Anti-Apoptotic Effect of Semelil on Testicular Germ Cells in Streptozotocin-Induced Chronic Diabetic Rats

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Abstract

Background: It is well known that diabetes is associated with impairment of testicular function. The present study aimed to investigate the effect of Semilil, an herbal medicine with angiogenic action, on testicular germ cell apoptosis in streptozotocin (STZ)-induced chronic diabetic rats.

Methods: Adult Male Sprague-Dawley rats were divided into 5 groups: (1) diabetic group, (2) Semilil-treated diabetic group, (3) insulin-treated diabetic group, (4) Semilil plus insulin-treated diabetic group, and (5) control group. Semilil was administered intraperitoneally at the dose of 13 mg/kg for 49 days. Apoptosis in testicular germ cells was determined by terminal-deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Apoptosis indices in testicular germ cells of animals were computed using the stereologic method.

Results: Body and testis weights were significantly decreased in all of experimental groups, except in controls (P<0.05 each). The apoptosis index was significantly increased in the diabetic dams when compared to control and insulin treated diabetic animals (P<0.01 and P<0.01, respectively). Although, the apoptosis index was markedly increased in Semilil-treated diabetic group compared to insulin treated diabetic group animals and controls (P<0.01 and P<0.001, respectively), there was a significant decrease in the apoptosis index of germ cells in the Semilil-treated diabetic group with respect to the diabetic group (P<0.01).

Conclusion: These results suggest that intraperitoneal administration of Semilil for seven weeks has a potentially beneficial role in reducing apoptosis in testicular germ cell in chronic diabetic adult rats, probably due to improve the angiogenesis.

Keywords: Apoptosis; Spermatogenesis; Diabetes

Introduction

Diabetes is associated with human reproductive dysfunction in both males and females [1-7]. The increasing incidence of diabetes diagnosed at reproductive age is an issue showing the importance of research on fertility in this group [1-3,6-7]. Several lines of studies demonstrate that diabetes affects male reproductive function at multiple levels due to its effects on the endocrine control of spermatogenesis, spermatogenesis itself or by impairing penile erection and ejaculation [1-8].

In previous studies, histopathological and histomorphometric alterations in seminiferous tubules have been reported in streptozotocin-induced diabetic animals [9-14]. In a study Guneli et al. [15] and coworkers showed a reduction in seminiferous tubule diameter, increased thickening of the basement membrane in seminiferous tubules and degenerated germ cells in diabetic rats. They also found that TUNEL-positive cells were significantly more numerous in diabetic rats than in controls. Moreover, Sainio-Pollanen et al. [16] and Cai et al. [11] revealed that diabetes increased apoptosis in testicular germ cells in mice.

The exact mechanism by which diabetes leads to male reproductive system dysfunction is not completely understood [1-3,17]. While, some human studies revealed that psychological issues and blood flow disruption are the main causes [3,8,18]. Numerous studies on rodents also suggest mechanisms including altered hormonal profiles, oxidative stress, DNA damage to sperm, and abnormal progression through spermatogenesis [3,5,10,15,18-21]. Diabetes also adversely affects seminiferous
epithelium including increased basement membrane thickness, degeneration and apoptosis of germ cells [5,9-13,16,21,22].

Semelil (ANGIPARS™), an extract of *Melilotus officinalis* (Yellow Sweet Clover), is a new herbal drug for diabetic foot ulcers management that has been formulated by Iranian scientists in recent years and according to the results of recent studies, this drug is effective and safe. Previous studies have reported a beneficial effects of *Melilotus officinalis* extract in micro-circulation improvement in chronic venous insufficiency and hydrocortisone-like anti-inflammatory effects. ANGIPARS™ also contains compounds such as 7-Hydroxycoumarin and flavonoids which have potent antioxidant effects [23-26]. Since a possible mechanism for this drug is angiogenesis and an increase in tissue blood flow and oxygenation; in this study, we assumed that the Semelil (ANGIPARS™) has a protective action on chronic diabetes induced apoptosis in germinal cells of seminifrous tubules as a result of improve in blood flow.

**Material and Methods**

**Animals**

**Study Design and Experimental Groups:** All procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals of Mashhad University of Medical Sciences, Mashhad, Iran. Sixty adult Sprague Dawley male rats (300-350g body weight, 6-8 weeks old) were purchased from Mashhad University of Medical Sciences Experimental Animal House (Mashhad, Iran). Animals were housed in individual cages at 22±2 °C with free access to pellet food and water and on a 12h light/dark cycle. They were fed a regular rat chow.

Diabetes was induced by intraperitoneal (i.p.) injection of 65mg/kg streptozotocin STZ, (Sigma, St. Louis, MO, USA) in 0.1M citrate buffer (pH 4.5). Two days after STZ injection, development of diabetes was confirmed by measuring glucose level in fasting blood samples taken from tail vein using Accu-Chek glucometer (Roche, Germany). Rats with blood glucose concentrations of 300mg/dl or higher were considered diabetic and included in the study.

The rats were randomly divided into five groups (n=12 each): (1) diabetic group, (2) Semilil- treated diabetic group, (3) insulin-treated diabetic group, (4) Semilil plus insulin- treated group, and (5) control group. Animals were kept diabetic for 56 days and treated the next 49 days with Semilil (13mg/kg; i.p.). Semilil (ANGIPARS™) was freshly diluted in sterile saline (0.9% sodium chloride) at 1:10. Animal weights were measured before and at the end of the treatment period using a digital scale.

**Tissue Sampling**

The treatment continued for 49 consecutive days, and then the animals were anesthetized with thiopental (40mg/kg, ip). The thorax of each animal was opened with surgical incision on the sternum and the perfusion was done from left ventricle and right atrium. A rinsing solution was perfused before the fixation solution (Bouin’s fluid). Due to the narrow testicular artery branches from the abdominal aorta near the renal artery, it is probably constricted and occluded during the perfusion process. Perfusion with rinsing solution helped overcome this problem. To make rinsing solution, 9.0g NaCl, 25g Polyvinyl Pyrrolidone, 0.25g Heparin, and 5.0g Procain-HCL were dissolved in one liter of water by thorough stirring.

The pH was adjusted to 7.35 with 1 N NaOH and twice filtered through Millipore filters of 3.0μm or less pore size. The perfusion of both solutions was performed by using a scalp vein attached to a 50cc syringe. Testes were immediately removed taking care to handle specimens gently to minimize trauma to the tissues prior to placement of each tissue into fixative solution. The tunica albuginea was shallowly pierced at each pole 5 times with a 21-gauge needle to aid in the penetration of the fixative solution. Fixation time was limited to 24 hours and tissues were transferred using 70% ethyl alcohol.

Alcohol was changed 3 times daily for 2 days before transferring the specimens to a saturated solution of 70% ethyl alcohol and lithium carbonate to neutralize the picric acid in Bouin’s fluid. The ethyl alcohol-lithium carbonate solution was changed 3 or more times until the yellow color of Bouin’s fluid was almost completely depleted from the tissue. Tissues were stored in 70% ethyl alcohol until they were processed [27,28]. The fixed tissues were dehydrated through a graded series of ethanol and embedded in paraffin according to standard procedures. Paraffin sections were placed on slides coated with poly-L-lysine and stored at room temperature until further processing. The 7μm thick paraffin sections were used for Terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) staining methods. The occurrence of apoptotic cell death in the testicular tissue was examined [22].

**In Situ Germ Cell Apoptosis Detection**

In situ cell death detection kit was purchased from Roche (Germany). Apoptosis process in testicular germ cells was examined following the manufacturer’s protocol. Seven micrometer thick sections were deparaffinized, rehydrated, washed in Phosphate Buffer Saline (PBS) and incubated with proteinase K (1 mg/ml) for 15–30 min at 18–24 °C. Peroxidase activity was quenched with the addition of 3% H₂O₂ in absolute methanol. Sections were washed with PBS and incubated with the TUNEL reaction mixture of terminal deoxynucleotidyl transferase plus the nucleotide mixture in reaction buffer in a moist chamber for 60 min at 37 °C in the dark.

Sections were washed with PBS then incubated with converter-AP containing fluorescein isothiocyanate (Fab fragment from sheep) conjugated with Alkaline Phosphates (ALP) in a humidified chamber for 30 min at 37 °C. PBS rinsing and incubation with substrate solution that contained 7mg diaminobenzidine (DAB) (Sigma) and 3 ml H2O2 in 10 ml of 50mM Tris-HCl, pH 7.4 for 10min at 15-25 °C in the dark followed. PBS rinsing and counterstaining with Hematoxylin at room temperature for 1 min was followed. Negative control
sections were processed with label solution (without terminal transferase) and positive control sections were incubated with micrococcal nuclease or recombinant DNase 1 to induce DNA strand breaks, prior to labeling.

The stained samples of testis were semi-quantitatively analyzed, so that the number of positive spermatogonia cells per 50 seminiferous tubules was counted in 50 randomly chosen fields in four testis cross sections per animal, and data were corrected for seminiferous tubule diameter. This value was expressed as an index which evaluates the changes in apoptotic activity. The index was calculated using the equation:

Where \( n (\text{tub+}) \) = percentage of positive tubules; \( n (\text{cell+}) \) = number of positive spermatogonia per tubule; \( n (\text{total}) \) = total number of tubules; \( d \) = seminiferous tubule diameter (mm) [23].

**Data Analysis**

Differences between groups were measured using an Analysis of Variance (one way ANOVA). Tukey-Kramer test was employed afterward, where appropriate. All data are presented as mean±S.D. Differences were considered significant at \( P<0.05 \).

**Results**

**Table 1:** Blood glucose concentrations, body and testis weights of animals in different studied groups. The data presented as mean±S.D. (n=12).

| Group                | Blood Glucose Level (Mg/Dl) | Animal Weight Before Starting the Experiments(G) | Animal Weight at the End of the Experiments | Testis Weight | Testis Weight/Body Weight Ratio |
|----------------------|-----------------------------|-------------------------------------------------|--------------------------------------------|---------------|----------------------------------|
| Control              | 109.1±6.6                   | 345.2±7.3                                      | 294.2±6.6                                  | 1.28±0.07     | 3.78±0.53                       |
| Diabetic             | 422.9±12.6**                | 342.1±7.4                                      | 238.2±7.2*                                 | 0.72±0.07**   | 3.02±0.82                       |
| Semilil Treated-Diabetic | 404.2±17.4**               | 350.1±9.3                                      | 287.1±11.1*                                | 1.08±0.09**   | 3.76±0.86**                     |
| insulin-treated diabetic | 128.8±8.1                 | 338.8±8.6                                      | 281.9±7.9*                                 | 0.94±0.09**   | 3.35±0.82**                     |
| Semilil plus insulin-treated diabetic | 124.3±5.3                | 345.2±7.3                                      | 294.2±6.57*                                | 1.11±0.08**   | 3.78±0.53**                     |

*Significant differences compared to before starting the experiment (\( P<0.05 \)).

**Significant differences compared to control (\( P<0.05 \)).

***Significant differences compared to diabetic group (\( P<0.05 \)).

Detailed data for blood glucose concentrations, body and testis weights of animals before and at the end of the experiments in different groups are presented in Table 1. As shown, blood glucose levels in diabetic and Semilil-treated diabetic animals were significantly increased when compared to controls (\( P<0.001 \) each). Moreover, body and testis weights were significantly decreased in all of experimental groups, except in controls (\( P<0.05 \) each). Testis weight/Body weight ratio also was strikingly increased in Semilil, Insulin, and Semilil plus Insulin-treated animals compared to untreated diabetic group (\( P<0.05 \) each). In our study, diabetic rats exhibited some symptoms commonly associated with diabetes (i.e. polyuria, polydipsia and diarrhea).

**Evaluation of TUNEL Staining**

Figure 1 represents the presence of TUNEL-positive cells in seminiferous tubules of rats in different groups. While, the number of TUNEL-positive cells in the control group was negligible (Figure 1); these cells were observed in all of the spermatogenic cell series in the seminiferous tubules of other experimental animals (Figure 1). Analyzing the apoptosis index, our results showed that the apoptosis index was significantly increased in the diabetic dams when compared to control, Semilil, Semilil plus insulin- and insulin-treated diabetic groups (\( P<0.001 \), \( P<0.01 \) and \( P<0.01 \), respectively; fig. 2). Although the apoptosis index was markedly increased in Semilil-treated diabetic group compared to controls (\( P<0.01 \); Figure 2), there was a significant decrease in the apoptosis index in the Semilil-treated diabetic group with respect to the diabetic group (\( P<0.001 \); Figure 2). Significant changes were determined in apoptosis index of the Semilil-treated diabetic group compared to the insulin treated group animals (\( P<0.01 \)).
Diabetes induces male reproductive structural and behavioral alterations by causing apoptotic cell death, atrophy of the seminiferous tubules, decreased tubule diameters and reduction of spermatogenic cell series [1,2,5,9,10,13,15,22]. Seminiferous tubule atrophy and the decrease in germinal cells were morphological indicators of spermatogenesis failure [5,9,10,13,15,22]. In humans also diabetes is associated with decrease of plasma levels of testosterone and gonadotrophins, and abnormal morphology of sperm [17,33]. Previous studies have shown that diabetes disturbs the proliferation and differentiation of Leydig cells, and changes the pituitary-testicular axis [7]. A reduction in testicular weight and atrophy of the seminiferous tubules were also observed in diabetic rats [5,11]. In this study, we assumed that the lack of optimal blood supply to testis in diabetic animals may cause an increase in apoptosis in germ cells in seminiferous tubules.

Semilil (ANGIPARS™) is a novel herbal drug containing Melilotus officinalis extract that recently approved for treatment of chronic wounds, particularly diabetic foot ulcers [23-26]. In the recent investigations, ANGIPARS™ showed an evident angiogenic effect. So, this drug could significantly accelerate wound healing [23-26]. It was expected that ANGIPARS™ improve diabetic induced apoptosis in germlinal cells in testes.

Testicular germ cell apoptosis is consistent with morphological analysis in the seminiferous tubules [15,34]. In the present study, TUNEL-positive cells showed the typical morphological features of apoptosis including chromatin condensation, cytoplasmic budding and apoptotic bodies. The apoptosis index in the testicular germinal epithelium was significantly increased in diabetic male animals, indicating that germinal cell apoptosis was induced by diabetes in rat testes. There were markedly fewer TUNEL-positive cells in the diabetic rats treated with Semilil.

On the other hand, it has previously been shown that diabetes increases oxidative stress in the diabetic male testis [35-40]. Under an elevated oxidative stress status, reactive oxygen species cause cellular injury via several mechanisms including lipid peroxidation and oxidative damage of proteins and DNA [35, 41-43]. Oxidative stress is recognized as a strong mediator of apoptosis [41-43]. Mitochondria have a key role in the apoptotic process [42-45]. The mitochondrial dysfunction induced by oxidative stress can lead to the release of cytochrome c and then caspase activation, which results in apoptotic cell death [42-43,46].

In the present study, we demonstrated that Semilil prevents diabetes- induced germinal cell loss in the diabetic male rat. These findings indicate that the germinal cell-protective effects of Semilil may reflect its role as a free radical scavenger, an antioxidant, an antiapoptotic or an anti-inflammatory agent. The thickness of the seminiferous tubule basement membranes plays an important role in spermatogenesis. Several studies
have revealed that diabetes increases the thickening of the seminiferous tubule basement membranes [47,48], and this thickness is accompanied by a decreased rate of sperm production and overall reduction in the size of seminiferous tubules. In the present study, light-microscopic examination revealed that thickness of the seminiferous tubule basement membranes increased in diabetic rat testes, but Semilil reduced this increase in thickness.

In conclusion, Semilil improves the testicular damage in chronic diabetic male rats. To our knowledge, this is the first report indicating that Semilil improved diabetes induced testicular dysfunction in diabetic rats. Semilil can contribute to a balanced oxidant-antioxidant status and provide a useful therapeutic option to reduce the associated testes injury in patients with diabetes mellitus.

Conclusion

These results suggest that intraperitoneal administration of Semilil for seven weeks has a potentially beneficial role in reducing apoptosis in testicular germ cell in chronic diabetic adult rats, probably due to improve the angiogenesis.

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