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Effect of Testosterone on Intracellular Signaling Pathway of Angiogenesis in Sciatic Nerve of Male Diabetic Rats

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ABSTRACT

Induction of angiogenesis in the damaged area can be usefulin diabetic neuropathy. Various factors can be effective on angiogenesis,such as steroid hormones. We investigatedeffect of testosterone ona the signaling pathway of angiogenesis in sciatic nerve(AKT/ERK pathways).Twenty four wistar rats (250-300 g)were subdivided randomly in four groups (n=6): 1) diabetic (D),(streptozotocin,50 mg/kg, IP), 2) diabetic gonadectomized (D+GDX), diabetes was induced aftergonadectomy. 3) diabetic with testosterone (D+T), after induction of diabetes, testosterone(2mg/kg/day, SC, for 6 weeks). 4) diabetic gonadectomized with testosterone (D+GDX+T), after gonadectomy and induction of diabetes, testosterone was injected. ThenAKT and ERK proteins were measured. Forevaluation of angiogenesis was used from Immunostainingmethod(PECAM-1/ CD31).Testosterone decreased Akt protein in the diabetic and diabetic gonadectomized groupssignificantly ($p<0.05$).Also testosteronedecreased ERK protein in gonadectomized condition significantly($p<0.05$). Testosterone treatment or gonadectomy had no significant effect on angiogenesis, but combination of testosterone and gonadectomy showed significant effect onangiogenesis($p<0.05$).According to results, can be offered that testosterone decreased angiogenesis by reduction of AKT in sciatic nerve in diabetic condition.

Key words:Angiogenesis, Diabetic Neuropathies, Sciatic, Testosterone**.**

INTRODUCTION

Diabetes mellitus is one of the metabolic diseases that can be characterized by high blood sugar. In this situation, there is insufficient or lack of insulin production by pancreatic beta cells. High blood sugar provides many symptoms such as: polyuria, polydipsia and polyphagia[1]. Diabetes plays a paradox role on vessels. For example diabetes increases neovascularization in kidney and retina but it can inhibit angiogenesis in coronary heart disease and peripheral vascular disease [2]. Diabetic neuropathy is one of the microvascular disorders. It was known, microvascular and macrovascular diseases are the most important cause of morbidity and mortality in diabetic patients [3]. The secondary blood flow is reduced and improved by induction of angiogenesis in the damaged area [4]. Angiogenesis is an important process that increases density of blood vessels in injured regions. These blood vessels supply oxygen and nutrition into damage tissue[5]. This

process involves several stages such as endothelial cell proliferation, migration, sprouting and tube formation in the vessels [6]. Although angiogenesis occurs during embryonic development but it can be seen in adult humans in some physiological conditions such as reproductive cycle, wound healing[7] and pathological condition such as diabetes [1]. Several factors can stimulate angiogenesis including the growth factors, cytokines and lipid mediators [5]. Various factors that affect angiogenesis have been studied. One of these factors are hormones, such asestrogen [8],testosterone [9] and ghrelin[10] .

Testosterone is one of the androgenic steroids that secreted by the Leydig cells of the testes in males and produced with ovaries and placenta in females. In addition, the adrenal glands in males and females body release testosterone hormones[11]. However the effect of estrogen in angiogenesis process studied widely, but the influence of androgens in this process is not fully understood [12]. Treatment with androgen in hypogonadal type 2 diabeticmen can declines insulin persistenceand cures glucose levels, also can recover ischemia with sever angina[13].It is reported that injection of testosterone increases angiogenesis in the prostate tissuein gonadectomized male rats[14]. The PI3K/AKT(AKT) and RAS/RAF/MEK/ERK (MAPK) are two of the most well-known pathways that control cell proliferation [8] and angiogenesis [15].This pathways are also involved in diabetes [16]. It should be noted, there are not any studies about this signaling pathway in sciatic nerve. Therefore we decided to investigate a part of the signaling pathways of angiogenesis in this nerve. The AKT/ERK pathways were our aim.

MATERIALS AND METHDS

Animals

Adult Male Wistar rats (250-300 g) were achieved from upbringing colony of Tabriz University of Medical Sciences, Tabriz, Iran. The animals were retained under a 12:12 h light/dark cycle at 23±2æ%C with food and water ad libitum. Twenty-four hours before the beginning of the study, all of the animals were transported to the laboratory in order to adaptation with environment. The rats were subdivided randomly in four groups (n=6) including: 1) diabetic (D), 2) diabetic gonadectomized (D+GDX), At first gonadectomy was done then diabetes was induced. 3) diabetic with testosterone (D+T), after induction of diabetes, testosterone was injected. 4) diabeticgonadectomized with testosterone (D+GDX+T), after gonadectomy and induction of diabetes, testosterone was injected.

Induction of diabetes

For induction of diabetes, streptozotocin (STZ) was injected into rats (50 mg/kg, IP, single dose) (Sigma). After two days, the fasting blood sugar levels were tested by the glucometer device (Boehringer Mannheim Indian applis, IN). The rats that had blood glucose levels above 300 mg/dl were placed in diabetes groups [17].

Removal of the gonads (Gonadectomy)

Animals were anesthetized by subcutaneous injection of ketamine/ xylazine(60mg-2mg/kg)[10]. They were perched on a flat place, after creating a small incision (~2 cm) was made in the abdominal wall. Testes were removed through the incision. A cut through the epididymis was made to remove the testes

Drug prescription

The animals in testosterone groups (D+T and D+GDX+T) received testosterone (2mg/kg/day, SC) for 6 weeks. Testosterone was prepared from the Amino Acids, P.F, Tianjin, china. In order to avoid of hormonal disorder, testosterone ejection was started after gonadectomy immediately[18].

Tissue sampling and protein measurement

After the end of 6 weeks, animals were anesthetized with an i.p injection of ketamine (80 mg/kg) and xylasin (5 mg/kg) and sacrificed. Then sciatic nerve was removed and after quick freezing with nitrogen, were kept until AKT and ERK measurement in -70 °C. Samples were weighted, homogenized in PBS (PH: 7.2-7.4) and centrifuged for 20 min (*1600 g*) in 4°C temperature. Then supernatants were removed and AKT and ERK proteins were measured. AKT and ERK levels were measured using sandwich rat ELISA Kits according to the manufacturers protocol (Rat p-AKT, N-16 Torrance, USA, Lot: 20141111. ERK 1/2, abcam Lot: GR196140-1).

Immunostaining for PECAM-1/ CD31:Evaluation of angiogenesis

Sciatic nerve were fixed in 10% formalin and paraffin embedded. Then, serial 3ìm thick sections were cut from paraffin blocks and floated onto charged glass slides[19]. Tissue sections were deparaffinized in xylene and dehydrated in a graded series of ethanol. Slides were incubated consecutively in proteinase K and treated by 0.3% hydrogen peroxide for blocking endogenous peroxidase activity. Sections were overlaid by a marker of angiogenesis in the name of primary antibody CD31 (Santa Cruz, USA) and incubated at +4°C overnight. Then sections were washed and incubated with standard avidin–biotin complex (ABC; Santa Cruz) according to the manufacturer's instructions. Then slides were incubated in DAB (di-amino-benzidine, Santa Cruz) and counterstained with Mayer's hematoxylin. Finally, sections were cleared in xylene, mounted with Entellan and assessed by light microscope (Olympus BX 40, Japan). For evaluation of immunostaining, the intensity of the staining was scored as 0 (<10%), 1 (10-25%), 2 (25-50%), 3 (50 -75%) and 4 (75-100%)[10].

Statistical analysis

Data were analyzed by using SPSS version 16.0 software and after that were tested byone way ANOVA followed by LSD analysis. The results were reported as mean± S.E.M and the P value less than 0.05 was considered significant.

RESULTS

Effects of diabetes and testosterone on AKT protein levels in sciatic nerve

The effect of 6 weeks of testosterone (2mg/kg/day) treatment on AKT protein level in sciatic nerve in diabetic and diabetic gonadectomized rats showed that the amount of this protein in the diabetic group that receiving testosterone significantly decrease compare to the diabetic group ($p<0.05$). Also our results showed in the diabetic gonadectomized group that receiving testosterone, AKT level reduced compared with the group ($p<0.05$). Also diabetic gonadectomized had no significant difference with control group (Fig1).

Effects of diabetes and testosterone on ERK protein levels in sciatic nerve

As it can be seen in figure 2,gonadectomy or treatment with testosterone could not significantly change the levels of ERK in sciatic nerve compared with diabetic group. However level of the ERK protein in diabetic gonadectomized group significantly increased compared with the diabetic gonadectomized group that received testosterone.

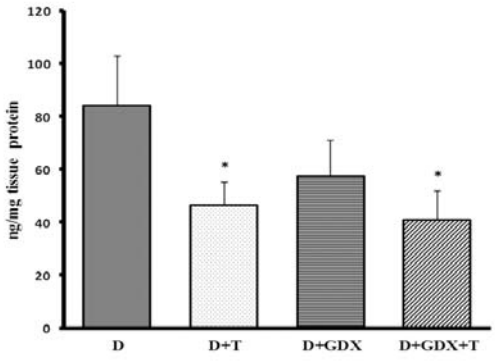

Fig. 1: Effect of testosterone (2mg/kg/day) on AKT level in sciatic nerve after 6 weeks in male rats(n=6). Diabetic (D), diabetic plus testosterone (D+T), diabetic gonadectomized (D+GDX) and diabetic gonadectomized recipient of testosterone (D+GDX+T) groups. Data are expressed as mean±SEM. * p<0.05 vs the diabete group

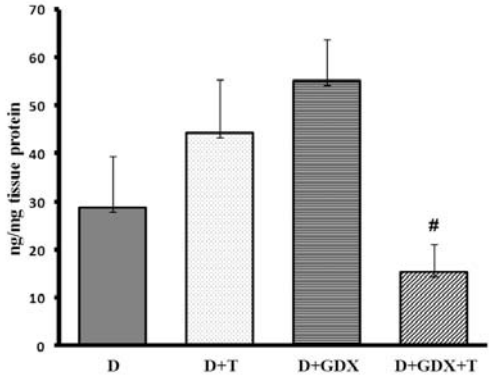

Fig. 2: Effect of testosterone (2mg/kg/day) on ERK level in sciatic nerve after 6 weeks in male rats (n=6). Diabete(D), diabete plus testosterone(D+T), diabetic gonadectomized (D+GDX) and diabetic gonadectomized recipient of testosterone (D+GDX+T) groups. Data are expressed as mean±SEM. # P<0.05 vs the diabetic gonadectomized

Effects of testosterone on angiogenesis in sciatic nerve

Brown stained area in sciatic nerves show density of endothelial cells in the vascular (Figure3). Testosterone treatment or gonadectomy had no significant effect on angiogenesis, but combination of testosterone and gonadectomy showed significant effect on angiogenesis($p<0.05$).

DISCUSSION

The finding of our study showed that removal of the gonads had no effect on expression of AKT and ERK protein, but testosterone administration in diabetic and diabetic gonadectomized condition reduced AKT level in sciatic nerve if the effects on the reduction of ERK was only in diabetic gonadectomized condition. Histological results also indicated that removal of the gonads increased the process of angiogenesis in the sciatic nerve and testosterone exacerbated this process in this situation. Diabetic neuropathy is one of the common complication in diabetes that observed following vascular injury and tissue hypoxia [20]. Tissue hypoxia can activate factors that eventually led to the formation of new blood vessels or angiogenesis in the tissue. Among these factors can be referred to VEGF. Revese et al. in their study show that the induction of diabetes by STZ, increased the expression of VEGF and treatment with insulin decreased the expression of VEGF [20].

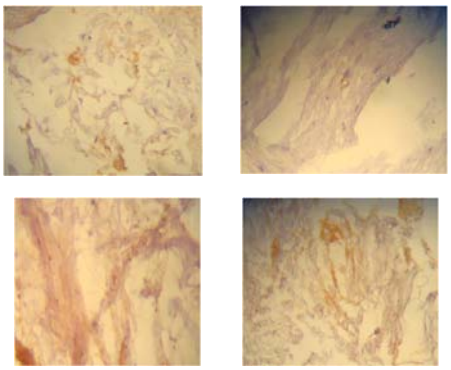

Fig. 3: Immunohistochemical diagnosis of CD31 in sciatic nerve. Brown stained tissues show CD31 immunostained endothelial cells in (A):diabetic, (B): diabetic+ghonadectomized, (C): diabetic+ghonadectomized+ testosterone, (D): diabetic + testosterone(40X).

Recently, Cheng et al. offered that the effects of diabetes on angiogenesis had a paradox situation [21]. In diabetic condition, some regions had uncontrolled angiogenesis such as retinal, whereas in some areas, angiogenesis decreased or not observed such as small blood vessels in peripheral tissues [21]. Jiann XU et al. reported several potential mechanism in relation to impaired angiogenesis in diabetes: oxidative stress, change in the expression of miRNA, lack of growth factors, inhibition the AKT pathway and changes in the VEGF receptors [22]. A variety of intracellular pathways have been identified in the process of angiogenesis that we study two signaling pathways involve in angiogenesis such as AKT protein through PI3K/AKT and ERK protein though RAS/RAF/MEK/ERK.

Several factors can affect the signaling pathways. Such factors include can be pointed male sex hormones such as testosterone. Existence androgen receptors on lumbar and sacral in rat sciatic nerves has been demonstrated[23]. Recently, Teubnera et al. investigated the effect of testosterone on angiogenesis in testes vessels for 12 weeks. Their results showed that testosterone increase vessels angiogenesis in testes. It should be noted that they attributed the enhancement of angiogenesis to increased expression of angiogenic factors (TGF-á and Ang2) and VEGF receptors [24]. Sieveking et al. found that the androgen hormones dose-dependent increase the production of mRNA VEGF and its receptor and the subsequent the angiogenesis process [12]. Our histological studies indicated that angiogenesis increased with testosterone in gonadectomized condition in sciatic nerve. So it can be suggested that other hormones that are secreted by the gonads and are eliminated during gonadectomy, contribute in process of angiogenesis and removing them increased this process. One study suggest that testosterone can active AKT in cancer and osteoblasts cells [25]. While our results showed that testosterone reduced AKT. This contradiction can be attributed to the diabetic status, type of tissue, the lack of gonads and dose of testosterone. Because diabetes itself leads to suppression of the AKT pathway [26]. However, studies have also demonstrated that insulin is activates PI3K/AKT pathway [27, 28]. Therefore, it is expected that reduction of insulin in diabetes also reduced the AKT. Also

mentioned earlier that activation of androgen receptors can decrease the phosphorylation of AKT. On the other hand the existence of androgen receptors in the sciatic nerve has been demonstrate, so activation of the receptors can reduce the AKT. Purves et al. showed that diabetes can activate ERK protein in heart tissue with two ways:1-direct way(increase blood sugar) 2-indirect way(enhancement oxidative stress)[26]. Briaud et al. suggested that glucose activates ERK in the â cells [29]. However, walker et al. referred to impact of testosterone on ERK phosphorylation in sertoli cells in the mice testes[30]. One study showed that there is a reverse relationship between testosterone and blood sugar[31]. Our results also showed that the effect of testosterone on decrease ERK can be seen only in terms of the removal of gonads. According to these results we can hypothesize that the impact of the exogenous testosteronewill be prominent on ERK when the gonads were removed and this condition followed by increasing the number or sensitivity of receptors. Also testosterone reduced ERK by reducing blood sugar and followed by a decrease in insulin [29].

Lissbrant et al. demonstrate that gonadectomy reduced VEGF expression in ventral prostate and testosterone increased VEGF expression [14]. The histological results showed that gonadectomy and testosterone enhance the angiogenesis in sciatic nerve. To justify this difference can cited the effect of diabetes condition and type of tissue. Probably in gonadectomized and removal testosterone condition, ERK was actived in sciatic nerve and increased angiogenesis whereas testosterone may be reduced angiogenesis with decreasing of blood sugar and then insulin and disabled AKT [27-29, 32]. Dimmeler et al showed that AKT can inhibit ERK pathway by phosphorylation of RAF in this pathway [27]. Therefore we can suggest that each factor increase AKT, it can reduce ERK in this way. According to results, can be offered that testosterone decreased angiogenesis by reduction of AKT in sciatic nerve in diabetic condition.

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