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Spermatozoa Characteristics of Streptozotocin-induced Diabetic Wistar Rat: Acrosome Reaction and Spermatozoa Concentration

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Streptozotocin으로 유발된 당뇨병성 Wistar Rat 정자의 첨체반응 및 수 변화 특성

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= 국문초록 =

당뇨병은 생식내분비 조직의 구조나 기능 변화를 유발하여 호르몬 합성 및 분비량의 변화를 야기하고, 정자의 운동성 등에 영향을 미치는 것으로 알려져 왔다. 그러나 부정소와 수정관내 정자의 농도 변화나 수정능력 획득 및 첨체반응에 미치는 영향은 잘 알려져 있지 않다. 본 실험에서는 Wistar 쥐에 streptozotocin을 투여하여 당뇨병을 유발시킨 후 3일과 14일에 부정소 각 부위와 수정관내 정자 농도 변화를 조사하고 부정소 꼬리와 수정관내 정자의 첨체반응 유도 실험 (acrosome reaction to ionophore challenge test, ARIC test)을 이용하여 정자의 수정능력을 평가하였다. Streptozotocin을 주사한 후 혈액내 인슐린 및 포도당 농도는 당뇨병 경과 기간이 길어짐에 따라 반비례 관계를 보였다. 부정소 머리와 몸통내 정자의 농도는 3일군에서부터 감소하기 시작하나 꼬리에서는 14일군 (15.2±2.1)에서 대조군 (28.1±4.0)이나 3일군에 (24.8±2.9) 비해 유의하게 감소하였다. 수정관내 정자 농도는 14일군이 0.025±0.013으로 대조군과 (0.108±0.03) 3일군에 (0.067±0.046) 비해 유의하게 감소하였으며, 3일군도 대조군에 비해 유의한 차이를 보였다. 자발적 첨체반응율은 대조군의 부정소 꼬리 정자는 37.1±2.4이고 수정관내 정자는 49.3±2.4로 두 부위간 유의한 차이를 보였다. 3일군과 14일군의 부정소 꼬리와 수정관내 정자의 자발적 첨체반응율은 대조군에 비해 유의하게 증가하였다. 한편 14일군의 수정관내 정자의 자발적 첨체반응율은 대조군이나 3일군에 비해 유의하게 증가하였다. ARIC test 결과 대조군과 3일군에서는 20% 이상 차이를 보였으나 14일군에서는 약 8.4% 차이를 보

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였다. 위의 결과가 부정소의 성숙 조절기능 이상 또는 정자형성 이상에 기인한 것인지는 더 연구되어야 하나 당뇨병 병력이 길어짐에 따라 정자의 수적인 감소, 자발적 첨체반응의 증가나 첨체반응의 약화가 유발되어 생식능력의 감소 원인으로 작용하는 것으로 사료된다.

Key Words: ARIC-test, Diabetes mellitus, Epididymis, Maturation, Spermatozoa

INTRODUCTION

Diabetes leads to metabolic abnormalities involving regulation of carbohydrate metabolism, and these abnormalities produce pathological changes in a variety of organ systems. It has two types, insulin-dependent diabetes (type 1) is believed to have a genetic predisposition activated by environmental events, which results in an autoimmune reaction to B-cell population. The pathogenesis of noninsulin-dependent diabetes (type 2) is less understood, but it is thought that insulin resistance precedes the disease. The results of studies about effect of diabetes upon male reproduction are inconsistent and nonspecific. Most of the attention and information concerning sexual function in the male diabetes has overwhelmingly been focused upon the diagnosis incidence, etiology, therapeutic intervention of impotence, and histology of endocrinological organs (Ali *et al.*, 1993; Cohen *et al.*, 1984; Dinulovic & Radonjic, 1990; Dunsmuir & Holmes, 1996; Faerman *et al.*, 1972; Garcia-Diez *et al.*, 1991).

The epididymal maturation and capacitation of the spermatozoa have an influence on male reproduction. The testicular spermatozoa are still immature and incapable of effective forward motility and lack the ability to fertilize oocytes. Spermatozoa acquire these fertilizing ability as they pass through the epididymis. The capacitated spermatozoa can bind to the receptors on the zona pellucida (ZP), and then acrosome reaction (AR) is induced. Through these events, the spermatozoa penetrate the ZP, fuse with oolemma, and form a male or female pronucleus. The spermatozoa which have uncompleted acrosome reaction, can't bind completely to the receptors on the ZP, so they can't accomplish the fertilization. Also a acrosome-reacted spermatozoa loss the ligands, so they cannot attend the fertilization (Parinaud *et al.*, 1995). The finding that the AR must be precisely timed with respect to spermatozoa-zona pellucida interaction to ensure zona pellucida penetration was behind the development of the AR to ionophore (Avrech *et al.*, 1997; Long *et al.*, 1996; Holt *et al.*, 1997).

Cauda epididymis has the great majority of spermatozoa which attain their full fertilizing potential and has the storage function. The vas deferens has the function of the absorption of the old spermatozoa (Eddy & O'Brien, 1994). The concentration of the cauda spermatozoa is concerned in the capability of reproduction of an individual. Therefore, in the experimental animal model, the decrease of the cauda epididymal spermatozoa is one of the criterias of fertility.

Diabetes mellitus has been known to alter the characteristics of semen, motility, morphology and concentration, but the results are still controversial (Bartük *et al.*, 1975; Rubin, 1962). However, the change in spermatozoa physiology as well as epididymal spermtozoa storage under diabetic condition was scarcely studied. This investigation was conducted to verify the changes in epididymal spermatozoa concentration and acrosomal reaction characteristics of streptozotocin (STZ) -induced diabetes Wistar rat.

MATERIALS AND METHODS

1. Experimental Animals

Adult (12 weeks old) Wistar male rats were maintained with food and water *ad libitum* in the environment controlled rearing system with 12L:12D cycle. The rats were maintained well

in the standard condition, temperature (20~22℃), and humidity (50%) in Asan Institute for Life Science.

2. Induction of Diabetes Mellitus

Diabetes was induced with STZ (70 mg/kg) which was solved at 1 mL citrate buffer solution (pH 4.5) and injected into intraperitonium. Experimental animals were sacrificed at 3 days and 14 days respectively. To confirm the induction of diabetes, the concentration of urine glucose was measured with Combur urine test strip. The concentration of blood glucose was measured with Glucose analyzer (Beckman Model 6517, USA) and the concentration of insulin in blood was measured with radio immuno-assay kit (Linco's rat insulin RIA kit, RI-13K). The none induced animals were discarded in this examination.

3. Preparation, Count, and Capacitation of spermatozoa

Experimental animals were anesthetized with ether and then blood were collected from the left atrium, and isolated the epididymis and vas deferens. The adipose tissue and blood was removed from those within PBS. Under the dissecting microscope, epididymis were separated to caput, corpus, and cauda epididymis. Each part was located at the 24 well culture dish, and then was scissored several times and added 2 mL 0.4% BSA BWW medium, and samples were incubated for 20 min at room temperature. The spermatozoa concentrations were measured with Maker chamber after mixed well. The incubation for spermatozoa in medium BWW lasted 5 h, the time required for the in vitro capacitation of rat spermatozoa (Oberländer *et al.*, 1996) at 37℃, 5% CO_2, and 100% humidity.

4. Acrosome Reaction Test

The procedures used to calcium ionophore A23187 challenge, lectin staining and categorization of staining patterns were used for acrosome reaction test, as previously described (Cheon *et al.*, 1998). Briefly, calcium ionophore A23187 (Sigma, St. Louis, MO, USA) was prepared as 5 mmol/L stock in dimethyl sulfoxide (DMSO). Before use, stock was diluted with protein-free Ham's F-10, giving a final ionophore concentration of 10 μmol/L. For each ionophore challenge, sperm suspension with 1 x 10^6 motile sperm/mL was used. After 30 min incubation in CO_2 incubator (5% CO_2, 37℃, 100% humidity), the sperm pellet was resuspended in 50 μl absolute ethanol at 4℃. The spermatozoa were fixed for at least 30 min at 4℃ before staining.

For staining of slides, stock solution of fluorescein isothiocyanate (FITC)-conjugated Pisum Sativum lectin (PSA: Sigma) was diluted 1 to 10 with ultrapure water and kept in the dark at 4℃. Each slide was covered with a 20 μl drop of FITC-PSA (100 μg/mL) and placed in a dark humidified chamber at 4℃ for 15 min. Excess stain was removed by ultrapure water and allowing to dry in dark chamber. Then the staining patterns were observed with fluorescence microscope (Olimpus, BX 70). The difference between the two (ionophore minus spontaneous) was considered to be the percentage of sperm in the population capable of responding to ionophore

5. Statistical Analysis

Data were examined using Exel (version 7.0). Where appropriate, nonpaired t-tests were used to compare sample means between experimental groups.

RESULTS

1. Concentration of Plasma Glucose and Insulin

The concentration of plasma glucose was increased in proportion to duration of diabetes compared to control as shown in Figure 1. However, the concentration of insulin in plasma was decreased showing inverse proportion

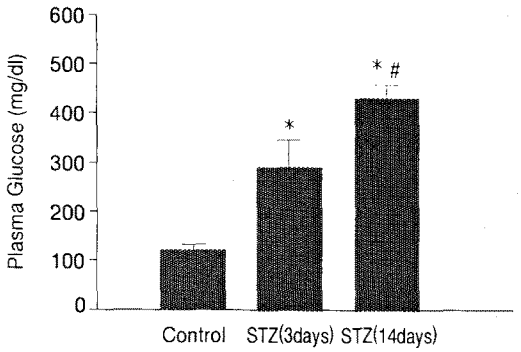

Figure 1. The changes of plasma glucose level in normal and STZ-induced diabetic Wistar rats. *; p<0.05 vs Control and #; p<0.05 vs STZ (3days).

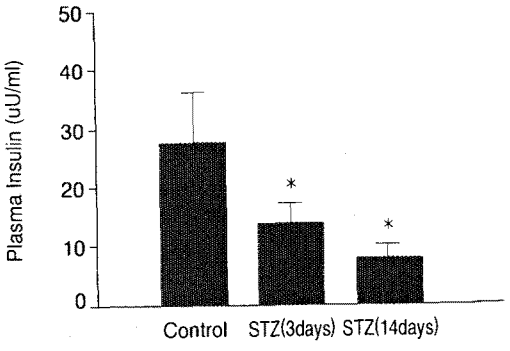

Figure 2. The changes of plasma insulin level in normal and STZ-induced diabetic Wistar rats. *; p<0.05 vs Control.

Table 1. Comparison of sperm concentration at epididymis and vas deferens in Wistar rat induced with streptozotocin

Duration of Diabetes (Days)	Concentration ($x10^6$)			
	Caput	Corpus	Cauda	Vas deferens
0 (Control)	23.9±1.6	13.3±1.7	28.1±4.0	0.108±0.030
3	13.3±4.0**	5.0±0.7**	24.8±2.9	0.067±0.046*
14	5.8±1.6** ##	4.7±1.2**	15.2±2.1** ##	0.025±0.013** ##

Concentration data are expressed as mean ± SE (n = 8).
*p<0.05, **p<0.001 versus control, ## p<0.001 versus 3 day

to the concentration of glucose (Figure 2).

2. Changes of spermatozoa concentration

Caput epididymis and vas deferens spermatozoa concentration were significantly decreased as the longer and longer the duration of diabetes. So the difference among control, 14 day and 3 day was significant, and the difference between the 3 days and 14 days was significant, too (Table 1). In corpus epididymis of diabetic rat, the concentraiton was significantly decreased compared to control, but there was no significance between 3 days and 14 days (Table 1). The spermatozoa concentration of cauda epididymis was not significantly changed 3 days after diabetes induction, but there was significant decrease at the 14 days (Table 1).

So to speak, in the diabetes-induced rat, the spermatozoa concentration was firstly decreased at the caput and corpus, but not decreased at cauda. However, with the lapse of diabetes duration, the decrease of spermatozoa concentration was showed both in epididymis and vas deferens. (Table 1).

3. Characteristics of Acrosome Reaction

It is reported that the spontaneous acrosome reaction rate is about 30%, but as shown in table 2, the spontaneous acrosome reaction rate was variable with position. The spontaneous acrosome reaction rate of cauda epididymal spermatozoa at the control was 37.1±2.4. On the other hand, spontaneous reaction rate of vas deferens was 49.3±2.4 which was significantly higher than that of cauda epididymal spermatozoa (Table 2). In 3 days group, spontaneous reaction rates of cauda epididymal and vas deferens spermatozoa were 46.9±3.5 and 55.9±7.0 respectively, showed significantly difference between them. These rates had significant difference compared to the control gro-

Table 2. Spontaneous and induced acrosome reactions and ARIC values at at 3 and 14days for streptozotocin-induced diabetic Wistar rat

Acrosomal factors measured	Cauda	Vas deferens	(cauda: vas deferens)
Spontaneous Reaction			
Control	37.1±2.4	49.3±2.4	p<0.001
3 days	46.9±3.5[a]	55.9±7.0[a]	p<0.05
14 days	64.2±4.5[ab]	60.1±0.4[ab]	p<0.05
Induced Reaction			
Control	66.8±6.7[c]	63.5±5.5[c]	no
3 days	69.0±3.5[c]	62.6±5.0[c]	p<0.05
14 days	72.6±5.4[c]	63.0±8.9	p<0.05

All results are presented as the mean ± SD (n = 8).
[a] p<0.05 versus control of spontaneous and induced reaction, respectively
[b] p<0.05 versus 3 days group of spontaneuou and induced reaction, respecively
[c] p<0.05 versus spontaneous reaction (control: control, 3 days: 3 days, 14 days; 14 days)

up (Table 2). In 14 days group, spontaneous reaction rates of cauda epididymal and vas deferens were 64.2±4.5 and 60.1±0.4 showed significance. Compared with control and 3 days group, those had significance (Table 2). Spontaneous rate was significantly increased with duration of diabetes in cauda epididymis and vas deferens but the width of rate between two part was decreased with duration of diabetes (Table 2).

When the acrosome reaction was induced with calcium ionophore at each experimental groups, the results as followed. The induced reaction rates of cauda epididymis and vas deferens in control were 66.8±6.7 and 63.5±5.5 respectively which showed no significance, but those rates were significantly higher than spontaneous rate in control (Table 2). In 3 days group, the induced reaction rates of cauda epididymis and vas deferens were 69.0±3.5 and 62.6±4.9 respectively which showed significant difference. Compared to control of 3 days, there was significant difference (Table 2). The induced reaction rate of cauda epididymis at 14 days group was significantly higher than 14 days control, but in vas deferens there was no difference between control and reaction rate. The difference of induced reaction rates between cauda and vas deferens had significance (Table 2).

DISCUSSION

The relationship between diabetes and male reproductive abnormality has been studied using congenital or induced with chemicals such as STZ diabetes (Murray *et al.*, 1983; Paz *et al.*, 1980). The structural or functional changes of testis or endocrine organs has been reported in diabetes mellitus patients, but the results is controversial (Sexton & Jarow, 1997) because of the various experimental groups especially by the duration and medical treatments.

Semen analysis according to their ages and duration of the disease has shown that the volume of ejaculate was decreased in the group of younger patients with longer duration of the disease. In the other groups, the volume of ejaculate was almost unaffected. In the type I patients, the number and motility of spermatozoa progressively decreased (Dinulovic & Radojic, 1990). In the STZ-induced diabetic Zucker rat, the concentration of spermatozoa at epididymis and vas deferens was decreased with the longer duration (Cheon *et al.*, 1998). It suggested that even if there are differences of sensitivity to the STZ between strains, the

number of spermatozoa in the epididymis decreased with diabetes.

Spermatozoa which are capacitated recognize the receptor on the ZP and penetrate and fuse with oolemma. Acrosome reaction occurred and completed signal transduction after binding to the receptor on the ZP (Melendrez *et al.*, 1994; Mendoza *et al.*, 1995; Roldan *et al.*, 1994). Therefore spontaneously acrosome-reacted spermatozoa cannot recognize the receptor so that they didn't take part in fertilization; besides, spermatozoa which have intact acrosome cannot bind to the oolemma (Liu & Baker, 1994). Therefore the following factors were analyzed: the spontaneous reaction rates (control); induced reaction rates (ionophore-challenged); and the difference between the two, being the proportion of spermatozoa in the population capable of reaction in response to calcium influx (acrosome reaction to ionophore challenge, ARIC) for predict the fertility (Cummins, 1994; Mortimer & Fraser, 1996).

With the use of the ARIC test, two type of AR pathology have been defined: AR insufficinecy (Tesarik & Mendoza, 1993) and AR prematurity (Tesarik & Mendoza, 1995). In human, AR insufficiency describes cases in which the difference in frequency of AR between ionophore-treated and untreated aliquots of a capacitated sperm population is below 15%, while AR prematurity is used for those cases in which the frequency of spontaneous AR is above 20% (Tesarik & Mendoza, 1995). So far, prediction of the fertility of the rat with the ARIC was reported by Cheon *et al* (1998) with Zucker rat. In Wistar rat, the spontaneous reaction rate was about 37% which is higher than human, and it is similar with Zuckers.

The spontaneous reaction rate of cauda epididymis was higher than reported rate (about 30%, Oberländer *et al.*, 1996). However, Cheon *et al* (1998) reported the spontaneous reaction rate of Zucker rat was 38%, similar with that of Wistar rat. Characteristics of acrosome reaction at vas deferens is not well known, but in the present study, the spontaneous reaction rate is higher than that of cauda epididymis. It is reasonable that such a acrosome reaction characteristics of vas deferens is due to the absorption function of vas deferens. Similar with the diabetes Zucker rat (Cheon *et al.*, 1998), after the induction of diabetes the spontaneous reaction rates were increased at the cauda and vas deferens, with maximal decrease in the longest diabetes duration. The spontaneous reaction rate of vas deferens was increased by longer and longer the duration but the difference with cauda was decrease. It is thought that this results may be from the loss of the function of cauda epididymis.

ARIC value of the cauda epididymal spermatozoa was 8.4% in the 14 days group, but in the control and 3 days group were at least 22%. In the spermatozoa of the vas deferens, the ARIC value was slowly decreased by the duration. These results indicate that the fertility of the diabetic Wistar rat is decreased and it is resulted from the abnormal function of the epididymis.

Anejeculation caused by diabetes mellitus can be managed by using assisted reproductive techniques with spermatozoa obtained by electroejaculation, or aspiration. The fertilization rate of oocytes following IVF is lower than the control (Denil *et al*, 1992; Hovatta & von Smitten, 1993), but using ICSI, showed normal fertilization, and pregnancy (Hovatta *et al.*, 1996). Therefore, spermatozoa of the diabetic patients have fertility less than normal, but have the capability of development. It is thought that the main factors of decreased fertility result from the deficiency of maturation of the spermatozoa, especially acrosome reaction.

In summary, the concentration of the spermatozoa at the epididymis and vas deferens was decreased with longer duration of the disease. The spontaneous reaction rate was significantly higher in the vas deferens. The cause leading to increase of the spontaneous reaction rate of spermatozoa of the vas deferens maybe due to the

absorption function of the vas deferens. The spontaneous reaction rate of the diabetic rat was higher than that of the control group. According the present study, diabetes leads to metabolic abnormality involving regulation of carbohydrate metabolism, so that induces the abnormal function of the epididymis. In the ARIC value, the fertility decreased with the longer duration of the diabetes. From the results, it is suggested that diabetes decrease spermatozoa concentration and induce abnormality of the maturation concerned to the acrosome reaction, so it decrease the fertility of the animals with diabetes.

SUMMARY

Some of the information concerning sexual function in the male diabetes has been focused upon the problems of endocrine or semen parameters. However, the characteristics of acrosome reaction and spermatozoa concentration at the epididymis and vas deferens have scarcely been studied, and the causes of the infertility has not been critically identified. So, we designed to inspect the spermatozoa concentration and the characteristics of acrosome reaction at epididymis and vas deferens of diabetic Wistar rat induced by streptozotocin (STZ, 70 mg/kg, ip). Experimental animal was sacrificed at 3 days and 14 days after the STZ injection. In the diabetes-induced rat, the levels of insulin and glucose had a pattern of inverse proportion. The spermatozoa concentrations in caput and corpus epididymis were significantly decreased in all diabetic condition. In cauda epididymis, however, there was significant decrease in sperm concentration at 14 days onward. In diabetic rat, the spontaneous reaction rate of spermatozoa of cauda and vas deferens were significantly higher than the control group. The ARIC (acrosome reaction to ionophore challenge) value of caudal sperm was 28.7 at control, 22.1 at 3 days, and 8.3 at 14 days. In the present study the spermatozoa concentration was decreased and the spontaneous reaction rate was increased by diabetes. In ARIC-test, it is revealed that the fertility of spermatozoa of 14 days group was lower than control or 3 days group. Diabetes mellitus may be provoke the decreased fertilization rate and subsequent infertility.

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