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Evaluation of the Effect of STZ-Induced Diabetes on Oocyte Morphology and Subsequent Embryo Development in Superovulated Mice

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Abstract: Type 1 diabetes was shown to affect oocyte quality and subsequent embryo development. In this study, we aimed to evaluate the effect of diabetes on oocyte quality based on morphological criteria and subsequent embryo development in a mouse model. Female CD 1 mice were used as the experimental animals for superovulation and male mice were used as the source of spermatozoa for IVF. Significant differences were observed in the morphological criteria such as perivitelline space, zona pellucida and polar bodies between both groups ($p<0.05$). Following superovulation and IVF, embryo quality and blastocyst development rate were compared between groups and it was observed that embryo quality and blastocyst development rate were superior in control group compared to diabetic one ($p<0.05$). These results suggest that even chemically-induced diabetes may show adverse effects on female reproductive cells and subsequent embryo development.

Key words: Streptozotocin, type 1 diabetes, superovulation, mice, Turkey

INTRODUCTION

Insulin-dependent diabetes mellitus is characterized by the increase in blood glucose levels which is a result of insufficiency of insulin production. This increase in glucose levels was shown to result in serious pathological complications such as neonatal diseases and death, missed abortions and congenital malformations (Colton *et al*., 2003). In humans, type 1 or insulin-dependent diabetes was shown to have negative effects on pregnancy outcome as a result of increased risk of congenital abnormalities and negative prenatal outcomes such as abortion (Greene, 1999; Farrell, 2002).

In streptozotosin and alloxine-induced hypoinsulinemic and hyperglisemic mice, zygotes have shown lower fertilization rates compared to nondiabetic mice 48 hours after human Chorionic Gonadotrophin (hCG) administration *in vivo* (Diamond *et al*., 1989). In previous studies, it was shown that 2-cell embryos which were obtained from diabetic mice developed up to blastocyst stage significantly later than in control mice (Colton *et al*., 2003; Chi *et al*., 2000). In the studies performed with rodents, it was observed that diabetes had a negative impact on embryos before and after implantation. In both chemically and naturally-induced diabetes, it was observed that embryos have shown more fragmentation and degeneration (Lee *et al*., 2001).

Diabetic state was shown to exert negative effects also on oocyte maturation and folliculogenesis. It was shown that diabetes exerted harmful effects on meiosis in superovulated mice (Bordignon *et al*., 1999; Diamond *et al*., 1989). Colton *et al*. (2002) have found that the oocytes that they isolated from diabetic mice showed a weaker maturation compared to the oocytes in control group. In addition to this, it was shown that diabetic mice have tended to develop less metaphase II oocytes compared to control group (Blyszczuk *et al*., 2003; Boiani and Scholer, 2005).

Based on these data in the literature, we aimed to evaluate the effects of streptozotocin-induced diabetes on oocyte quality based on morphological characteristics as well as subsequent embryo development in a mouse model *in vitro*.

MATERIALS AND METHODS

Ethical approval of this study was taken from Animal Research Ethics Committee of Kocaeli University Faculty of Medicine (approval no:12/3-2009).

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Experimental animals: In this study, CD1-type female mice of 8-10 weeks old and weighing between 18-26 g were used as the experimental animals to induce superovulation. All animals used in the study was composed of 31 female and 10 male mice. Mice were provided free access to food and water and were kept in a 12 h light dark^{-1} cycle (lights were on at 06:00 h).

Induction of diabetes: Diabetes was induced in female mice by intraperitoneal administration of streptozotosin (Sigma-Aldrich, STEINHEIM). Amount of streptozotocin to inject was calculated according to the average body weights of female mice and injections were done according to these concentrations. This amount was calculated by using low and high dose application protocol of Animal models of diabetic complications consortium. In our study, 100 mg kg^{-1} streptozotocin was administered intraperitoneally. Before injection, mean blood glucose levels of mice were measured as 75 mg dL^{-1}. About 4 day after the injection, their blood glucose levels were measured by taking a blood sample from their tail veins. The mice with a glucose level of 200 mg kg^{-1} were considered as diabetic. Mean blood glucose level following injections was measured as 267 mg dL^{-1} in our study. Mice were kept at 12 h dark light^{-1} cycle for one month in order to observe a possible effect of diabetes on female reproductive system. Following this period, blood glucose levels were measured again and superovulation was performed.

Superovulation and IVF protocol: Superovulation was achieved with a subcutaneous injection of 5 IU PMSG/animal (Sigma-Aldrich, Germany). It was followed by 5 IU hCG (Pregnyl: Organon) subcutaneously 48 h later. Following 13-15 h after hcg injection, female mice were sacrified. Oviducts and ovaries were removed and transferred into the culture medium (10 mL Hepes-m HTF buffer+1 mg BSA mL^{-1}). Oocytes were torn out of ampullae and cumulus-oocyte complexes were removed by denudation inside hyaluronidase enzyme (80 IU mL^{-1} Hyaluronidase, Vitrolife, Sweden). Quality assessment of the denuded oocytes was done as described below. Then, they were transferred into fertilization dish including fertilizasyon media (10 mL mHTF +4 mg BSA mL^{-1}).

In each experiment, one 12-16 week old male mouse was sacrified by cervical dislocation 1 h before oocyte collection. Cauda epididymis and vas deferentia were excised and placed in a dish containing 1 mL of capacitation medium (10 mL mHTF buffer +9 mg BSA mL^{-1}) covered with mineral oil. With the aid of insulin needles, the tissue was minced and kept in an incubator (Sanyo 18 MCM, Japan) under the conditions of 37°C and 5-6% CO_2 for 10 min to allow the spermatozoa to swim-out. Then the tissues were removed and spermatozoa were kept in the incubator for an hour for capacitation. After one hour of capacitation, 10 μL drop of spermatozoa was put into the fertilization drop with the oocytes and left for fertilization in the same incubator. The number of spermatozoa was adjusted to 1-25 million/mL per 10 oocytes.

Embryo culture: After gamete co-incubation for nearly 6 h, zygotes were washed in KSOM culture medium supplemented with 1% non-essential and 2% essential amino acids (Gibco) (KSOMaa) and gently pipetted to remove spermatozoa and attached cumulus cells. Zygotes were then transferred to 500 μL of KSOMaa medium and cultured in a humidified incubator under the conditions of 37°C and 5-6% CO_2. Day of fertilization was considered as day 1. Blastocyst development was monitored on day 3.5-4.5.

Study groups: In the study, female mice were classified into two groups including:

- Control group (n = 8): mice in this group underwent superovulation. Superovulation was performed as described above. This group was used as the control for diabetic group
- Diabetic group (n = 10): mice in this group underwent superovulation after the confirmation of a blood glucose level of >200 mg dL^{-1}

Assessment of oocyte morphology: The morphology of the oocytes were evaluated based on four criteria as shown in Table 1.

Assessment of embryos and blastocysts: Embryos with >6 and even-sized blastomeres and <10% fragmentation

Table 1: Morphological criteria of oocytes

Parameters	Normal	Abnormal
Zona Pellucida (ZP)	Thin and clear, colorless, regular and continuous	Thick (>20 μ) and/or dark
Cytoplasm	Bright and/or diffused granulation	Dark and dense granulation
Perivitelline Space (PVS)	Normal	Large
Polar Body (PB)	Regular and unfragmented	Irregular and/or fragmented

were defined as grade A embryos by Gustava. Blastocyst development rate was calculated by dividing all blastocysts expanded until the end of day 4.5 by total number of zygotes.

Statistical analysis: In this study, SPSS program version 13.0 was used for statistical analysis. For numerical variables, nonparametric Mann-Whitney U test was used for comparisons between groups. The χ^2-test was used for the comparison of categorical variables between groups. The $p<0.05$ was considered as statistically significant.

Table 2: Demographics of the groups

Parameters	Control (n = 8)	Diabetic group (n = 10)	p-values
Body weight (g)	23.6	22.9	0.08
Number of oocytes retrieved (n)	125.0	108.0	0.07
Number of MII oocytes (n)	110.0	85.0	0.06

Table 3: Comparison of variables regarding oocyte quality between both groups

Parameters	Control group	Diabetic group	p-values
Large perivitelline space (%)	26±2.1	59±0.6	0.02*
Abnormal ZP (%)	22±1.8	58±2.8	0.01*
Ratio of abnormal cytoplasm (%)	52±3.5	55±4.7	0.06
Ratio of abnormal polar bodies (%)	24±2.2	60±1.2	0.02*

All values were expressed as mean±SD; $p<0.05$ was considered as statistically significant

RESULTS AND DISCUSSION

Demographics of the animals were given in Table 2. Body weight of the animals were similar between groups ($p>0.05$). The number of oocytes retrieved and number of MII oocytes were increased in control group compared to diabetic group, however, the difference was not statistically significant (125 vs. 118 and 110 vs. 85, respectively) ($p>0.05$). All the variables of the oocytes in both groups were given and compared in Table 2. The images of normal and abnormal oocytes based on some morphological citeria was given in Fig. 1.

There were statistically significant differences between both groups in terms of PVS, ZP thickness and ratio of abnormal PBs ($p<0.05$ for each). However, no statistically significant difference was found between groups in terms of the ratio of abnormal cytoplasm ($p> 0.05$) (Table 3).

In our study, a significant difference was found between groups in terms of Grade A embryos (76±1.389 vs. 53±2.5%; $p<0.02$). It was observed that embryo quality was better in control group compared to diabetic group. The rate of blastocyst development was compared between control and diabetic groups and it was observed that there was a significant difference between both groups in terms of blastocyst development (61.88±8.425% in control group vs. 42.4±17.379% in diabetic group ($p<0.02$)). In addition, fertilization rate was significantly decreased in diabetic group compared to control group (65% vs. 85%; $p<0.05$) (Fig. 2).

It was shown in numerous studies that meiotic maturation and subsequent developmental potential were negatively affected when oocytes were exposed to diabetic conditions (Diamond *et al*., 1989; Colton *et al*., 2002; Moley *et al*., 1991). The number of metaphase II oocytes and germinal vesicle breakdown (Colton *et al*., 2002; Chang *et al*., 2005; Kim *et al*., 2007; Ratchford *et al*., 2007) were found to be decreased in diabetic mice; indicating that cytoplasmic maturation was abnormal in superovulated oocytes which were obtained from diabetic mice (Goud *et al*., 2006; Wang *et al*., 2009). In our study, we found that the ratio of MII oocytes was lower in diabetic group compared to controls; however, the difference was not statistically significant; showing that STZ-induzed diabetes did not have an adverse effect on oocyte maturation.

Good-quality mature human oocytes are thought to have a clear, moderately granular cytoplasm, a small PVS, a clear, colorless ZP and contain a single unfragmented PB (Veeck, 1994). Dysmorphic oocytes are commonly classified as having abnormal cytoplasm (dark cytoplasm or cytoplasmic granularity), cytoplasmic inclusions (vacuoles, refractile bodies or Smooth Endoplasmic Reticulum (SER) clustering), an abnormal ZP an abnormal PVS (e.g., a large PVS or debris in the PVS) and an abnormal PB (Rienzi *et al*., 2011, 2003). Previous reports also showed that diabetes exerted negative effects on oocyte quality. Wang *et al*. (2010) have studied the effect of maternal diabetes on oocyte quality with a focus on mitochondrial dysfunction and identified an impaired oocyte quality. Wang and Moley (2010) also suggested that maternal diabetes led to oocyte defects by two routes including meiotic spindle and chromatin defects and structural and functional abnormalities of mitochondria. In our study, we compared four characteristics of mature oocytes in order to determine the effects of diabetes on oocyte quality. Our findings were similar to many studies reporting that diabetes showed a negative effect on oocyte quality. These negative effects were mostly seen on the morphological characteristics including zona pellucida, perivitelline space and polar bodies in our study.

In both chemically-induced and spontaneous diabetic models, significant delays were observed in preimplantation embryo development (Diamond *et al*., 1989; Vercheval *et al*., 1990; Beebe and Kaye, 1991; Moley *et al*., 1991) and also high incidence of degenerated and fragmented embryos were observed (Diamond *et al*., 1989; Hertogh *et al*., 1992; Pampfer *et al*., 1990; Lea *et al*., 1996). Blastocysts which were obtained

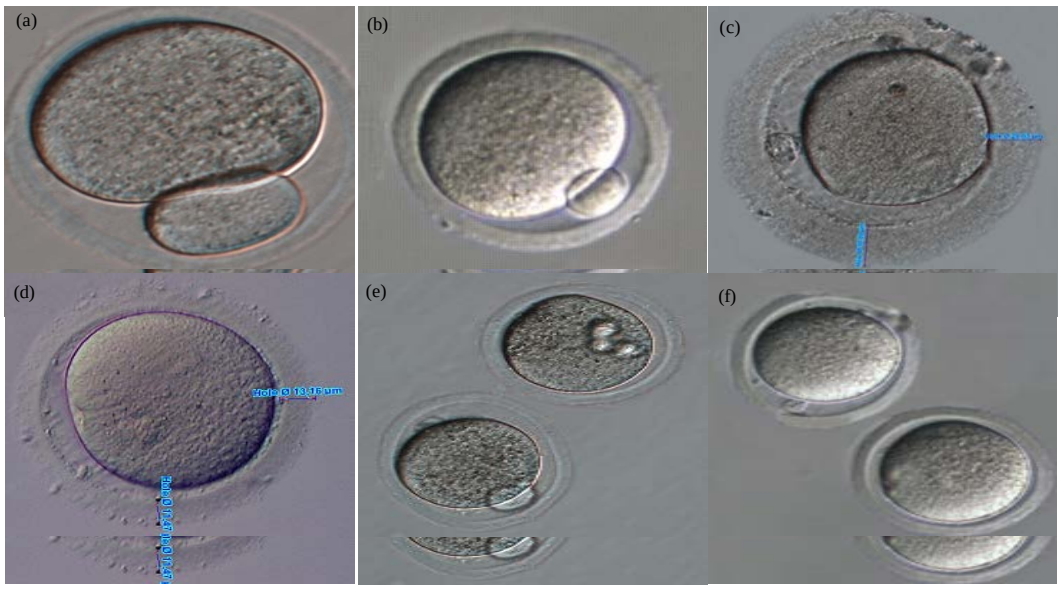

Fig. 1: Denuded MII oocytes in diabetic and control groups (at 40X magnification): a) MII oocyte with a large PB (in diabetic group); b) MII oocyte with a normal sized PB (control group); c). MII oocyte with a thick and irregular zona pellucida and large PVS (diabetic group); d) MII oocyte with a regular and normal zona pellucida (control group); e) MII oocytes with a granular cytoplasm, large PVS and fragmented polar body (diabetic group) and f) MII oocytes with a normal cytoplasm (control group)

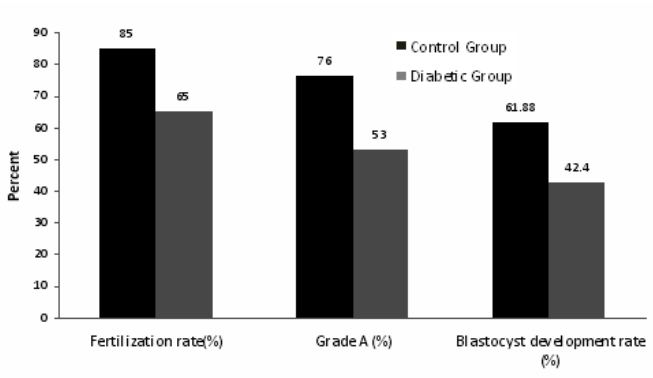

Fig. 2: Effect of diabetes on fertilization rate, embryo quality assessment and blastocyst development rates. At the end of culture period, the embryos were assessed for development up to the blastocyst stage. All of these parameters were found to be significantly different between both groups ($p<0.05$)

from diabetic animals were shown to contain fewer cells than those from nondiabetic controls (Hertogh *et al*., 1992; Pampfer *et al*., 1990; Lea *et al*., 1996). Likewise, numerous reports have suggested that the diabetic condition adversely affected the development of pre and post implantation embryos in rodents (Diamond *et al*., 1989; Vercheval *et al*., 1990; Moley *et al*., 1991; Wyman, 2008). Earlier studies have shown that *in vitro* cultured two-cell embryos that were retrieved from diabetic mice experienced significant delays in their progression up to the blastocyst stage (2). Vesela *et al*. (1994) found that about 50% of two-cell embryos isolated from subdiabetic rats were unable to develop to the eight-cell stage, even in a nondiabetic tract. All these *in vivo* studies on embryo development clearly demonstrated that zygotes and embryos that were exposed to diabetic conditions showed abnormalities and delays in subsequent development during preimplantation period. In our study, we classified cleavage-stage embryos based on the report by Gustava and found that the number of grade A (good quality)

embryos was higher in control when compared to diabetic group as similar to previous data in the literature. However, in those studies, zygotes and embryos were exposed to maternal diabetes *in vivo* and directly affected by diabetes. We performed IVF in our study, means that zygotes and embryos were not directly exposed to diabetic state any more following oocyte collection. Therefore, we may suggest that these indirect negative effects of diabetes on embryo development may be derived from its direct negative effects on oocyte quality.

CONCLUSION

Based on our results, we can suggest that diabetes exerts negative effects on the parameters which are indicators of oocyte quality and on subsequent embryo development *in vitro*. Thus, we suggest that it will be better to take possible negative effects of diabetes into consideration during assisted reproduction treatment in order to increase oocyte and embryo quality and to focus on the treatment of diabetes in women to improve the success in these techniques.

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