A research on the effects of electrical intensity on wound healing in streptozotocin induced acute diabetic male rats
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Abstract
Diabetes is a type of disease which adversely affects the circulation of blood at the tissues and eventually causes tissue damage because of vascular complications. The tissue damage, which is caused by diabetes, has negative effects on some of the important organs such as eyes and kidneys, and as well as on the skin. High level of blood sugar, which is encountered in unregulated diabetes, affects skin surgeries (i.e: skin flaps) negatively. TENS method (Transcutaneous Electrical Nerve Stimulation), which is used for treatment of pain, pruritus, neuropathy, etc., since many years, is also used for problems such as wound healing and insufficient blood flow in skin. In our study, TENS' effect on reduction of flap necrosis was investigated on Wistar albino rats, which were experimentally induced diabetes via intraperitonal injection of single dose of 60 mg/kg Streptozotocin (STZ). As a result of the experiments, it was observed that, TENS current reduced flap necrosis in control group. However, in the rats which were induced experimentally via STZ, a significant flap healing wasn’t observed. Also, in our study, it was found that, inflammation decreased in diabetic rats which were exposed to TENS.

Key words: Diabetes Mellitus, Streptozotocin, TENS, Skin Flaps, Rat

1. Introduction
Diabetes mellitus is a chronic hyperglycemic disease of metabolism, that leads defects in carbohydrate and lipid metabolism due to a decrease in secretion of the hormone insulin or absolute or relative rareness of insulin effect. Clinically, it can be diagnosed by common symptoms such as polydipsy, polyuria, polyphagy, pruritus, weight loss, and by disease specific complications like retinopathy, neuropathy and nefropathy.

Insulin hormone provides the penetration of blood sugar into the cells. Thus, energy is provided for surviving of the cells and also blood sugar level is stabilized at regular (normal) levels. When measured at any hour of the day, if the blood sugar measure is >200mg/dl, it indicates the existence of diabetes. If the result(measurement) is between 140-200 mg/dl, it is required to investigate the existence of the disease. Blood sugar should be below 140 mg / dL, two hours after meals (Korugan et al., 1999).

Around the world, millions of people are affected by diabetes mellitus, it causes deaths in many countries because of its complications. Its complications are seen with acute and chronic periods and both increases the morbidity and mortality of the patients. As this suggests, diabetes mellitus is not only a metabolic disease, rather, it is the name given to a group of diseases which have different etiologies (Greenberg and Sacks, 2002). About %10 of the world population has hidden or undiagnosed diabetes (Öztürk 1999). Mainly, two types of diabetes have been determined and among them Type I is dependent on insulin. Insulin, which is secreted from beta cells of the islets of pancreas (an organ that has settled behind the stomach because of immunologic, toxic or various environmental reasons) decreases with time and finally disappears. In this type of diabetes, especially patients under the age of 35, should be given insulin hormone in treatment. Patients with Type II diabetes have insulin, even, although there is more than the required level, blood sugar level rises above the required level, since it couldn’t transport blood glucose to the target cells because of various reasons (Büyükdevrim, 1989).

Micro and macro vascular effects of diabetes and related complications emerge at the end of a long process. Insulin consumption delays and gradually decreases these effects (LeRoith et al. 2005). Diabetes is important for our
era, because it is among the leading causes of blindness and end-stage renal diseases, and it is the main chronic syndrome that causes hospital admissions, injuries and premature (early) death. The most serious finding about diabetes is that its fatality cannot be limited.

Although the etiopathogenesis of these changes which develop due to insulin in diabetes, is not known exactly, some viruses such as Coxsacki B, Epstein-Barr (Barrett-Connor, 1985), Langerhans islets that recreate insulin, and/or auto-immunous reactions against insulin reseptors constitute experimental evidences hypothetically.

Among systemic diseases, diabetes mellitus is one of the issues that dermatologists deal a lot, because of skin complications and various (skin) associations it causes directly or indirectly. 80% of the skin symptoms are observed (encountered) in the first five year of the diabetes, artery complications are subsequent symptoms (Tüzün 1994).

The negative effects of diabetes on wound healing has been known for a long time. In diabetic patients wound healing is slower and complications occur occasionally. The reason of slowness (disablelty) in wound healing is selective inhibition of collagen synthesis (Spanheimer et al., 1988). In animals which are induced diabetes pharmacologically, 30 % less collagen content was determined compared to control group (Andreassen et al., 1988). It has been shown that there is a positive correlation between decreased collagen and increased catabolism in diabetic animals (Leung, 1986; Hennessey et al., 1990).

Another reason for negative impact of diabetes on wound healing, is activation of glucocorticoid mechanisms which causes reduction in wound tension force. It has been shown that, in diabetic rats, wound tension force increases after adrenalectomy (extraction of adrenal grands) or via usage of glucocorticoid receptor blocks (Bitar, 1998). Most common diabetogenic agents that were used in experimental diabetes models have been Streptozotocin and Alloxan (Batisel, 1997).

2. Materials and methods

2.1. Material

In our study, 40 male Wistar Albino rats, each having approximately 200-240 g weight, which were supplied from Eskişehir Osmangazi University Medical and Surgical Experimental Research Center (TICAM), were used. Rats were divided into 4 groups, each containing 10 animals, kept under standart conditions with free access to food and water (ad libitum). All of the animals were treated appropriately in terms of ethical rules (Özelmas, 1995). Also before the beginning of the study, approval of Eskisehir Osmangazi University Local Ethical Council was obtained.

60 mg STZ (Sigma Chemical Co., St. Louis Missouri, ABD) which is dissolved in pH 4.5 = 0.1 M citrate buffer was given intraperitonally to diabetic rat groups. To control groups, just pH 4.5, 0.1 M citrate buffer was given.

Group 1. A: Diabetic male; STZ was given by dissolving in citrate buffer, skin flaps were removed and replaced later (n=10).

Group 2. B: Diabetic male; STZ was given by dissolving in citrate buffer, skin flaps were removed and replaced later, TENS was applied (performed) (n=10).

Group 3. C: Normal male; was given citrate buffer, skin flaps were removed and replaced later (n=10). Being first control group, was primarily compared to group B (n=10).

Group 4. K: Normal male; was given citrate buffer, skin flaps were removed and replaced later. Being second control group, was primarily compared to group A (n=10).

Since reproduction period, birth, parental care and lactation period affects the experimental results, male rats were preferred in our study.
2.2. Method
2.2.1. Forming experimental diabetes

Before the experiment, rats were left to starving for one night. Then their glucose levels were measured and 60 mg/kg streptozotocin (STZ) which is dissolved in pH=4.5 0.1 M citrate buffer was given intraperitonally to form diabetes (Öztürk 1999). 48 hours after the streptozotocin injection, glucose levels were measured again via blood samples which were taken intravenously from tails. For glucose measurement, Prestige IQ (Home Diagnostics Inc., USA) glucose meter and compatible test strips were used.

2.2.2. Surgical operation

Rats were anesthetized with intraperitoneal (ip) cetamine chlorid (Ketalar, Pfizer) (100 mg/kg). Flap region was shaved and cleaned with Povidone Iodine (betadine). Skin flaps with 2x7 cm base were drawn on dorsal skins of the rats with methylene blue (Figure 2). Flaps were removed, including Panniculus carnosus (Figure 3). Later, flaps were replaced to their location by sewing with 4.0 chronic cat-gut. During the entire surgical operation, sterile techniques were used. The area of the surgical operation was sprayed with Opsite (spray), medical dressing was performed with gauze bandage.

Figure 2. Skin flaps with 2x7 cm base were drawn on dorsal skins of the rats with methylene blue.

Figure 3. Drawings of the dorsal skin flap replaced.

2.2.3. TENS application

After surgical operation, the rats of group B and C were taken and TENS application began immediately. Electrodes of TENS device (6 cm diameter) were fixed to the rat’s dorsal in such a way that its center resides on the flap base. 0.2 ms, monopolar, 20 mA electric current with 80 Hz frequency was applied for 1 hour in a day, throughout a week, with TENS device (Omron E4 HV- F-128-E TENS Massager, Japan). During the application, Rats were sedated with cetamine hydrochlorid (50 mg/kg, i.p) and they were put into restrictive cages as shown in Figure 4.

2.2.4. Necrosis Measurements and Biopsy

After the operation, on the 7th day, rats were sacrificed by injecting 100 mg/kg high dose of sodiumpentothal intraperitonally. Flaps and necrosis zones were drawn to acetate paper, then they were measured planimetrically and were photographed. The surface areas of necrosis regions of flaps were calculated in mm² and % percent values. After all of the rats from each group were sacrificed, incisional biopsy of 1x0.5 cm dimensions was taken from the necrosis and solid border, up to the muscle layer including skin and underskin, and histological examination was performed.
2.2.5. Histological technique and investigation

Received tissue samples underwent routine tissue processing in Eskisehir Osmangazi University Medical Faculty, Department of Histology. The tissue samples that were taken from the animal via biopsy, were fixed in 10% formalin for 1-2 days. Then they were washed in running water for 3-4 hours. Samples which were undergone 70, 80, 90, 96 I and 96 II % ethyl alcohol solutions respectively were dehydrated. Then they were treated with xylol I and II for 20 minutes each, and (thus) transparency was provided. In order to remove the xylol and to replace paraffin, tissue pieces were left to I, II, III paraffins respectively for 1 hour and inclusion was provided. For smooth and comfortable sectioning, paraffine (kerosine) which has been hardened in room temperature, was poured in liquid form onto the (tissue) pieces that were put into prismatic moulds. After waiting for its hardening, 4-5 μm thick sections were taken from the parafin blocks via using microtome (Leica RM 2145) and they were left to 40-45 °C of water bath (Leica HI 1210). After the tissue laps were cleared, sections were taken on to the glass slide. After the parafin remnants were melted and cleared (purified), tissue sections were stained with Hemotoksilin-eosin (HE) method. After the last Xylo, an adhesive substance named Entella was dropped onto the slide glass, it (slide glass) was enclosed with lamella and left to drying at room temperature. Finally, tissues which were examined via light microscope (Olympus BH2-RFCA 1.25x) were photographed (Olympus DP70 attachment). In this process 3.3 NFK lens was used with the objective.

2.3. Statistical evaluation

Statistical evaluation of the blood glucoses and necrosis measures of the control and experimental groups (K,A,B,C) were performed with One Way Variance Analysis (One Way ANOVA.). To determine which groups are different from each other, “éscheffe” multiple comparison test was used. Also, comparison of criteria which were determined histologically was done via Kruskal-Wallis non-parametric one-way variance analysis. Paired comparison of the groups was also done via K-W multiple comparison test.

3. Results

3.1. Diabetical findings

During surgical operation and investigation period after the operation, among rats, there hasn’t been any death case encountered. With respect to groups, blood glucose values (mg/dL) were measured 48 hours after STZ and citrate buffer injection, and just before removing flaps.

3.2. Pathological findings

The average flap necrosis and standard deviations are determined as 555.8 ± 54.7; 210.3 ± 33.0; 821.6 ± 66.4 and 787.0 ± 85.2 mm² for K; C; A and B groups respectively.
3.3. Histological findings

On the tissue samples which were taken at the 7th day from all experiment animals, epithelium defects, tissue regeneration, hyperemia, fiber increase and cell infiltration parameters were measured histologically.

K: Insufficient tissue regeneration, middle level hyperemia and minimal cell infiltration was observed. Fiber increase was determined again in minimum level. Epithelial defects were again scarcely observed.

A: In this group of subjects, beside intense epithelial damage, extensive cell infiltration was observed. Intense vein hyperemia and significant fiber increase was encountered (observed). Significant tissue regeneration could not be determined.

B: In incision region, fiber increase and cell infiltration was seen significantly. Epithelium defects were determined in the specimens of this group, though not as much as in A. No signs of tissue regeneration was observed (found). Again, similar to group A, intense hyperemia was observed.

C: Regenerate epithelium and collagen tissue repair was completed, hyperemia and cellular infiltration has not been observed (found) in the preparate. In terms of fiber increase, it has been observed that it is lower compared to group K. In group C, epithelium and collagen tissue repair has been completed and compared to group K, less fibrillar growth has been observed. (In the non-diabetic rats, which were applied TENS, flap recovery is higher/better.)

In groups A and B (with and without TENS), intense epithelium defects were observed. In all of the groups, there hasn’t been observed (found) any increase in vascularity. This is because of TENS’ vasodilation effect, rather than its effect on vascularity.

Figure 6. Group C (healthy, TENS applied control), histopathological assessment (Ep: Epithelium, CT: Connective tissue, H: Hypremic vein) (HEx890)
Table 1. Histological evaluation criteria and criteria the number of observed subjects

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Figure 7. Group A (experimental diabetes) histopathological assessment (DEp: Damaged Epithelium, İ: Infiltration) (HEx440)

Figure 8. Group B (Diabetic, TENS applied experimental) histopathological assessment (DEp: Damaged Epithelium, İ: Infiltration) (HEx440)
3.4. Statistical findings

In our study, blood glucose measurements were done 48 hours after injecting STZ solution to experiment groups and citrate buffer to control groups. It was found that glucose measurements differ from each other in 4 groups, via applying One Way Variance Analysis. When Groups A and B are compared to Control (K) group, statistically a very significant difference (P<0.001) was observed (found) in blood sugar measurement. However, between group K and group C, statistically a very significant difference was not found (P>0.05). When group A and group B’s blood sugar measurement was compared statistically, a significant difference wasn’t found (P>0.05). On the other hand, when group A and group C’s blood sugar measurements are compared statistically, a significant difference (P<0.001) was found.

4. Conclusions

C There are various opinions about etiologies of the complications seen in Diabetes mellitus which is a systemic disease caused by insulin deficiency or abnormalities in insulin synthesis and immunity formation against insulin. Depending on the metabolic disorders, and as especially observed on uncontrolled diabetes charts (tables), highness of blood glucose level increases oxidative activity and thus increases free radical formation and proteins’ glycosylation (Bayness, 1991). If plasma and cell membrane proteins are exposed to high concentration of glucose for a long time, glucose is fastly bound to amino groups of the proteins with a non-enzymatic way. The newly formed glycosylation products reach to an equilibrium proportional to blood glucose concentrations and a little more stable early (initial) glucolization products appear (form). Free radicals are produced since these are exposed to oxidation, and finally molecular level defects occur in diabetes (Walter et al., 1991).

Flaps are frequently used in removing tumors, covering tissue defects that occur due to trauma related tissue losses. In spite of the great advances in flap surgery, the flap necrosis which appears on distal region is still a serious clinical problem. To prevent from this, many physical and chemical treatment methods are being tested. No matter which method is used, it is aimed to increase blood flow to the flap or ischemia tolerance. One of the most convenient ways to increase ischemia tolerance is surgical delay phenomenon (Milton, 1969). This method is used for a long time and its mechanism has not been fully understood. According to recent studies, flap necrosis was reduced by the increase of anastomosis between choke vessels (Callegari et al., 1992; Taylor et al., 1992; Yang and Morris, 1998).

Inhibition of coagulation factors also reduces the acute ischemia related necrosis in flaps. In a study about inactivation of FVIIa, without any effect on systemic hemostasis, at flap’s distal ends trombus was prevented (prohibited) and flap viability(survival) was increased (Alizadeh et al., 2004). To study flap viability(survival), there has been studies with many pharmacologic agents. Efficiency of sympathycolitics (Suarez et al., 1992), smooth muscle relaxants (Suarez et al., 1992; Smith and Dolan, 1999), rheological agents (Takayanagi and Ogawa, 1980) and free radical blockers (Stewart et al., 1994) were investigated. In recent years, studies have focused on increasing flap viability(survival) by giving endogen factors from outside (Tellioğlu et al., 2001; Pang et al., 2003; Gürlek et al., 2004).

To reduce and prevent (prohibit) flap necrosis, physical methods are also used. Providing a humid medium and heating the flap are frequently used clinical methods. TENS (Transcutaneous Electrical Nevre Stimulation) is also one of these physical methods. Accordingly, TENS, closes the spinal gate by stimulating thick fibers continuously, and prevents from pain via presynaptic inhibition by increasing endorphine secretion (Walsh and Baxter, 1996). By various researchers, it has been shown that, TENS have effects on the skin blood flow (circulation). According to a clinical research which was conducted by forming bulla on the skin of volunteers, it has been shown that high frequency TENS current increases microcirculation (Wikström et al., 1999). According to a clinical study done by forming bulla on skins of volunteers, it has been shown that, creating high-frequency TENS increases flow microcirculation (Wikstrom et al., 1999). With a study conducted among volunteers, Cramp and colleagues (2000) have shown that low frequency TENS increases skin blood circulation (flow). Sherry and colleagues (2001) have determined a temporary increase on local blood circulation(flow) at stimulation levels which provide muscle contraction (sherry et al., 2001).

In our study, parallel to previous studies, it has been shown that, in non diabetic rats, TENS provides a statistically significant increase in flap’s viability(survival) area. This study is investigating the effects of TENS on flap survival in acute diabetic rats. In literature, about this subject, there isn’t any experimental study encountered. There are clinical and experimental studies in which TENS’ effect on healing of diabetic ulcer is investigated. According to the results of these studies, TENS has positive effects on healing of diabetic wound healing (Lundeberg et al., 1992; Baker et al., 1997; Peters et al., 1998; Thawer and Houghton, 2001).

However, in this study, it was determined that, TENS application didn’t reduce the flap necrosis in diabetic rats compared to non-diabetic ones. Insulin deficiency may be the reason for this. In many studies, which show that electrical stimulation has positive effect on wound healing, animals which have regulation on blood sugar were involved(used) Lundeberg and colleagues have determined that electrical stimulation increases healing of ulcer in diabetic patients. In another study, providing proper wound care and prevention from weight to feet, electrical stimulation increases wound healing in diabetics (Peters et al. 1998).
In the studies they’ve done with STZ induced diabetic rats, Babovic and colleagues (1994) have shown that, viability (survival) of epigastric isle flaps in diabetic animals decreases (reduces) after secondary ischemia. When insulin supplements were given to diabetic rats, flap’s ischaemia tolerance and flap vitality (survival) has increased. (Babovic et al., 1994). It is known that suitable insulin treatment prevents from the complications of diabetes. In our study diabetic rats were not given insulin treatment. This study, even though indirectly, shows that, for treating circulatory disorders in diabetics, insulin treatment is more preferable than the other treatment methods. Lack of decrease of flap necrosis in diabetic rats is may be since blood sugar regulation can’t be done with insulin. Moreover, our study consists of diabetic’s acute phase and hyperglycemic conditions.

In a study in which electrical stimulation’s effect on the histological features of wounds in diabetic rats was examined; it has been determined that, low-voltage electrical stimulation has increased collagen storing in nondiabetic rats, while it has no effect on diabetic rats. To obtain same variations (changes) in diabetic rats, lower voltages were needed. It was concluded that electrical stimulation has different effects on diabetic and non-diabetic animals (Thawer and Houghton, 2001).

In our study, although TENS hasn’t decreased distal flap necrosis in rats in which acute diabetes was formed macroscopically, it has decreased inflammation in histological examinations. In the group to which TENS was applied, a middle level inflammation was determined, while, in acute diabetic rats which were not applied TENS, intense inflammation findings were detected. In literature, a study, which shows that inflammation increases in acute diabetes, was encountered (Komesu et al., 2004). In our study, in the acute diabetic rats which were applied TENS, existing inflammation decreased (reduced) compared to control group diabetics which were not applied TENS. In addition to this, TENS’ effect on reducing (decreasing) inflammation on non-diabetic rats has supported the macroscopical findings in literature histologically (Lundenberg et al., 1998; Kjartannsson et al., 1988; Kjartannsson and Lundenberg, 1990; Im et al., 1990; Atalay, 2003).

In this study, the effects of TENS on flap viability (survival) of acute diabetic rats were investigated in terms of macroscopic measurement of necrosis and histological findings. Studies will go on (continue) with Laser Doppler Flowmeter and with investigation of biochemical parameters which show reperfusion of ischemia.

As a result, TENS current significantly reduces the flap necrosis area in non-diabetic animals. However, on acute diabetes formed animals, which do not take insulin treatment, positive effect on flap survival hasn’t been determined.

References


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