Vol.55, n. 5: pp. 695-703, September-October 2012
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Effect of Oral Supplementation of the Linoleic and Gammalinolenic Acids on the Diabetic Pregnant Rats

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ABSTRACT

The aim of this work was to evaluate the direct protective action of oral fatty acid supplementation against the deleterious effect of hyperglycemia on maternal reproductive outcomes; fetal growth and development on female Wistar rats. The animals were distributed into four experimental groups: G1= non-diabetic without supplementation (Control group); G2= non-diabetic treated with linoleic (LA) and gammalinolenic acid (GLA) (1 mL of Gamaline-V/day); G3= diabetic without supplementation and G4= diabetic treated with LA and GLA. Diabetes was induced by streptozotocin (40 mg/kg). At day 21 of pregnancy, the gravid uterus was weighed and dissected to count the dead and live fetuses, resorption, implantation, and corpora lutea numbers. The fetuses were analyzed for external and internal anomalies. The treatment with Gamaline-V supplementation to diabetic rats interfered in the maternal reproductive outcome (reduced number of live fetuses and embryonic implantation); however, it protected the deleterious on the incidence of congenital anomalies caused by hyperglycemia.

Key words: diabetes, rat, pregnancy, anomalies, fatty acid supplementation, Gamaline-V

INTRODUCTION

Before insulin discovery, diabetic patients were dying before reaching the reproductive age, those who did not suffered a nearly 50% mortality rate during pregnancy. Despite the introduction of insulin and the great advances in the understanding of the pathogenesis of *Diabetes mellitus*, it is still associated with a high risk of complications, especially in the women with suboptimal glycemic control (Vitoratos et al. 2010).

Diabetes mellitus is one of the most common maternal illnesses resulting in anomalous offspring (Queisser-Luft et al. 2002; Farrel et al. 2002). Evers et al. (2004) found that the rate of congenital malformations was 8.9% in 324 women with type-1 *Diabetes mellitus* and 2.3% in 200,679 women of the general pregnant population. In another recent study, the incidence of congenital malformations was 37 per 1.000 in 372 women having type-1 *Diabetes mellitus*, in comparison with 14 per 1.000 in non-diabetic pregnant women (Kinsley et al. 2007). The main factors contributing to an increased perinatal mortality in diabetic pregnant women are the congenital anomalies of the fetus. Yang et al. (2006) reported

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that in 13 perinatal deaths, 5 (38%) were due to congenital anomalies of the neonate. These anomalies have become a serious problem with both the social and financial implications. Despite extensive human and animal studies, the precise pathogenesis remains unknown. Fetal malformations seen in diabetic pregnancies can be severe and affect various organs such as the eyes, ears, gastrointestinal system, urinary system, heart, and nervous system (Bartha et al. 2003). Experimental results support this notion of hyperglycemia as a teratogen, since high glucose levels (Dienelt and Zur Nieden 2010) or maternal diabetes *in vivo* as well as exposure to high glucose concentration or diabetic serum *in vitro* cause embryonic maldevelopment. In our laboratory, several studies using laboratory animals have been developed to study the pathophysiological mechanisms of severe diabetes in pregnant rats using a single dose of STZ (40 mg/kg) in the adulthood of the animal. Among these studies, it was found that diabetic rats showed hyperglycemia (glycemia > 300 mg/dL) (Damasceno et al. 2002, 2004; Rudge et al. 2007; de Souza et al. 2010), increased rates of loss of embryos after implantation (Rudge et al. 2007; de Souza et al. 2009, 2010), increased incidence of fetal abnormalities (Damasceno et al. 2002) and alterations antioxidant defense system (Volpato et al. 2008). Furthermore, rats with severe diabetes had increased levels of DNA damages (higher genotoxicity) in the presence or absence of pregnancy (Lima et al. 2007, 2008).

The precise cellular mechanisms causing diabetic embryopathy have not been completely clarified; however, several suggestions concerning the etiology of diabetic embryopathy have been proposed, including increased oxidative stress, decreased antioxidant defense, or both conditions existing simultaneously (Ornoy et al. 2010).

Some investigations reveal metabolic changes in diabetes associated to the production of prostaglandins. An imbalance in the synthesis of prostaglandins causes disturbances in fertility and teratogenesis (Higa et al. 2010). The higher biological activity of prostaglandins is derived from arachidonic acid, an essential fatty acid highly required throughout the gestation (Herrera, 2002). This component can be obtained from the diet or the synthesis from its precursor, the linoleic acid. The linoleic acid is synthesized only by the plants and is the most common polyunsaturated fatty acid found in nature. The first step of its biological activity is the delta-6-desaturation and production of gammalinolenic (GLA), which is rapidly converted in dihomogammalinolenic acid (DGLA). This, in turn, is converted more slowly in arachidonic acid. There is evidence that diabetic status slows the conversion of linoleic acid into their products, with consequent changes in the synthesis of prostaglandins (Eriksson and Borg 1991).

Currently, many benefits have been described for the linoleic acid for the animals and humans, such as in the treatment of cancer, oxidative stress, atherosclerosis, bone formation and composition in obesity, diabetes and immune system (Silveira et al. 2007). However, no studies showing the prophylactic action of fatty acids in diabetes-induced teratogenesis in the humans was investigated. Experimental studies are needed to approaching the clinical conditions to establish its application in a secure way. Therefore, the present study was designed to evaluate the direct protective action of oral fatty acid supplementation against the deleterious effect of hyperglycemia on maternal reproductive outcomes, fetal growth and development of the rats.

MATERIALS AND METHODS

Animals

Twelve-week-old female Wistar rats, weighing approximately 190-220g were obtained from UNESP - Univ Estadual Paulista. During the two-week acclimatization and the experimental exposure periods, the rats were maintained in an experimental room under controlled conditions (temperature of 22 ± 2ºC, relative humidity of 50 ± 10%, and a 12-hlight/dark cycle starting at 7:00 AM), with food (Purina® rat chow Brazil) and tap water *ad libitum*. The Ethics Committee for the Experimental Animal Research of the local Institute approved the protocols used in this study.

Experimental Procedure

Diabetogenesis period

Diabetes was induced by streptozotocin (STZ - SIGMA Chemical Company, St. Louis, Millstone). STZ was dissolved in citrate buffer (0.1M, pH 6.5) and administered by intravenous route at a dose of 40 mg/kg body weight. Non-diabetic rats only received citrate buffer. For inclusion criteria, the diabetic state was confirmed by glycemia > 300 mg/dL seven days after STZ injection by a One-

Touch Ultra Johnson & Johnson® glucometer (de Souza et al. 2010).

Experimental Groups
After diabetic state was confirmed, virgin female Wistar rats were mated overnight with non-diabetic male Wistar rats. The morning on which sperm were found in the vaginal smear was designated as day 0 of pregnancy (Damasceno et al. 2008). Pregnant rats were randomly distributed into four experimental groups (n minimum= 15 animals/group): G1= non-diabetic without supplementation (Control); G2 = non-diabetic treated with linoleic (LA) and gammalinolenic acid (GLA); G3 = diabetic without supplementation; and G4= diabetic treated with LA and GLA. The supplementation was orally given by gavage (1mL of Gamaline-V/day – Herbarium, containing 400mg LA and 180mg GLA) from day 0 to 14 of pregnancy. The control group received saline solution in similar condition to treatment of other experimental groups.

Course of pregnancy
Glycemia was measured at days 0, 7, 14 and 21 of pregnancy in all the experimental groups. At day 21 of pregnancy, the dams were anesthetized by sodium pentobarbital and humanely killed. The gravid uterus was weighed and dissected to count dead and live fetuses, resorption, implantation, and corpora lutea numbers. The number of implantation sites was determined by the Salewski method (Salewski 1964). The rate of pre-implantation loss was calculated as: total number of corpora lutea − total number of implantations ×100/total number of corpora lutea, and post-implantation loss rate was calculated as: total number of implantations − total number of live fetuses × 100/total number of implantations (Damasceno et al. 2008). The term fetuses were removed and weighed. The fetuses were classified by the mean ± 1.0 standard deviation (SD) according to the mean values of fetal weights of the control group: as small for pregnancy age (SPA) when weight was smaller than control mean minus (-) 1.0 SD; appropriate for pregnancy age (APA) when weight was included in control mean ± 1.0 SD; and large for pregnancy age (LPA) when weight was greater than control mean plus (+) 1.0 SD. The placental index was calculated by the placental and fetal weight rate (Calderon et al. 1992).

Fetal anomaly analysis
The fetuses were weighed and analyzed for the incidence of external anomaly. After external analysis, half of the fetuses were fixed in Bodian's fluid and serial sections were prepared for visceral examination as described by Wilson (Wilson, 1965). The remaining fetuses were prepared for skeleton examination by the staining procedure of Staples and Schnell (Staples and Schenell 1964).

Statistical Analysis
The data were presented as mean ± standard deviation. F test was applied to compare the mean glycemia and maternal weight among the groups. Chi-square test was applied to compare the proportion data and Tukey test for the maternal reproductive and fetal development parameters. For all the tests, the limit of significance established was $p<0.05$ (Zar 1999).

RESULTS

In the beginning of the experiment, there were 294 rats, but at the end, only 64 rats were pregnant at term. There was loss of 78% of female rats in function of lack of mating of the diabetic rats (G3 and G4) (data not shown). The maternal glycemic mean of diabetic groups (G3 and G4 groups) was higher than 300 mg/dL, confirming the hyperglycemic state. The fatty acid supplementation (Gamaline-V) did not interfere on the maternal glycemia of the rats (G2 and G4 groups). The fetal glycemic mean of the rats from G3 and G4 groups was higher than 300 mg/dL (Fig. 1) and it was correlated with the maternal glycemia.
The mean number of corpora lutea was lower in the diabetic group treated with LA and GLA (G4) in relation to the non-diabetic treated group (G2). The number of embryonic implantations was lower in the diabetic rats (G3 and G4 groups) as compared with G2 group. There was no statistical difference on the losses before and after implantation among the four experimental groups. The mean number of live fetuses per litter was lower in the diabetic group treated with fatty acids (G4) compared with non-diabetic groups (G1 and G2). No significant difference in the number of dead fetuses and in the number of resorptions was observed (Table 1). The fetal sex ratio did not present statistical difference when the diabetes status was induced and/or when there was

treatment with the fatty acid supplementation. The initial maternal weight was similar in all the groups, but at term pregnancy, the G4 group presented a lower final weight as compared with G1 and G2 (data not shown).

The litter weight (uterus with fetuses andattachments – gravid uterus weight) was significantly lower in the diabetic groups (G3 and G4) as compared with the non-diabetic groups (G1 and G2) (Table 1).

The maternal weight gain during pregnancy from G4 group was statistically lower in relation to other experimental groups (Fig. 2).

In G3 and G4 groups, the mean of fetal weight was lower and the mean of placental weight was higher. The placental index was consequently higher in the G3 and G4 groups. Higher incidence of SPA fetuses and lower rate of APA and LPA fetuses was observed in the G3 and G4 groups in relation to the G1 and G2 groups (Table 1).

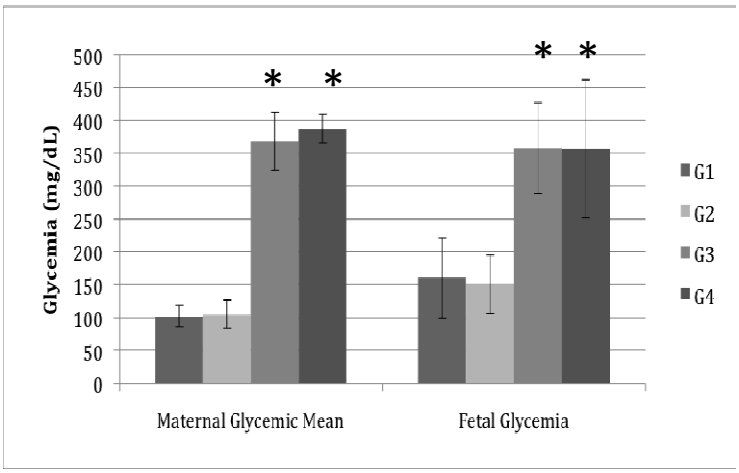

Figure 1 - Glycemic mean from non-diabetic and diabetic rats exposed or not to oral supplementation with fatty acids and from their respective fetuses. *p<0.05 - statistically significant difference as compared to G1 and G2 (F test).

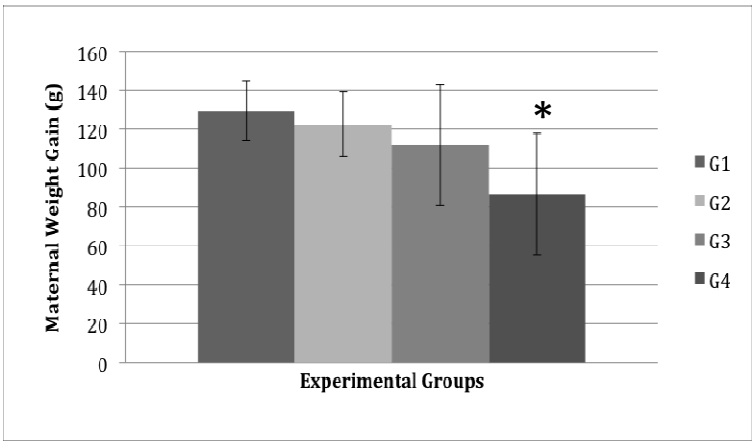

Figure 2 - Maternal weight gain mean from non-diabetic and diabetic rats exposed or not to oral supplementation with fatty acids. *p<0.05 - statistically significant difference compared to G1 and G2 (F test).

Table 1 - Maternal reproductive outcomes from non-diabetic and diabetic rats exposed or not to oral supplementation with fatty acids.

	Groups			
	G1	G2	**G3**	**G4**
Virgin rats (n)	35	40	107	112
Diabetic rats (n)	-	-	100	105
Mated rats (n)	21	20	20	19
Pregnant females (n)	17	15	17	15
Corpora lutea				
Total (n)	225	209	209	173
Mean ± SD	13.2 ± 1.8	13.9 ± 1.9	12.3 ± 1.8	11.5 ± 2.0$^{\#}$
Implantation				
Total (n)	211	195	186	159
Mean ± SD	12.4 ± 1.4	13.0 ±2.5	0.9 ± 1.3$^{\#}$	0.9 ± 1.1$^{\#}$
Live fetuses				
Total (n)	205	182	175	145
Mean ± SD	12.0 ± 1.3	12.1 ± 2.2	10.2 ± 1.8	9.6 ± 2.8*$^{\#}$
Dead fetuses				
Total (n)	1	0	2	1
Mean ± SD	0.06 ± 0.24	0.00 ± 0.00	0.12 ± 0.49	0.07 ± 0.26
Resorptions				
Total (n)	6	13	11	14
Mean ± SD	0.35 ± 0.61	0.87 ± 1.13	0.65 ± 1.00	0.93 ± 0.88
Pre implantation loss (%)[a]	6.2	6.7	11.0	8.1
Post implantation loss (%)[a]	2.8	6.6	5.9	8.8
Gravid uterus weight (g)	85.6 ± 9.6	84.5 ± 14.4	70.7 ± 11.8*$^{\#}$	63.1 ± 17.4*$^{\#}$
Fetal body weight (g)				
Mean ± SD	5.35 ± 0.33	5.30 ± 0.42	4.70 ± 0.60*$^{\#}$	4.40 ± 0.44*$^{\#}$
SPA Fetuses (%)[a]	18	26	66*$^{\#}$	88*$^{\#}$
APA Fetuses (%)[a]	60	58	31*$^{\#}$	11*$^{\#}$
LPA Fetuses (%)[a]	22	16	3*$^{\#}$	1*$^{\#}$
Placental weight (g)				
Mean ± SD	0.57 ± 0.07	0.55 ± 0.09	0.67 ± 0.11$^{\#}$	0.72 ± 0.21*$^{\#}$
Placental Index (g)				
Mean ± SD	0.106 ± 0.014	0.103 ± 0.012	0.146 ± 0.035*$^{\#}$	0.164 ± 0.049*$^{\#}$

Values expressed as mean ± standard deviation (SD) and proportion (%). Legend: G1 = non-diabetic without supplementation (Control group, n = 17); G2 = non-diabetic treated with linoleic and gammalinolenic acid (n = 15); G3 = diabetic without supplementation (n = 17); and G4 = diabetic treated with LA and GLA (n = 15).
*$p<0.05$ – statistically significant difference in relation to G1 group (Tukey test; [a]Chi-square test).
$^{\#}p<0.05$ – statistically significant difference in relation to G2 group (Tukey test; [a]Chi-square test).

In the external examination, 188 fetuses were analyzed in the G1 group, 167 in G2 group, 158 in G3 and 130 fetuses in G4 group. Of all of these, 113, 106, 95 and 72 fetuses were respectively analyzed for skeletal anomalies. There was a higher incidence of skeletal anomalies in the rats from G3 groups as compared with the control group (G1). The reduction in the number of skeletal abnormalities in the diabetic treated with LA and GLA (G4) was not sufficient to determine significant statistically differences as compared with the G3 group. The occurrence of cranial anomalies showed the predominance of G2 over the other groups. The number of malformed sternebrae was higher in G3 and G4 in relation to G2. The occurrence of anomalies in vertebrae was higher in G3 as compared with non-diabetic groups (G1 and G2) (Table 2).

In the G1 group, 75 fetuses were analyzed for visceral anomalies, 61 in G2, 63 in G3 and 58 in G4 group. There were losses of fetuses in all the experimental groups due to technical reasons which impaired internal analysis. Fetuses from G3 group presented a significant increase in the

incidence of visceral anomalies in relation to the G1, G2 and G4 groups. There was the presence of two hydropic fetuses in one rat from G3 group. The dams from G3 group presented fetuses with gastroschisis and cordis extrophya, hydrocephalia, cardiomegalia, septal defect and hydronephrosis. The supplementation with Gamaline-V in the diabetic rats (G4) decreased the incidence of these anomalies, approximating of those observed in the G1 and G2 groups (Table 2).

Table 2 - Frequency of fetal anomalies from non-diabetic and diabetic rats exposed or not to oral supplementation with fatty acids.

	Groups			
	G1	G2	G3	G4
External anomalies				
Number fetuses examined (litter)	188 (17)	167 (15)	158 (17)	130 (15)
Total number of fetuses with alteration	0 (0.0%)	0 (0.0%)	4 (2.5%)	1 (0.7%)
Hydropsia	0 (0.0%)	0 (0.0%)	2 (1.2%)	1 (0.7%)
Gastroschisis	0 (0.0%)	0 (0.0%)	1 (0.6%)	0 (0.0%)
Cordis extrophya	0 (0.0%)	0 (0.0%)	1 (0.6%)	0 (0.0%)
Skeletal anomalies				
Number fetuses examined (litter)	113 (17)	106 (15)	95 (17)	72 (15)
Total number of fetuses with alteration	59 (52.2%)	88 (83.0%)	86 (90.5%)	67 (93.1%)
Incomplete ossification of skull	12 (10.6%)	59 (55.7%)*	23 (24.2%)	22 (30.6%)
Dumbbell ossif. of vertebral centrum	7 (6.2%)	14 (13.2%)	42 (44.2%)*$^{\#}$	27 (37.5%)*$^{\#}$
Bipartite ossif. of vertebral centrum	12 (10.6%)	13 (12.3%)	35 (36.8%)*$^{\#}$	13 (18.1%)
Small rib	1 (0.9%)	1 (0.9%)	4 (4.2%)*$^{\#}$	2 (2.8%)
Supernumerary rib	9 (8.0%)	22 (20.8%)	45 (47.4%)*	33 (45.8%)*
Incomplete ossification of rib	9 (8.0%)	22 (20.8 %)	44 (46.3%)*	33 (45.8%)*
Incomplete ossification of sternebrae	23 (20.4%)	17 (16.0%)	56 (59.0%)$^{\#}$	54 (75.0%)$^{\#}$
Visceral anomalies				
Number fetuses examined (litter)	75 (17)	61 (15)	63 (17)	58 (15)
Total number of fetuses with alteration	1 (1.3%)	1 (1.6%)	8 (12.7%)*$^{\#\$}$	1 (1.7%)
Cardiomegaly	0 (0.0%)	0 (0.0%)	4 (6.3%)	0 (0.0%)
Septal deffect	0 (0.0%)	0 (0.0%)	1 (1.6%)	0 (0.0%)
Hydrocephaly	0 (0.0%)	0 (0.0%)	1 (1.6%)	0 (0.0%)
Hydronephrosis	1 (1.3%)	1 (1.6%)	2 (3.2%)	1 (1.7%)

Values expressed as proportion (%). Legend: G1 = non-diabetic without supplementation (Control group, n = 17); G2 = non-diabetic treated with linoleic and gammalinolenic acid (n = 15); G3 = diabetic without supplementation (n = 17) and G4 = diabetic treated with LA and GLA (n = 15).

*$p<0.05$ – statistically significant difference in relation to G1 group (Chi-square test).

$^{\#}p<0.05$ – statistically significant difference in relation to G2 group (Chi-square test).

$^{\$}p<0.05$ – statistically significant difference in relation to G4 group (Chi-square test).

DISCUSSION

In the present study, the negative effects of diabetes on the reproductive physiology have been studied. It was found that the diabetic group free of oral supplementation of fatty acid did not present alterations on the indicators of fertility. These parameters were similar to those from the control group. These findings contradict most of the experiments, which described an increased incidence of resorptions (early embryonic death) in the diabetic rats (Saito et al. 2010). However, Uriu-Hare et al. (1985) also found no interference of streptozotocin-induced diabetes in the number of resorptions corroborating with the present results. There was no alteration in the number of dead fetuses in the diabetic rats of this work. Padmanabhan et al. (1988) observed divergent results. Despite using the same strain of as in the present experiment, these authors applied the diabetogenic drug during the pregnancy, which might explain this difference.

The incidence of losses before and after the embryonic implantation observed in the diabetic and non-diabetic groups was consistent with the literature data (Saito et al. 2010; de Souza et al. 2009). The low incidence of these losses in the diabetic rats indicated unaltered reproductive performance in these groups of animals, impacting directly on the pregnancy rate. The post-implantation losses are common in women with

type-1 *Diabetes mellitus*, which presents no adequate glycemic control in periconceptional period. This fact is evidenced by higher rates of early pregnancy losses in these patients (Golbert and Campos, 2008).

The supplementation of LA and GLA (Gamaline-V) caused reduction in the number of corpora lutea, implantations and live fetuses per litter in diabetic dams. It seemed valid to speculate that the fatty acid supplementation could alter the process of ovulation and embryonic fixation in the uterus, impeding fetal development in these dams. However, Gamaline-V treatment did not damage the maternal reproductive outcome in the non-diabetic rats.

The final maternal weight and weight of the uterus may suffer interference from the number of fetuses obtained at the end of pregnancy. Then, the treatment with Gamaline-V had negative effects on maternal weight. In contrast, Poulos et al. (2001) observed no changes in weight gain in the rats with daily supplementation of a conjugated linoleic acid (CLA) diet (6.5 g/100g soybean oil and 0.5 g/100g CLA) from days 7-21 of pregnancy.

In this study, severe diabetes determined a decrease of fetal weights and higher incidence of fetuses small for pregnancy age (SPA). Other experiments showed lower body weight of fetuses in the diabetic group (de Souza et al. 2009). The severe hyperglycemia exhausted fetal pancreatic-endocrine activity, reducing insulin secretion, and consequently impaired fetal growth, which led to intrauterine growth restriction (IUGR) at the end of pregnancy. The present study showed an increased placental weight in the diabetic animals, corroborating with studies in the literature (de Souza et al. 2009; Iessi et al. 2010). The effects of maternal diabetes on the placental flow suggested that the fetus developed into hypoxia and malnutrition. The increase in the placenta would encourage the supply of oxygen and nutrients, increasing the area of maternal-fetal exchanges. However, Calderon et al. (1992) demonstrated in histological analysis that the increase in the placental weight was associated to impairment of fetal nutrition by increasing the thickness of the placental membrane. The present results also showed that diabetic rats showed a dysfunction in the placental index, confirming literature data (de Souza et al. 2010). After treatment with the gamaline-V, the non-diabetic rats presented low maternal weight gain that was associated to a poor maternal adaptation, damaging fetal development.

This finding might explain the high incidence of cranial anomalies in G3 group. However, this explanation was not valid to justify the anomalies found in the sternebrae. In this region, as well as the skull, there was a higher incidence of reduced ossification, but the involvement of sternebrae was more common in the diabetic groups. The supernumerary rib was most frequent anomaly in this region and, in the vertebrae, there was the occurrence of bipartite ossification centers. These anomalies were more common in the diabetic dams. The visceral anomalies were more frequent in the diabetic rats, similar to results observed in other studies (Wilson 1985; Padmanabhan and Al-Zuhair 1988). The use of the gamaline-V decreased the incidence of these anomalies in the fetuses from G4 group, which showed similar data to those observed in the control groups.

This study demonstrated a reduction in the incidence of congenital anomalies in the experimental diabetes after the administration of gamaline-V. The literature also showed that the supplementation of arachidonic acid or its precursors in the diabetic rats not only prevented the malformations in embryos (Reece et al. 2006), but also in the fetuses at term.

In conclusion, this study demonstrated that the treatment with the fatty acid supplementation (gamaline-V) to the diabetic rats interfered in the maternal reproductive outcome (reduced number of live fetuses and embryonic implantation). However, it reduced the incidence of congenital anomalies caused by the hyperglycemia.

ACKNOWLEDGMENTS

The authors acknowledge José Alexandre do Prado and Biologist Azize Cristina Capelli Nassr for their technical assistance.

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Received: December 16, 2010;
Revised: June 06, 2011;
Accepted: January 15, 2012.

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