HGF/C-MET PATHWAY HAS A ROLE IN TESTICULAR DAMAGE IN DIABETES INDUCED BY STREPTOZOTOCIN

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Abstract

Objective. The aim of the study was to investigate the role of Hepatocyte Growth Factor (HGF)/c-Met pathway in testicular damage provoked by streptozotocin (STZ)-induced diabetes and the effects of insulin treatment on the HGF/c-Met pathway.

Methods. Total 21 paraffin-embedded testicular tissues of control (n=7), streptozotocin (STZ)-induced diabetic (n=7) and insulin-treated diabetic (n=7) Wistar albino rats were used in this study. Testicular damage was examined histologically and by Johnsen's score was also evaluated. Immunohistochemical stainings of HGF and c-Met were analysed by using antibodies against HGF and c-Met.

Results. We found the degeneration in seminiferous tubule epithelium and disorganization of spermatogenic cell series in testis tissues of diabetic rats. We also determined decrease both in seminiferous tubule diameter and Johnsen's scores in diabetic group. The expressions of HGF and c-Met in seminiferous tubule epithelium and in spermatogenic cells (especially spermatocytes and spermatids) were significantly increased in diabetic rats compared to those of control. Insulin treatment significantly reduced the diabetes-induced morphological changes and HGF/c-Met over expressions in the diabetic rat testis.

Conclusion. HGF/c-Met pathway might have a role in diabetes- induced testicular damage. Drugs acting on this pathway might be effective to prevent or delay the testicular damage induced by diabetes.

Key words: diabetes, HGF/c-Met, insulin, testis, testicular damage.

INTRODUCTION

Diabetes mellitus, a chronic metabolic disease, is increasing worldwide. According to World Health Organisation (WHO), diabetes prevalence was 8.5% among the adult population in 2014 (1). Diabetes has many complications because of the increased glucose levels (2). Infertility is one of the important

complication of diabetes especially in young man. Many experimental animal studies report that diabetes causes testicular damage and testicular dysfunction by decreasing spermatogenesis (3-5). Also in human studies decreased sperm motility and semen volume were reported (6-8).

Because of the defects in insulin secretion in diabetes, insulin is the main treatment in the patients who have a pancreatic β cell damage (9). Insulin decreases high blood glucose levels in diabetic patients and reduces microvascular and cardiovascular complications in those with type 1 diabetes (10). The metabolic effects of insulin are well known in diabetes but the effects on testicular damage related by STZ induced diabetes are limited (11, 12).

Hepatocyte Growth Factor (HGF) is a cytokine that stimulates mitogenesis, morphogenesis and angiogenesis and have a regulator role in many biological processes such as embryogenesis, homeostasis, tissue regeneration. The multiple actions of HGF occur by its receptor c-Met which has a tyrosine kinase activity. The effects of HGF were investigated in different disease models in experimental studies and it is a new biological drug candidate. It is reported that the HGF is one of the growth factors which regulate the testicular development (13). The role of HGF/c-Met signalling system in germ cell homeostasis and male fertility was shown (14). But the role of HGF/c-Met system in testis tissues of diabetic rats is unknown.

Therefore, in this study, we aimed to demonstrate the role of HGF/c-Met pathway in the testis tissues of diabetic rats and the effects of insulin treatment on the HGF/c-Met signalling system and also testicular dysfunction in diabetic rats.

METHODS

Archival paraffin-embedded animal testicular samples were used in this study. These archival tissues

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were obtained from our previous study (61/2009). After ethical approval from Dokuz Eylul University, School of Medicine Animal Use Committee, some animal tissues including testis tissues were collected as paraffin-embedded blocks and stored for using for the future studies in the archive of Histology and Embryology Department (657/2010). To use these testis tissues in the present study was also approved by the Dokuz Eylul University, School of Medicine Animal Use Committee (55/2016). Total 21 paraffin-embedded testicular tissues of control (n=7), streptozotocin (STZ)-induced diabetic (n=7) and insulin-treated diabetic (n=7) rats were used in the present study. These tissue samples were collected in our previously reported study (15) from male Wistar albino rats exposed to the single intraperitoneal (i.p.) injection of 45 mg/kg STZ with or without insulin treatment (6 U/kg/day NPH insulin, s.c.). The age-matched control group received citrate buffer only. Rats were sacrificed by cervical dislocation after 8 weeks treatment. Then some tissues including testis etc. were removed and fixed in 10% formalin and embedded in paraffin blocks and stored in the Histology and Embryology Department archives.

Histological examination

After routine histological procedure, five-micron-thick sections were stained with haematoxylin and eosin (H&E). Images were analysed by using computer-assisted image analyser system having a microscope (Olympus BX50, Tokyo, Japan). Images were transferred into the digital video camera (DP71, Olympus, Japan).

Examination of Spermatogenesis

We used to Johnsen's score to categorize the spermatogenesis by the following criteria from 1 to 10. 10 = complete spermatogenesis and perfect tubules; 9 = many spermatozoa present and disorganized spermatogenesis; 8 = only a few spermatozoa present; 7 = no spermatozoa but many spermatids present; 6 = only a few spermatids present; 5 = no spermatozoa or spermatids but many spermatocytes present; 4 = only a few spermatocytes present; 3 = only spermatogonia present; 2 = no germ cells but only Sertoli cells present; 1 = no germ cells and no Sertoli cells present (16, 17).

Measurement of seminiferous tubule diameter (STD)

The 10 most circular seminiferous tubules were randomly identified in each section of the testis. The diameters of the seminiferous tubules were

measured with an ocular micrometer using the x10 lens. The mean STD in micrometers was determined for each testis (17). All sections were viewed under a microscope with an attached video camera and image analyzer system (UTHSCA Image Tool for Windows version 3.0 software).

Immunohistochemical Assessment

Immunohistochemical staining was performed using the streptavidin/biotin method. The immunohistochemistry procedure for c-Met (sc-161, Santa Cruz Biotechnology, Inc, 1/100 dilution) and HGF- β (sc13087, Santa Cruz Biotechnology, Inc, 1/100 dilution) were performed. Sections were incubated at 60°C overnight then dewaxed in xylene for 30 min. After rehydrating through a decreasing series of alcohol, sections were washed in distilled water for 10 min. They were then treated with trypsin at 37°C (Cat No: 00-3008 Digest All 2A, Zymed, San Francisco, CA, USA), to unmask antigens by heat treatment. Then slides were washed in deionized water three times for two minutes. Sections were incubated in a solution of 3% H₂O₂ for 15 min to inhibit endogenous peroxidase activity. They were then incubated with normal serum blocking solution for 30 minutes. Sections were then incubated in a humid chamber for overnight at +4°C with antibodies. Afterwards, they were washed three times for 5 min each with PBS, followed by incubation with biotinylated IgG and then with streptavidin-peroxidase conjugate (Invitrogen, Histostain- Plus Broad Spectrum, 85-9043). After washing, three times for 5 min with PBS, sections were incubated with DAB substrate containing diaminobenzidine (1718096, Roche) for 5 min to detect immunoreactivity and then with Mayer's hematoxylin. Sections were covered with mounting Entellan (Merck, Germany) and were analyzed on a light microscope with a DP70 digital video camera (Olympus, Tokyo, Japan). The percentage of positive cells was calculated at five areas per sections. Immunolabelling intensities of HGF- β and c-Met were graded (with semiquantitative assay) independently by two observers (I.T. and S.C.M.) blind to the experimental conditions.

Evaluation of immunostaining

The intensity of cytoplasmic or membranous staining was scored from 0 to 4. Extent of staining was scored from 0 to 4+ (0: negative; 1+: 0–25%; 2+: 26–50%; 3+: 51–75%; 4+: 76–100%) according to the percentage of the positively stained areas.

Statistical analysis

All values were expressed as the mean±standard error (mean±SEM) by using GraphPad Instat™, 1990-1994, GraphPad Software V2.05a 9342, USA. Differences among groups were performed using one way analysis of variance followed by Tukey-Kramer multiple comparison tests for blood glucose level, body weight and seminiferous tubules diameter. The Kruskal Wallis and Mann Whitney-U test were used to compare staining intensity among groups. p values of <0.05 were considered to be statistically significant.

RESULTS

Evaluation of Hematoxylin-Eosin Staining

Normal histological structure was observed in H&E stained testis tissue of the control group. Seminiferous tubules epithelium were in normal histological appearance with including the cells at different stages of spermatogenesis in this group (Fig. 1a). The seminiferous tubule structure in the

diabetic rats was found to be disrupted (Fig. 1b). Spermatocytes and spermatids have been reduced and disorganized of cell lines. The histological structure in the insulin-treated diabetic group resembled that of the control group. While the number of spermatogenic cells (spermatocytes and spermatids) was increased, the number of atrophic - seminiferous tubules was decreased in insulin-treated diabetic rats compared to the diabetic group (Fig. 1c). Also, there was an improvement in the seminiferous tubule structure and spermatogenic cell series became regular in insulin-treated diabetic group.

The Mean Seminiferous Tubule Diameter and Johnsen's Criteria

The seminiferous tubule diameter and Johnsen's criteria values of each group are shown in Table 1. It was shown that both were significantly decreased in diabetic rats compared to the control group ($p<0.0001$). However, these values were increased in the insulin-treated diabetic rats compared with the diabetic rats

Figure 1. Hematoxylin&Eosin(H&E) and HGF and c-Met immunohistochemical staining of testis tissues (C: Control, D: Diabetes, D+I: Diabetes+insulin).

($p < 0.001$). In the insulin-treated diabetic group, while seminiferous tubule diameter values were close to the control group, but Johnsen's criteria values were significantly lower than in control group ($p < 0.005$).

Immunohistochemical Analysis

Immunohistochemical examination indicated a statistically significant increase in the expressions of HGF and c-Met which were increased in seminiferous tubule epithelium and spermatogenic cells (especially in spermatocytes and spermatids) in diabetic group compared to control group ($p < 0.001$, the arrows show the immunopositive staining, Fig. 1d,e,g,h, Table 2).

However, immunoreactivity of HGF and c-Met were decreased in the insulin-treated diabetic rats compared with the diabetic rats ($p < 0.01$, Fig. 1f, i). While the increase of immunoreactivity of HGF and c-Met were reduced by insulin treatment, c-Met expression was higher than those of control in the insulin-treated diabetic group ($p < 0.001$, Table 2).

DISCUSSION

This study indicated that diabetes caused testicular damage in adult rats, and insulin treatment improved the changes of testicular morphology by providing protection against the degeneration of seminiferous tubuli and the loss and disorganization of spermatogenic cell series. Additionally, the expressions of HGF and c-Met in the seminiferous tubule epithelium were significantly increased in the diabetic rats, these increases might be an indication of the role of HGF/c-Met signalling system in diabetes-induced testicular damage. As the increase of immunoreactivity of HGF and c-Met were reduced by insulin treatment supports

this hypothesis.

Diabetes is a frequent metabolic disease causes many important complications such as endothelial dysfunction, cardiomyopathy, nephropathy, retinopathy as well as male infertility (1). Several experimental studies have shown that diabetes leads to a marked impairment in fertility by altering the morphology and function of sperm cells as well as increasing seminal plasma abnormalities (18). It is well reported that diabetes leads to testicular dysfunction by causing apoptotic cell death in germ cells, atrophy of the seminiferous tubules, decreased tubule diameters and disorganisation and reduction of spermatogenic cell series (3,19).

In present study, we verified the testicular damage by histologically and we also measured the Johnsen's scores of the testis tissues for assessing spermatogenesis. We observed the degeneration in seminiferous tubule epithelium and disorganization of spermatogenic cell series in testis tissues of diabetic rats. Our results are consistent with those of previous studies (17). In accordance with previous studies, in our study, Johnsen's scores of diabetic rats were lower than 6, which indicates the presence of testicular damage (16, 17). Additionally, seminiferous tubule diameter (STD) was decreased in our diabetic group. By all these findings demonstrating that testicular damage was successfully occurred in STZ-induced diabetes in our animal model.

Hepatocyte Growth Factor (HGF) is a cytokine which has a regulator role in many biological processes such as embryogenesis, homeostasis and tissue regeneration. HGF acts by its receptor c-Met, which has a tyrosine kinase activity. HGF is also one of the growth factors that has a role in testis development

Table 1. Seminiferous tubule diameters (STD) and Johnsen's scores of groups

	C	D	D+I	
Seminiferous tubule diameters	292.3±41.2	153.4 ±43.3*	290.2± 44.8**	(*), $p < 0.0001$; versus C (**), $p < 0.001$ versus D
Johnsen's scores	9.2± 0.2	5.5± 0.2*	7.7± 0.3**,#	(**), $p < 0.001$ versus D (#), $p < 0.005$ versus C

(C:Control, D: Diabetes, D+I: Diabetes+insulin)

Table 2. Immunohistochemical staining scores of groups

	C	D	D+I	p
HGF	4.4±0.7	11.3±0.9*	6.6±0.9#	(*), $p < 0.001$; versus C (#), $p < 0.01$; versus D
c-Met	3.9 ±0.7	32.1 ± 13.3*	15.4 ±1.1**,#	(**), $p < 0.01$; versus C (##), $p < 0.001$ versus D

(C:Control, D: Diabetes, D+I: Diabetes+insulin)

(13) and its role in modulating the functional activities of somatic and germ cells of the rat was shown (20). There is a limited number of studies related to the role of HGF/c-Met pathway in some diabetic complications but there is no study in diabetes-induced testicular damage in our knowledge (21). Increased HGF/c-Met expressions were also shown in the heart, aorta and kidney tissues of diabetic rats (15, 22, 23).

In our study, we found the expressions of HGF and c-Met in seminiferous tubule epithelium and in spermatogenic cells (especially spermatocytes and spermatids) were significantly increased in diabetic rats compared to those of control. Although we could not study functional activities HGF and /or c-Met on testicular function in diabetes, Catizone *et al.* reported that HGF modulates germ cell metabolic activities *in vitro* non-diabetic model, positively regulating germ cell proliferation and decreasing the number of apoptotic germ cells (13, 20).

Oxidative stress is one of the important pathogenic factors in diabetes related complications and the role of oxidative stress in diabetic rat testicular damage was reported in many studies (23, 24). It is well known that HGF/c-Met pathway is involved in oxidative stress. However, there is no report about testicular damage due to HGF-induced oxidative stress in diabetic or non-diabetic rats. Li *et al.* reported the HGF treatment decreased the high glucose induced oxidative stress and c-Met expressions were increased in rat mesangial cells in oxidative state (25, 26). Although we could not measure the oxidative stress parameters, we showed the diabetes-induced testicular damage histologically and the increase in the immunohistochemical staining of HGF/c-Met in seminiferous tubule epithelium of diabetic rats. These results might be related to the development of oxidative stress due to high glucose levels, as we have examined only the expression of HGF/c-Met level and not the oxidative status in our study. Therefore, further analysis is definitely required and this is a limitation of our study.

Insulin is the major treatment of diabetic patients who have pancreatic cell damage. It decreases blood glucose levels and prevents or delays metabolic complications. Therefore we treated the STZ-induced diabetic rats by insulin for 8 weeks. Insulin treatment led to a decrease in the blood glucose levels and an improvement in the diabetes-related reduction in body-weight, as expected. In the literature there is a limited number of studies related to the insulin effects on testis tissue of diabetic animals (11, 12). Some *in vitro* studies

showed that treatment of seminiferous tubule segments in culture with insulin stimulates spermatogonial DNA synthesis in rats (27) and insulin treatment also promotes spermatogonial differentiation of primary spermatocytes (28). Schoeller *et al.* reported that insulin had improved that impaired spermatogenesis especially *via* the hypothalamic-pituitary-gonadal axis in Akita diabetic mice and restored male fertility. They also reported the regeneration of the seminiferous tubuli by the insulin treatment in diabetic rats (11, 12). Similarly, in our study, testicular damage due to diabetes was reduced, atrophic seminiferous tubule number decreased and spermatogenic cell series became regular by the insulin treatment in STZ-induced diabetic rats. We also found that increase in both the mean seminiferous tubule diameter and Johnsen's criteria values of diabetic rats were reduced by insulin treatment. However, the Johnsen's score of insulin-treated diabetic group was still lower than those of the control group suggesting that the insulin treatment is not completely effective to reverse testicular damage induced diabetes alone.

In addition to systemic effects of insulin, it has been shown the importance of local insulin signaling in the testis; however, its specific role has not yet been determined. Therefore, we focused on the relation between insulin and HGF/c-Met signalling pathway in testicular dysfunction of diabetic rats. We found that in testis tissues, immunohistochemical staining of HGF/c-Met was decreased significantly by the insulin-treated diabetic rats compared to those of untreated-diabetic group. However, c-Met expression was still higher in insulin-treated diabetic rats compared to control rats. These results suggest that protective effects of insulin on the diabetes-induced testicular damage, not only its effect on the hyperglycemia, might also be due to inhibition of HGF/c-Met over expressions in testis tissues. To our knowledge, no study has investigated the insulin effects on HGF/c-Met expressions in testis tissues of diabetic rat so far; thus, we believe our results are important as being the first to examine this issue.

In conclusion, these results suggest that although insulin is essential treatment for type 1 diabetes but it might be inadequate to prevent diabetes-induced testicular damage and infertility. New treatment approaches possibly target on HGF/c-Met pathway might be promising on diabetes-induced testicular damage. However, further studies are necessary to understand the role of HGF/c-Met signalling pathway in diabetes-induced testicular damage.

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Conflict of interest

The authors declare that they have no conflict of interest concerning this article.

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