produced by diabetes (Reece et al., 1996). Finally, the positive correlation between glycated hemoglobin and the percentage of malformed fetuses supports the theory proposing glycation as a mechanism for hyperglycemia-induced dysmorphogenesis. The first support for this theory came from Pollak et al. (1988) who

Table 2. Incidence of malformations.

	Group					
	N	NG1	NG2	D	DG1	DG2
External abnormalities						
Litters (affected/total)	1/10	0/10	0/10	0/1	1/5	2/5
Fetuses (affected/total)	1/110	0/128	0/116	0/4*	1/51	4/46
Hematoma	1	0	0	0*	1	4*
Exencephalia	0	0	0	0	0	1
Visceral malformations						
Litters (affected/total)	1/10	1/10	2/10	1/1	4/5*	4/5*
Fetuses (affected/total)	1/77	1/88	2/80	1/3	12/35*	15/32*
Cleft palate	0	0	0	1*	6	9
Hydronephrosis	1	1	1	1	3	13
Renal hypertrophy	0	0	1	0	0	0
Renal atrophy	0	0	0	0	1	0
Ectopic kidney	0	0	0	0	1	0
Pelvic kidney	0	0	0	0	1	0
Hydrocephalia	0	0	0	1	0	0
Anophthalmia	0	0	0	0	0	1

Normal (N) and diabetic (D) mice were treated with 1% or 2% glycine (NG1 and DG1, or NG2 and DG2, respectively). * $p < 0.001$ vs N.

demonstrated a correlation between the extent of glycation of fetal tissue and a higher incidence of major congenital anomalies. More recently, Kubow et al. (1993) related glycosylated embryonic proteins to hyperglycemia-induced dysmorphogenesis using whole embryo cultures. This explained the fact that protein synthesis is accelerated in embryos. Thus the greater availability of lysyl groups leads to an enhanced glycation in hyperglycemic conditions. Glycation not only modified the structural and biochemical properties of proteins and consequently their biological role in physiologic processes (Brownlee et al., 1984), but glycation of DNA has also been proposed to be associated with dysmorphogenesis in rodent models exposed to diabetic pregnancy or high-glucose cultures (Lee et al., 1995, 1999).

In conclusion, this paper shows that maternal treatment of glycine reduces the embryo lethal effect and maternal toxicity of streptozotocin-induced diabetes in pregnant CF-1 mice. Further research regarding other possible mechanism(s) of protection by this amino acid and its teratogenic action, if any, is necessary.

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