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Silymarin ameliorates uterine and ovarian damage in streptozotocin induced diabetic rat model

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Diabetes is a systemic disease that affects microvasculature in almost all organs. Uterus and the ovaries may also be a target for diabetes. We investigated effects of diabetes on the uterus and the ovaries and the role of silymarin treatment on the effects of diabetes on uterine and ovarian microenvironment in a diabetic rat model. Seven non-diabetic (control) and fourteen diabetic female mature Sprague-Dawley albino rats were used. Diabetes was induced by intraperitoneal injection of 60 mg/kg streptozotocin and 100 mg/kg oral silymarin and was administered for four weeks to 7 of diabetic rats. After the treatment, blood samples were collected; hysterectomy with bilateral oophorectomy was performed for histopathological examination. Stromal degeneration, follicular degeneration, stromal fibrosis scores of the ovary and gland degeneration and stromal fibrosis scores of endometrium were significantly decreased after silymarin treatment. Silymarin treatment significantly decreased plasma TGF-β levels and increased plasma AMH (Anti-Müllerian hormone) levels with respect to saline-treated group. This study suggests that silymarin ameliorates the uterine and ovarian damage in a diabetic rat model.

Keywords: Anti-Mullerian hormone, Follicular degeneration, Stromal fibrosis

Diabetes mellitus is a chronic systemic disease characterized by insulin resistance, altered insulin secretion, and hyperglycemia[1]. Its incidence is increasing gradually due to changes in eating habits and lifestyle; thus more patients are diagnosed with diabetes in younger ages and in reproductive period[2]. Diabetes mellitus alters the functions of most of the organ systems such as cardiovascular[3], neuronal[4], and excretory systems[4] as well as the reproductive system[5,6]. As a result of hyperglycemia, both ovarian and uterine microenvironments are affected. Impaired folliculogenesis and steroidogenesis, anovulation, endometrial glandular degeneration, stromal fibrosis and spontaneous abortion were demonstrated as consequences of diabetes[5,6].

Main complications of hyperglycemia are attributed to its micro and macro-vascular effects[7,8]. In a hyperglycemic environment, non-enzymatic glycation of biological proteins provokes the irreversible formation of advanced glycation end products (AGEs)[9,10]. The AGEs and the increased expression AGE receptors (RAGE) generate reactive oxygen species (ROS) and inflammatory cytokines such as tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interleukin-1β (IL-1 β)[11,12]. These mediators modulate the signaling pathways such as protein kinase C, mitogen-activated protein kinase and p47phox and activates nuclear factor- kappa B (NF-κB) transcription factor; this cascade influences the synthesis of downstream mediators[12]. Hyperglycemia also inhibits deglycation systems and promotes accumulation of AGE in endothelial cell, which in turn may lead to fibrosis in different organ systems[13]. It was recently demonstrated that AGE also had an important role in female reproduction[14].

With an increased interest in phytochemicals, flavonoids are very popular with their therapeutic effects in several diseases with their antioxidant and free radical scavenger properties[15]. Silymarin, extract of milk thistle (*Sylibum marianum*), has been known for a long time for its antioxidant, anti-inflammatory and anti-hyperglycemic features[16]. It was also

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demonstrated that it has nephroprotective[17], hepatoprotective[18], neuroprotective[19] effects. It was recently reported that it protects against the progression of insulin resistance in type 2 diabetes mellitus by hampering the oxidative process and improving hepatic metabolism[20]. In another recent study, Soto *et al.*[21] reported that silymarin improved the reduction in the β pancreatic cell by inducing β-cell neogenesis in a pancreatectomy model.

The effects of silymarin on the reproductive system in diabetes mellitus have yet to be studied therefore, we investigated whether silymarin may improve the ovarian and uterine microenvironments in diabetic rats.

Materials and Methods

Animals

21 female Sprague-Dawley albino mature rats, weighing 200-220 gm were used. Animals were fed *ad libitum* and housed in pairs in steel cages having a temperature-controlled environment (22 ± 2°C) with 12 h light/dark cycles. The Committee for Animal Research of Ege University approved the experimental procedures. All animal studies are strictly conformed to the animal experiment guidelines of the Committee for Human Care.

Experimental protocol

Diabetes was induced by intraperitoneal (*i.p.*) injection of streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO, USA) (60 mg/kg in 0.9% NaCl, adjusted to a pH 4.0 with 0.2 M sodium citrate) in 14 rats[6]. No drug was administered to the remaining rats exhibiting blood glucose levels were under 120 mg/dL (n=7) (control group, Group-1). Diabetes was verified after 24 h by evaluating blood glucose levels with the use of glucose oxidase reagent strips (Boehringer-Mannheim Corp, Indianapolis, USA). The rats with blood glucose levels 250 mg/dL and higher were included in this study as diabetic rat group (n=14). Then, 14 diabetic rats were randomly divided into 2 groups; group-2 (diabetic control group, 7 rats) was given no medication and received 2 mL saline by oral gavage. Group 3 (silymarin group, 7 rats) were given 100 mg/kg/day silymarin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline byoral gavage for four weeks[17,22]. Then, the animals were euthanized and blood samples were collected by cardiac puncture for biochemical analysis and hysterectomy and bilateral oophorectomy was performed for histopathological examination.

Histopathological examination of the uterus

Two centimeter uterine tissues from each corn were embedded into paraffin. Two formalin-fixed uterine sections from each corn (4 μm) were stained with hematoxylen & eosine. All sections were photographed with Olympus C-5050 digital camera mounted on Olympus BX51 microscope (Olympus Corp, Tokyo, Japan). 3 photos from each section were taken with 10, 20 and 100 X magnification.

Endometrial gland degeneration, stromal fibrosis were scored from 0 to 3 according to the injury severity, where 0 represented no pathologic findings, and 1, 2 and 3 represented pathologic findings of less than 33%, 33% to 66%, and more than 66% of the uterine section, respectively[6].

Histopathological examination of the ovary

Both ovaries were embedded into paraffin. Three formalin-fixed ovary sections (4 μm) from each ovary were stained with hematoxylen & eosine. All sections were photographed with Olympus C-5050 digital camera mounted on Olympus BX51 microscope (Olympus Corp, Tokyo, Japan). 3 photos from each section were taken with 10, 20 and 100 X magnification

Follicular degeneration, stromal degeneration, and stromal fibrosis were scored from 0 to 3 according to the injury severity, where 0 represented no pathologic findings, and 1, 2 and 3 represented pathologic findings of less than 33%, 33% to 66%, and more than 66% of the ovarian section, respectively[6].

Measurement of plasma TGF-β

Plasma *TGF*-β was measured using commercially available enzyme-linked immunosorbent assay (ELISA) kit (Biosciences, Aalst, Belgium). *TGF*-β levels were expressed in pg/mL.

Measurement of plasma AMH levels

Plasma AMH levels were measured by using commercially available enzyme-linked immunosorbent assay (ELISA) kit (Biosciences, Aalst, Belgium). The plasma samples were diluted 1:2 and AMH was determined according to the manufacturer's recommendations. AMH levels were expressed in ng/mL.

Statistical analysis

Data analyses were performed using SPSS version 15.0 for Windows. The groups of parametric variables were compared by Student's T-test and analysis of variance (ANOVA). The groups of nonparametric variables were compared by Mann Whitney U test. Results were given as mean ± standard error of the mean (SEM). A value of $P< 0.05$ was accepted as

statistically significant. $P< 0.001$ was accepted as statistically highly significant.

Results

Stromal degeneration, follicular degeneration, stromal fibrosis scores of the ovary and glandular degeneration and stromal fibrosis scores of the endometrium were investigated in control group (n=7), in untreated (saline) diabetic rat group (n=7) and in silymarin administered diabetic rat group (n=7) (Table 1). Also plasma glucose, TGF-β and AMH levels were compared between the same groups (Table 2).

Endometrial gland degeneration score was significantly higher in saline-treated group than zin control group ($P = 0.00007$) whereas it was significantly lower in silymarin treated group than in saline-treated group ($P = 0.003$). Similarly, endometrial stromal fibrosis score was significantly higher in saline-treated group than in control group ($P = 0.00002$) whereas it was significantly lower in silymarin treated group than in saline-treated group ($P = 0.004$) (Fig. 1B, D &F) (Table 1).

Ovarian stromal degeneration score, follicle degeneration score and stromal fibrosis score were significantly higher in saline-treated group than in control group ($P = 0.00006$, $P = 0.00002$, $P = 0.000001$, respectively) whereas they were significantly lower in silymarin treated group than in saline-treated group ($P = 0.017$, $P = 0.03$, $P = 0.002$, respectively) (Fig. 1A, C & E) (Table 1).

Silymarin had no effect on plasma glucose levels since it was 432.2 ± 43.9 mg/dL before the treatment and 435.6 ± 40.5 mg/dL after the treatment in

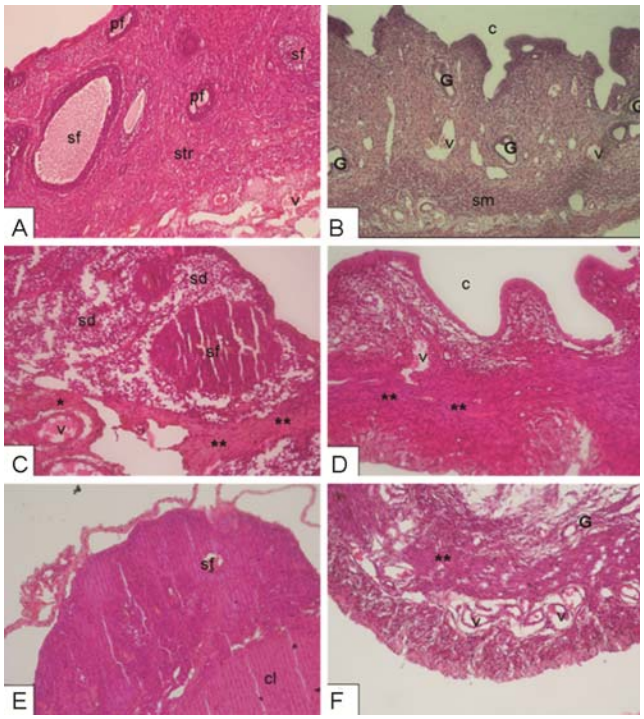

Fig. 1—Hematoxylen &Eosine (H & E) staining of sections from rat ovary (× 20 magnification) **[**A, C & E**]**; [A] Control group (Group-1), ovarian sections showed normal stroma (str), primary follicle (pf), secondary follicle (sf), vessels (v); [C] Saline treatment in diabetic rats (Group-2), ovarian sections showed fibrotic ovarian stroma (**), perivascular fibrosis (*), stromal degeneration (sd); [E] Decreased ovarian stromal fibrosis, perivascular fibrosis (*), stromal degeneration (sd) by silymarin treatment in diabetic rats; corpus luteum (cl) (Group-3). Hematoxylen&Eosine (H & E) staining of sections from rat uterus(x 20 magnification) **[**B, D & F**];** [B] Control group (Group-1), smooth muscle (sm),vessels (v), uterine cavity (c), endometrial gland (G); [D] Saline treatment in diabetic rats (Group-2), uterine sections showed fibrotic uterine stroma and decreased endometrial gland. (**); [F] Decreased uterine fibrosis and increased endometrial gland (G) by silymarin treatment in diabetic rats (Group-3).

Table 1— Ovarian and endometrial histopathological scores in experimental groups

	Control group	Diabetic rats with saline treatment	Diabetic rats with silymarin treatment
Endometrial gland degeneration score	0.25 ± 0.16	2.37 ± 0.18*	1.6 ± 0.18$^{\#}$
Endometrial stromal fibrosis score	0.25 ± 0.16	2.75 ± 0.16*	1.76 ± 0.17$^{\#\#}$
Ovarian stromal degeneration score	0.37 ± 0.18	2.37 ± 0.26*	1.6 ± 0.26$^{\#}$
Ovarian follicle degeneration score	0.52 ± 0.27	2.62 ± 0.18*	1.9 ± 0.22$^{\#}$
Ovarian stromal fibrosis score	0.26 ± 0.16	2.1 ± 0.22*	1.3 ± 0.18$^{\#}$

* = $P< 0.0001$, $^{\#}$ = $P< 0.05$, and $^{\#\#}$ = $P< 0.01$

Table 2—Biochemical parameters in experimental groups

	Control group	Diabetic rats with saline treatment	Diabetic rats with silymarin treatment
Glucose before treatment, mg/dL	110.1 ± 4.9	468.7 ± 36.5 *	432.2 ± 43.9
Glucose after treatment, mg/dL	120.2 ± 7.4	453.8 ± 41.7 *	435.6 ± 40.5
TGF-β, pg/mL	6.1 ± 0.7	25.2 ± 3.6 *	17.1 ± 1.5 $^{\#}$
AMH, ng/mL	2.4 ± 0.24	0.9 ± 0.06 *	2.03 ± 0.23 $^{\#\#}$

*$P< 0.0001$, $^{\#}P< 0.05$, and $^{\#\#}P< 0.01$

silymarin treated diabetic rat group (P = 0.916). Plasma TGF-β levels were significantly higher in saline-treated diabetic rat group than in control group (P = 0.000012), silymarin treatment decreased TGF-β levels significantly when compared with the non-treated group (P = 0.023). Diabetes Mellitus caused significantly decreased serum AMH levels in saline-treated diabetic rat group compared with controls (P = 0.00003). Silymarin treatment increased serum AMH levels significantly when compared with the non-treated group (P = 0.001) (Table 2).

Discussion

To investigate the effects of silymarin on uterine and ovarian microenvironment in a diabetic rat model, we demonstrated that stromal degeneration, follicular degeneration, stromal fibrosis scores of the ovary and gland degeneration, and stromal fibrosis scores of endometrium were significantly decreased after silymarin treatment. Additionally, silymarin treatment significantly decreased plasma TGF-β levels and increased plasma AMH levels with respect to saline treated group.

Although there are still ongoing studies regarding the mechanism of diabetic complications today, formation of advanced glycation end products is one of the reasons for the involvement of most of the organ systems in diabetes mellitus. AGE upregulate its receptor (RAGE) in case of chronic hyperglycemia[11]. The RAGE gene promoter contains NF-κB binding sites, which modulate RAGE expression and link RAGE to the inflammatory response[23]. Activation of NF-κB pathway leads to inflammation, smooth muscle cell (SMC) proliferation, and endothelial dysfunction that increase the permeability of the vascular wall to macromolecules[24]. Like many organ systems affected from the chronic hyperglycemic state, the female reproductive system may also be affected. In a recent study, Merhi[14] reported that AGE-RAGE system had an important role on a female reproductive system such as granulosa cell dysfunction, adipocyte pathophysiology, obesity, and insulin resistance. In our previous report, we demonstrated that streptozotocin-induced diabetes caused glandular degeneration and stromal fibrosis in the rat endometrium[6]. Erbas *et al.*[25] recently reported that follicular degeneration, stromal degeneration, stromal fibrosis and NF-κB immuno-expression were significantly increased in diabeticrat's ovary compared with control group.

So far, many studies investigated the effects of silymarin for its anti-oxidant features[20,26]. Bouderba *et al.*[20] reported that silymarin significantly improved the total antioxidant status and decreased malondialdehyde (MDA) levels in a metabolic syndrome model. In a similar study, Wu *et al.*[16] demonstrated that silymarin treatment significantly inhibited expression of inflammatory mediators in S100b-stimulated monocytes, which is a ligand of RAGE that initiates the complex interactions of oxidative stress and inflammatory reactions. In a very recent study, silymarin was found beneficial for preventing the side effects of liver and kidney induced by chronic isotretinoin therapy[26]. Moreover, silymarinhas been studied for its anti-diabetic properties and positive effects on diabetic complications. In a randomized, double-blind, placebo-controlled clinical trial,the results showed a significant decrease in HbA1c, FBS, total cholesterol, LDL, triglyceride, SGOT and SGPT levels insilymarintreated type-2 diabetic patients[27]. Soto *et al.*[28] reported that silymarin treatment significantly increased serum insulin levels and decreased plasma glucose levels in a rat pancreatectomy model. They showed that silymarin increased the insulin and Pdx gene expression and caused β-cell proliferation. The same study group reported in another study that silymarin induced the expression of a pancreatic Nkx6.1 transcription factor that specifically directed the differentiation of the insulin-secreting β-cell[21]. In our study, plasma glucose levels did not significantly change after silymarin treatment in diabetic rats. One of the most important reasons could be the dose of the drug, since in the previous studies, the silymarin dose used were double. Secondly, the rats used in previous studies were males. Future studies with different gender may provide us better results.

Menstrual abnormalities, impaired folliculogenesis, decreased fecundity, premature menopause and spontaneous miscarriages are some examples[5,29,30] of reproductive outcomes of diabetes mellitus as reported in many clinical trials and animal model studies. Histopathological changes due to diabetes in ovary and endometrium may cause such problems. Endometrial glandular and stromal changes may cause implantation failures, fetal losses, and advanced pregnancy outcomes. Additionally, ovarian tissue injury may also be responsible for the impaired fertility outcomes. Fibrotic changes due to AGE accumulation and inflammatory reactions occurred in

the ovary and uterus may involve other organs such as liver[31]. Jeong*et al.* reported that TGF- β played an important role as a profibrogenic factor in chronic liver disease, triggering the expression of procollagen-I and tissue inhibitor of metalloproteinases-1 (TIMP-1) which were key effectors of fibrogenesis[32]. They also reported that silymarin treatment significantly reduced hepatic fibrosis in carbon tetrachloride (CCl_4) administered rats *via* down-regulation of TGF- β expression. In another study, silymarin significantly reduced serum TGF- β levels and improved nephropathy in type-2 diabetic patients[33]. Similarly, in our study, we demonstrated that plasma TGF- β levels were significantly increased in diabetic rats with respect to controls and silymarin treatment significantly decreased plasma TGF- β levels and stromal fibrosis in both uterus and ovary.

As mentioned before, impaired folliculogenesis and decreased ovarian reserve are the results of follicular degeneration in chronic hyperglycemia. Anti-mullerian hormone (AMH), a glycoprotein produced by granulosa cells of ovary, is the excellent indicator of ovarian reserve and granulosa cell injury. It was reported that follicular degeneration causes a significant reduction in plasma AMH levels in diabetic rats[34]. We are in the same line with the previous studies that diabetes caused a significant reduction in plasma AMH levels in the diabetic rats. Bur silymarin treatment significantly increased plasma AMH levels and improved ovarian reserve.

This study has some limitations such as the silymarin treatment in a dose-dependent manner may give better results regarding the optimal dose of the drug. Secondly, insulin levels besides plasma glucose levels may elucidate about the anti-glycemic effect of silymarin. Moreover, parameters regarding oxidative status such as serum/tissue MDA, glutathione peroxidase, myeloperoxidase, and superoxide dismutase can be further evaluated. Larger studies with greater sample size may be more helpful. Moreover generalizing an animal model study to human beings is impossible. So, clinical studies are necessary that supports our findings. Also, the long-term effects of silymarin on ovarian reserve and fecundity can be further studied.

In conclusion, these findings demonstrated that silymarin treatment in diabetic rats significantly improved histopathological parameters in ovary and uterus, increased plasma AMH levels and decreased plasma TGF- β levels. These data suggest that silymarin could be a potential therapeutic agent for its positive effects on the reproductive system in diabetes mellitus. However, further clinical studies are needed to support our findings.

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