INFLUÊNCIA DO MEIO HIPERGLICÊMICO INTRAUTERINO EM DIFERENTES FASES DE VIDA DOS DESCENDENTES DE RATAS

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Dissertação apresentada à Faculdade de Medicina de Botucatu – Unesp, Programa de Pós-Graduação em Ginecologia, Obstetrícia e Mastologia. Área de concentração: Tocoginecologia, para obtenção do título de Mestre.

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"[...]Pode ser que um dia tudo acabe... Mas, com a amizade construiremos tudo novamente, cada vez de forma diferente. Sendo único e inesquecível cada momento que juntos viveremos e nos lembraremos para sempre..."

Albert Einstein

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SOMATOSTATIN, GLUCAGON AND INSULIN IN DIABETIC PREGNANT RATS AND THEIR OFFSPRING

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ABSTRACT

Background: We hypothesized that not only insulin is relevant for embryofetal development and diabetes-derived alterations as well the glucoregulatory hormones glucagon and somatostatin. To investigate this possibility in rat pregnancy and in their offspring, we have compared glycemia, insulin, glucagon and somatostatin in late pregnancy and postnatal period to associate with maternal reproductive outcomes of diabetic rats.

Methods: Wistar rats were randomly assigned into: Non diabetic (C); Mild diabetes (MD); and Severe diabetes (SD). Diabetes was induced in rats by streptozotocin. The female rats were mated and two experiments to evaluate pancreatic hormone were conducted: death of dams and their respective fetuses at day 21 of pregnancy and at day 10 postpartum (newborn assessment).

Results/Discussion: The SD and MD rats presented impaired reproductive performance. In general, the rats presenting severe diabetes and their offspring showed metabolic desarrangement confirmed by reduced body weight and alterations in the pancreatic hormone levels. In the presence of mild diabetes, the offspring presented similar metabolic response to those of their dams against maternal hyperglycemic insult.

Conclusion: The diabetes decreased embryofetal competence and caused alterations in the glucoregulatory hormones (glucagon and somatostatin), which showed that somatostatin might be the hormone more susceptible to these changes. This study demonstrates the importance in studying glucagon and somatostatin more profoundly, because of these hormones could be predictor factors of the adverse results in the adult life.

Key words: diabetes, insulin, glucagon, somatostatin, rat, pregnancy

INTRODUCTION

Diabetes mellitus (DM) is a group of diseases characterized by high blood glucose levels that result from defects in the ability of body to produce and/or use insulin [1]. There is evidence that the diabetic intrauterine milieu and early postnatal nutritional, metabolic, and hormonal environment may cause predispositions to the development of disorders and diseases in later life [2-7].

The intrauterine environment balanced is essential for normal development of the fetuses. Different mechanisms (gene expression, epigenetic, metabolic pathways) may act in the embryonic environment in early development, compromising their future health [8]. Epigenetic modifications of the genome provide a mechanism that allows the stable propagation of gene-activity states from one generation of cells to the subsequent. Epigenetic states can be modified by environmental factors, which might contribute to the development of abnormal phenotypes. The preimplantation embryo is particularly sensitive to epigenetic modifications that might permanently alter the phenotype in the adult [9].

Pregnancy is characterized by the complex hormonal interaction, which are important environment-dependent organizers of the developing neuro-endocrine-immune network that finally regulates all fundamental processes of life. There are evidences that cell-to-cell interactions are required for the normal secretory function of the endocrine pancreas [10]. When present in non-physiological concentrations during 'critical periods' of perinatal life, induced by alterations in the intrauterine or neonatal environment, hormones can act as 'endogenous functional teratogens' [11]. Studies in offspring of diabetic mothers have paradigmatically contributed to the perception of this developmental principle and understanding of causal mechanisms [6].

In vitro studies demonstrate that the administration of analogues of the pancreatic hormones (insulin, glucagon and somatostatin) are used to investigate the mechanisms involved in the synthesis and secretion of these hormones individually for use in treating diseases such as cancer and diabetes [12-14]. However, the mechanisms as these hormones interact under the endogenous point of view are still unclear. We hypothesized that not only insulin is relevant for embryofetal development and diabetes-derived alterations as well the glucoregulatory hormones glucagon and somatostatin. To investigate this possibility in rat pregnancy and their offspring, we have compared

glycemia, insulin, glucagon and somatostatin in late pregnancy and postnatal period to associate with maternal reproductive outcomes of diabetic rats.

MATERIALS AND METHODS

Animals and experimental design

Female and male Wistar (CEMIB – UNICAMP, Campinas – São Paulo State – Brazil) rats, 12-13 weeks of age, were housed in a certified animal care and food and water were provided *ad libitum*. All experimental procedures were approved by the Ethics Committee on Animal Experiments of the Botucatu Medical School – UNESP (Protocol Number 789). Wistar rats were maintained under controlled conditions (temperature 22±2°C, humidity 55±5% and 12h light/dark cycle). A total of 436 rats were randomly assigned into three experimental groups: Non diabetic (Control group – C); Mild diabetes (MD); and Severe diabetes (SD).

Parental non-diabetic female rats were mated with non-diabetic males to obtain newborns. For the induction of mild diabetes (glycemia 120-300 mg/dL), newborn female rats received streptozotocin (STZ - SIGMA Chemical Company, St. Louis, MO, USA), a beta (β)-cytotoxic agent, diluted in citrate buffer (0.1 M; pH 4.5) at a dose of 100 mg/kg on the first day of life by subcutaneous administration [15]. Newborn rats remained with their mothers until day 21 of life (weaning period). Glucose tolerance test (GTT) was performed at day 75 of life according to Campos et al. [16] to assess the development of altered glucose metabolism and used as a criterion for maintaining the rats in the respective group. For MD rats that presented glycemia higher than 140 mg/dL in more than two timepoints during GTT continued in the experiment. Glucose responses during the GTT were evaluated by estimation of the total area under the curve, using the trapezoidal method [17]. SD was induced in the adult rats with STZ and the drug was administered by intravenous injection at a dose of 40 mg/kg body weight. For inclusion criteria, the diabetic state was confirmed by a blood glucose concentration >300 mg/dL 7 days after STZ injection by a One-Touch Ultra glucometer (Johnson and Johnson®). In the C group, female received only citrate buffer and rats with glycemia below 120 mg/dL were considered non-diabetic and were included in this study.

In adult life, all groups (C, MD and SD) were mated overnight with non-diabetic male rats. The morning when spermatozoa were found in the vaginal smear was designated gestational day 0. On days 0, 7, 14 and 21 of pregnancy, maternal body

weights and glycemia were determined. Blood glucose concentrations were measured by a One-Touch Ultra glucometer (LifeScan, Johnson and Johnson®, Milpitas, CA, USA). Values were expressed in mg/dL. Because of the limited availability of plasma, it was necessary to conduct two experiments to evaluate pancreatic hormones: Experiment I – the rats were killed at day 21 of pregnancy (at term) and Experiment II – the rats were killed at day 10 postpartum.

On day 21 of pregnancy, fed rats were anesthetized with sodium thiopental (Thiopentax® 50 mg/kg) and decapitated for the collection of maternal blood for the assessment of glycated hemoglobin (HbA1c), plasma somatostatin, glucagon and serum insulin levels. Immediately following exploratory laparotomy, all viable fetuses and placentas were weighed for determination of placental index (placental weight/fetal weight). In the lack of visible implantation sites, the uterine corns were stained with a preparation of 10% ammonium sulphate [18]The fetuses were anesthetized with sodium thiopental and decapitated for the collection of blood for the assessment of same parameters of their mothers. Besides, the pancreas was then carefully removed, weighed on a balance sensitive and minced with scissors in about 1 mL of acid-alcohol before being sonicated for 10 seconds and subsequently extract was centrifuged at 2000 x *g* for 2 min and the supernatant removed and stored at -20°C until assayed for hormone content.

Assay for HbA1c determination

The maternal blood samples were collected in tubes containing EDTA tubes and analyzed by method of high-performance liquid chromatography in a specialized laboratory.

Assay for insulin, somatostatin and glucagon determinations

Samples were collected from maternal, fetal (at term pregnancy) and newborn (day 10 postpartum) blood in anticoagulant-free tubes to obtain serum and in other one containing EDTA and aprotinin to obtain plasma. Serum samples were used to measure the levels of insulin (kit protocol number: 90060; Crystal Chemical®, USA) and plasma samples were used for determination of levels of somatostatin (kit protocol number: 601971; Phoenix Pharmaceuticals, Inc.®, California, USA) and glucagon (protocol number: 602308; Phoenix Pharmaceuticals, Inc.®, California, USA) by ELISA according to their respective protocol.

Statistics

Results were presented as mean ± standard error. Gamma and Poisson distributions were performed when the data presented no normal distribution distribution (Gauss curve) such as number of corpora lutea, implantation, dead fetuses, fetal and placental weight, placental index, maternal, fetal and newborn glycemia, total pancreatic insulin, GTT and glycated hemoglobin. For comparison of the percentage of fetal viability and number of rats presenting post or pre implantation embryonic loss among the groups, Fisher's Exact Test was applied. Student Newman Keuls Test was performed to compare the number of live fetuses, litter weight and maternal, fetal and newborn serum insulin levels. Test of Multiple Comparison of Tukey was applied for comparison of the maternal weight gain, maternal, newborn and pancreas weight, and maternal, fetal and newborn plasma glucagon and somatostatin levels. For comparison of the of the fetal classification among the groups, Chi-square test was applied. A p value of less than 0.05 was considered significant.

RESULTS

Figure 1 displays the glycemic curves for oral glucose tolerance test (GTT) and the area under the curve (AUC) from different groups at day 17 of pregnancy. Experimental groups, mild diabetes (MD) and severe diabetes (SD), presented a significant increase ($p<0.05$) in the mean glycemia values at all timepoints measured. The AUC was higher in MD and SD groups compared with that of C group.

Figure 2 shows of the maternal glycated hemoglobin levels from different experimental groups. It was observed that the SD group presented higher glycated hemoglobin level in relation to that from C and MD groups.

Table 1 represents reproductive outcomes of rats with positive diagnosis of pregnancy of MD, SD and C groups. The dams of SD group showed a significant increase in the number of rats presenting post and pre implantation embryonic loss compared with those of MD and C groups.

Table 2 shows maternal reproductive performance of MD, SD and C groups. No significant statistically difference was evidenced in the corpora lutea and implantation number among different experimental groups. The rats of the SD group presented an increased number of dead fetuses and decreased number of live fetuses in relation to C

group and it was observed that the rats of the SD and MD groups presented decreased rate of the fetal viability in relation to those of C dams. The maternal weight gain of the SD rats was significantly lower compared with thouse of other groups (MD and C). There was a decrease in the litter weight of the MD and SD groups when compared with that of C group. The fetal weight mean of the SD rats was lower compared to mean of MD and C groups. The MD group showed a decrease of placental weights compared with those of the C group. There was significant increase of placental weights and indexes in the SD group relation to those found in the MD and C groups.

The SD group showed a significant increase in the proportion of fetuses classified as small for pregnancy age (SPA) and significant reduction of fetuses classified as appropriate for pregnancy age (APA) in relation with those found in the MD and C groups. There were decreased rates in fetuses classified as large for pregnancy age (LPA) in the SD group compared with those of other groups (MD and C). MD group showed an increased rate of fetuses SPA and decreased percentage of fetuses APA compared to those of C group (Figure 3).

The body weight of the MD dams was significantly decreased when compared with C group. In the dams, fetuses and newborns of the SD group, the body weights were significantly lower compared with those of the MD and C groups. The maternal and fetal glycemia of SD group showed a significant increase and serum insulin was decreased in relation to those of the other groups. At day 10 post partum, the newborn presented decreased blood glucose level and unaltered serum insulin levels compared to those of the MD and C groups. The total pancreatic insulin levels were reduced in the newborn of SD dams compared to those of other groups. The plasma glucagon and somatostatin levels were decreased in the MD dams and fetuses when compared with those of C group. In the newborns there was no change in the plasma glucagon levels but the plasma somatostatin levels decreased compared to those of C group. In the SD group the maternal plasma somatostatin and glucagon determinations were unaltered. In their fetuses, only glucagon level reduced, and in the newborn the somatostatin levels decreased compared to those of C group. The pancreas weight were not significantly different among experimental groups (Table 3).

DISCUSSION

In this study, glucose tolerance testing (GTT) demonstrated that rats with diabetes induced at adulthood had severe hyperglycemia (glycemia > 400 mg/dL – SD group), and glucose intolerance that led to increase in the area under the curve (AUC). On the other hand, despite not showing mean glycemia between 120 and 300 mg/dL, the rats with diabetes induced at birth (MD group), had glycemia > 140mg/dL on at least 2 timepoints and larger AUC compared to control confirming glucose intolerance. These results are in line with previous studies conducted at our laboratory [15, 18-25] and other literature reports [26-28], as well as those obtained in women with overt and/or gestational diabetes showing abnormal glucose levels in GTT during pregnancy [29].

Maternal hyperglycemia during the early stages of embryo development can cause physiological and metabolic changes that may be responsible for abnormalities that manifest later as adverse outcomes in diabetic pregnancies [30]. The reproductive performance of diabetic women before use of insulin does not differ from that of laboratory animals [31]. In this study, the number of of live fetuses was smaller, and the rates of embryo loss before and after implantation were higher in the SD group. Among MD rats, although only glucose intolerance was present, the rate of fetal viability was also reduced. Women with poorly controlled diabetes have a much higher incidence of reproductive problems, and neonatal morbidity and mortality [32-34]. High abortion rates are associated with the toxic effect of high glucose levels on fetal viability. In this study, maternal hyperglycemia reduced weight gain in rats with severe diabetes, as well as in the offspring of both diabetic groups, as reported by Souza et al. [35] and Sinzato [36].

Among severely diabetic rats, placental weight was higher resulting in increased placental index. The increase in placental weight might have served as a compensatory mechanism to ensure maternal-fetal exchange. However, it did not contribute to improve fetal development. As a matter of fact, the fetuses from this group showed intrauterine growth restriction, evidenced by the percentage of fetuses classified as small for pregnancy age (SPA). In the MD group, there was no difference in fetal weight and placental index, but placental weight was lower and the number of fetuses classified as SPA was increased. This is likely to be due to hyperglycemia-related changes in placental development that affect placental function and cytoarchitecture,

and thus impair maternal-placental-fetal exchanges. Despite several differences in morphology and development, the changes induced by maternal diabetes in rodent and human placentas are compatible. The degree of fetal damage, placental dysfunction, and availability/use of substrates by the fetus may lead to macrosomia or intrauterine growth restriction [37], as observed herein. Therefore, it is important to highlight that controlling glycemia since early pregnancy is essential for the development of the placenta and embryo-fetal growth.

Maintaining glycated hemoglobin (HbA1c) levels within the normal range is one of the major goals in diabetes control [38]. Several works have demonstrated the importance of HbA1c assessment in determining the risk of chronic diabetes complications [39]. HbA1c levels were higher in the SD group compared to other groups, and were associated with maternal reproductive performance complications.

Changes in the intrauterine environment, such as diabetes, can also have long-term effects on the function and structure of pancreatic islets [40,41]. In the severely diabetic rats studied here, serum insulin levels were reduced while plasma glucagon and somatostatin levels remained unchanged, which might have aggravated hyperglycemia. On the other hand, in the rats with mild diabetes, no changes in glycemia and serum insulin levels were observed by the end of pregnancy, but plasma glucagon and somatostatin levels were lower. It is possible that, as streptozotocin altered the beta cells, the other cells (alpha and delta) of the pancreatic endocrine tissue underwent rearrangement changing the cytoarchitecture of the islet cells reducing paracrine signalling among them.

In the offspring of rats with severe diabetes, plasma somatostatin levels were unchanged whereas plasma glucagon and serum insulin (hypoinsulinemia) levels were decreased. Indeed, fetal hypoinsulinemia results from beta-pancreatic cell exhaustion due to maternal and fetal hyperglycemia [42] that are associated with increase in the rate of intrauterine growth restriction as observed in this study. The weight reduction seen in the offspring of rats with severe diabetes persisted through the neonatal period. The offspring of rats with MD, in turn, showed unchanged serum insulin levels while plasma glucagon and somatostatin levels were decreased. The mechanisms that might explain these findings remain unclear.

Ten days after birth, serum insulin and plasma glucagon levels did not differ between both diabetic groups. However, the total concentration of pancreatic insulin was decreased in the SD group, once more highlighting the role of insulin as a growth

hormone. Plasma somatostatin levels remained reduced in the offspring of diabetic rats (MD and SD). Somatostatin is indirectly involved in glycemia regulation by allowing fine adjustments in the control of insulin and glucagon secretion [43].

In conclusion, diabetes decreased embryofetal competence and caused alterations in glucoregulatory hormones (glucagon and somatostatin), especially in somatostatin which seemed to be more susceptible to these changes. Overall, the rats with severe diabetes and their offspring showed different metabolic changes in response to hyperglycemia while rats with mild diabetes and their offspring responded similarly. Further studies focusing on glucagon and somatostatin are necessary as these hormones may be used as predictors of adverse effects at adulthood.

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TABLES

Table 1. Reproductive development of rats with positive diagnosis of pregnancy of mild (MD) and severe (SD) diabetic and control (C) groups.

	Groups		
	C (n=62)	**MD (n=32)**	**SD (n=122)**
Number of rats presenting post implantation embryonic loss	4	9	43*#
Number of rats presenting pre implantation embryonic loss	0	0	27*#

Values expressed as total number.
*p<0.05 - statistically significant difference in relation to C group; # statistically significant difference in relation to MD group (Fisher Exact Test).

Table 2. Reproductive maternal performance at the end of pregnancy, fetal and placental weight and placental index of mild (MD) and severe (SD) diabetic and control (C) groups.

	Groups		
	C (n=30)	**MD (n=20)**	**SD (n=48)**
Number corpora lutea[a]			
Total	367	242	576
Mean ± SD	12.23±1.55	12.10 ± 1.55	12.00 ± 2.09
Number implantation[a]			
Total	341	219	505
Mean ± SD	11.37 ± 1.52	10.95 ± 3.15	10.52 ± 2.89
Number of live fetuses[b]			
Total	330	195	456
Mean ± DP	11.00 ± 1.60	9.75 ± 2.73	9.50 ± 3.11*
Number of dead fetuses[a]			
Total	0	2	11*
Fetal viability (%)[c]	96.77%	89.04%*	90.29%*
Maternal weight gain (g)[d]	119.77 ± 30.98	101.50 ± 19.65	73.02 ± 28.82*[#]
Litter weight (g)[b]	82.16 ±14.45	69.55 ± 18.71*	66.16 ± 22.85*
Fetal weight (g)[e]	5.39 ± 0.39	5.10 ± 0.46	4.27 ± 0.64*[#]
Placental weight (g)[e]	0.54 ± 0.13	0.47 ± 0.09*	0.74 ± 0.22*[#]
Placental index (g)[e]	0.10 ± 0.02	0.09 ± 0.02	0.18 ± 0.06*[#]

Values expressed as mean ± standard deviation and proportion (%).
*$p<0.05$ – statistically significant difference in relation to C group; [#]statistically significant difference in relation to MD group. [a] Poisson Distribution; [b]Student Newman Keuls Test; [c]Fisher Exact Test; [d]Test of Multiple Comparison of Tukey; [e]Gamma Distribution.

Table 3. Body and pancreas weight, glycemia, prancreatic hormone concentrations (serum insulin, total insulin and plasma glucagon and somatostatin) of dams, fetuses and newborns of mild (MD) and severe (SD) diabetic and control (C) groups.

	Groups		
	C	**MD**	**SD**
Maternal			
Body weight (g)[a]	363.74±31.30	338.05±25.88*	303.77±37.61*[#]
Glycemia (mg/dL)[b]	84.93±10.88	86.10±16.14	562.29±73.92*[#]
Serum insulin (ng/mL)[c]	1.37±0.09	1.33±0.24	0.75±0.03*[#]
Glucagon (ng/mL)[a]	3.34±0.45	0.50±0.12*	2.44±0.30[#]
Somatostatin (ng/mL)[a]	0.85±0.18	0.30±0.05*	0.88±0.22[#]
Fetal			
Body weight (g)[b]	5.39 ± 0.39	5.10 ± 0.46	4.27 ± 0.64*[#]
Glycemia (mg/dL)[b]	86.58±24.66	61.50±8.93	340.02±33.42*[#]
Serum insulin (ng/mL)[c]	2.72±0.56	2.06±0.62	0.21±0.90*[#]
Glucagon (ng/mL)[a]	1.46±1.07	0.16±0.07*	0.34±0.14*
Somatostatin (ng/mL)[a]	0.60±0.11	0.10±0.04*	0.54±0.04[#]
Newborn			
Body weight (g)[a]	20.15±3.07	18.18±2,94	12.96±2.39*[#]
Glycemia (mg/dL)[b]	124.21±16.10	121.43±13.08	98.9±26.09*[#]
Serum insulin (ng/mL)[c]	0.25±0.05	0.37±0.13	0.31±0.05
Total pancreatic insulin (ng/mL)[c]	4.69±0.61	4.08±1.03	2.78±0.42*[#]
Glucagon (ng/mL)[a]	1.68±0.68	1.37±0.46	1.71±0.73
Somatostatin (ng/mL)[a]	2.22±0.21	1.57±0.16*	1.47±0.04*
Pancreas weight (g)[a]	0.04±0.02	0.04±0.01	0.03±0.01

Values expressed as mean ± standard deviation.
*$p<0.05$ – statistically significant difference in relation to C group. [#]statistically significant difference in relation to MD group. [a]Test of Multiple Comparison of Tukey; [b]Gamma Distribution; [c]Student Newman Keuls Test.

FIGURES

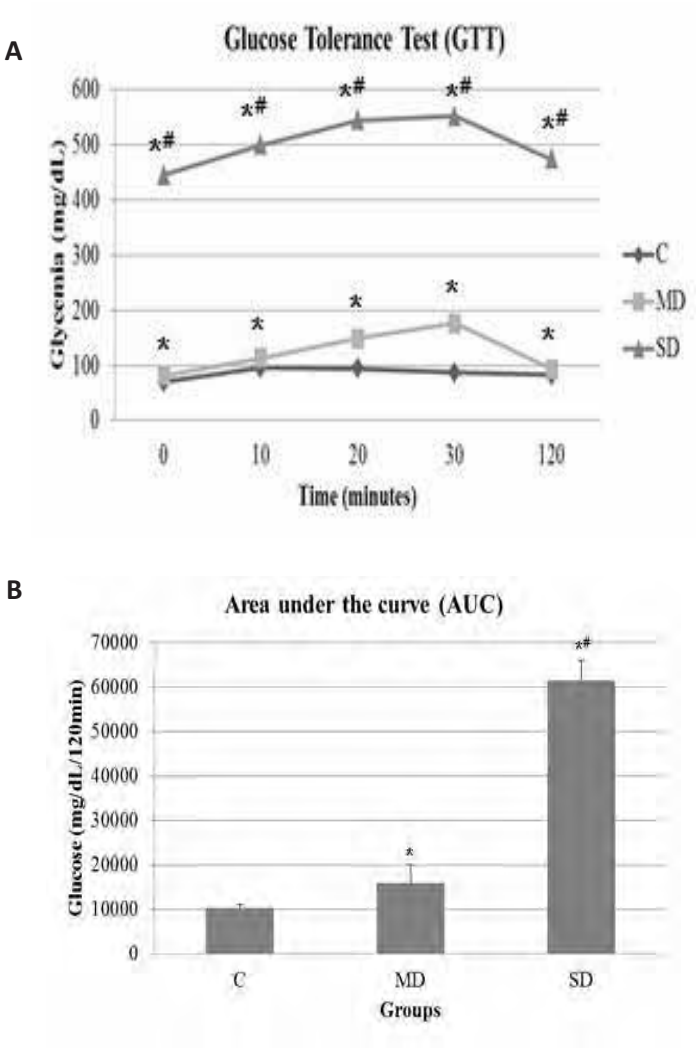

Figure 1. Oral glucose tolerance test (GTT – Figure A) and the area under the curve (AUC – Figure B) of control (C), mild diabetes (MD) and severe diabetes (SD) groups on day 17 of pregnancy. Data were reported as mean ± standard deviation. *$p<0.05$ - statistically significant difference in relation to C group; $^{\#}$statistically significant difference in relation to MD group (Gamma Distribution).

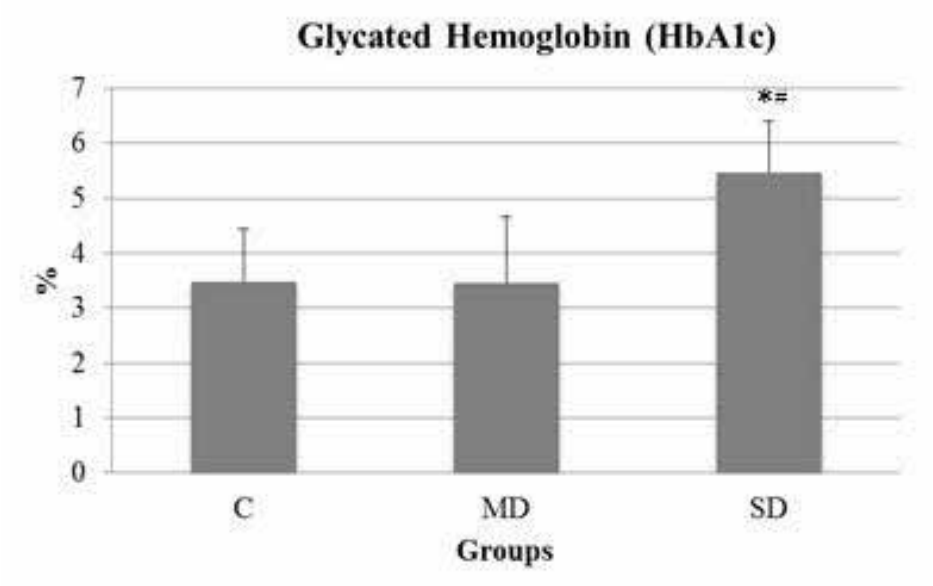

Figure 2. Percentage of glycated hemoglobin of mild (MD) and severe (SD) diabetic and control (C) groups.Values expressed as mean ± standard deviation. * $p<0.05$ - statistically significant difference in relation to C group. $^{\#}$statistically significant difference in relation to MD group (Gamma Distribution).

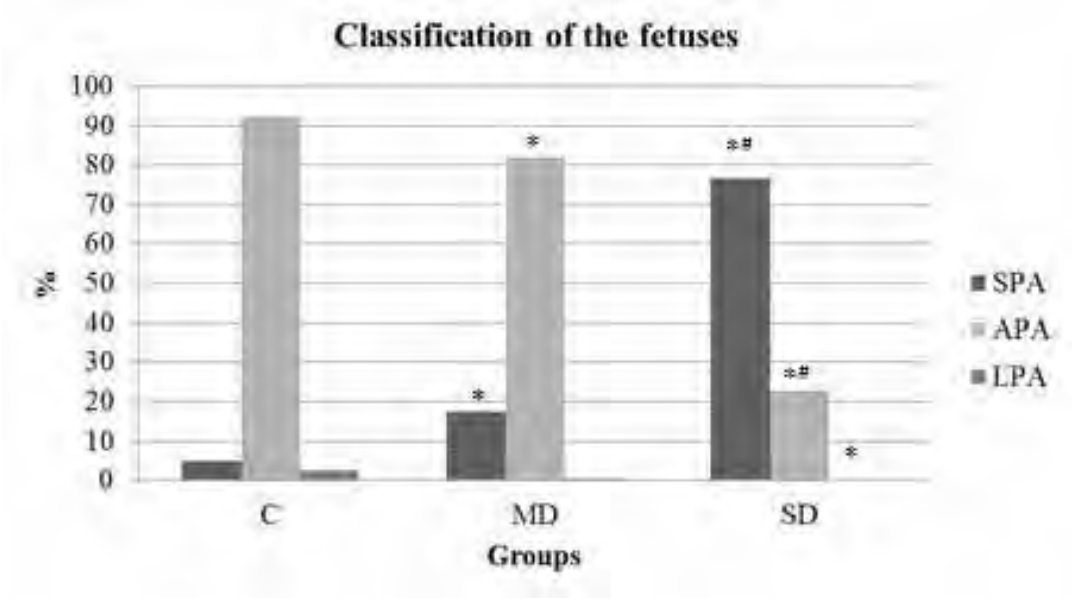

Figure 3. Proportion (%) of fetuses classified as small (SPA), appropriate (APA) and large (LPA) for pregnancy age at term pregnancy of mild (MD) and severe (SD) diabetic and control (C) groups. *p< 0.05 - statistically significant difference compared to control group, #statistically significant difference in relation to MD group (Chi Square Test).