

Paternal effect on embryo quality in diabetic mice is related to poor sperm quality and associated with decreased glucose transporter expression

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Abstract

The objective of this study was to determine whether sperm quality, fertilization capacity, and subsequent embryo development are altered in diabetic male mice and whether differences in facilitative glucose transporter (GLUT; now known as solute carrier family 2, SLC2A) expression in the testis and sperm exist. Using two type 1 diabetic mouse models, SLC2A expression in the testis and sperm was determined by western immunoblotting and immunofluorescence staining. To address sperm quality and fertilization capacity, computer-assisted sperm analysis and *in vitro* fertilization were performed. SLC2A1, SLC2A3, and SLC2A5 did not change in expression in the testes or sperm between diabetic and non-diabetic mice. SLC2A8 and SLC2A9b were less expressed in the testes of both diabetic models versus controls. SLC2A9a was not expressed in the Akita testis or sperm when compared with strain-matched controls. 3 β -hydroxysteroid dehydrogenase (HSD3B) expression was significantly decreased in the Leydig cells from the diabetic mice. Sperm concentration and motility were significantly lower in both the diabetics when compared with the control. These parameters normalized in Akita diabetic males treated with insulin. In addition, fertilization rates were significantly lower in the Akita group (17.9%) and the streptozotocin (STZ)-injected male group (43.6%) when compared with the normal group (88.8%). Interestingly, of the fertilized zygotes, embryo developmental rates to the blastocyst stage were lower in both diabetic models (7.1% Akita and 50.0% STZ) when compared with controls (71.7%). Male diabetes may cause male subfertility by altering steroidogenesis, sperm motility, and SLC2A expression. This is the first study to link a paternal metabolic abnormality to a sperm effect on cell division and subsequent embryonic development.

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Introduction

Diabetes has been associated with reproductive impairments in both men and women. Male reproductive alterations have been widely reported in individuals with diabetes. Many studies have documented abnormalities in testicular function and spermatogenesis in diabetic animals (Murray *et al.* 1983, Seethalakshmi *et al.* 1987, Scarano *et al.* 2006). In men affected by insulin-dependent diabetes, sperm have severe structural defects (Baccetti *et al.* 2002), significantly lower motility, and lower ability to penetrate hamster eggs (Shrivastav *et al.* 1989). The administration of high doses of streptozotocin (STZ) to male rodents induces a decrease in testicular testosterone production (Sanguinetti *et al.* 1995, Scarano *et al.* 2006).

Glucose is an important energy substrate for most mammalian cells. The entry of glucose into the cells is facilitated by a family of glucose transporters (GLUTs; now known as solute carrier family 2, SLC2A) that are characterized by the presence of 12 membrane-spanning helices and several conserved sequence motifs. Currently, there are 13 members of the facilitative glucose transporter

family: SLC2A1–12 and SLC2A13, which was previously known as the H⁺ coupled myo-inositol-transporter (HMIT; Joost *et al.* 2002, Wood & Trayhurn 2003). The SLC2A family of proteins has been subdivided into three classes: class I consists of SLC2A1–4; class II consists of SLC2A5, SLC2A7, SLC2A9, and SLC2A11; and class III consists of SLC2A6, SLC2A8, SLC2A10, SLC2A12, and SLC2A13 (Joost & Thorens 2001, Joost *et al.* 2002). SLC2As exhibit a high degree of sequence homology; however, they differ in their substrate specificity, kinetic characteristics, and tissue and subcellular distribution, and their response to extracellular stimuli (Joost *et al.* 2002, Riley *et al.* 2006).

SLC2A8 is a recently cloned member of the class III facilitative transporters and is expressed in the blastocyst stage embryo, heart, skeletal muscle, brain, spleen, prostate, intestine, and testis (Carayannopoulos *et al.* 2000, Doege *et al.* 2000, Gomez *et al.* 2006). Currently, we have confirmed that SLC2A8 is highly expressed in the Leydig cells as well as in the innermost cells of the seminiferous tubules in the mouse testis and localizes in the acrosome, midpiece, and principal piece of the mouse

sperm (Kim & Moley 2007). Human SLC2A9 was originally cloned from human kidney cDNA (Phay *et al.* 2000) and we have identified and cloned mouse SLC2A9 (Carayannopoulos *et al.* 2004). Sequence analysis has shown that SLC2A9 is most similar to SLC2A11, and both are categorized to the class II subgroup that also includes SLC2A5 and SLC2A7. A unique feature of mSLC2A9 is the existence of two predominant splice variants: mSLC2A9a and mSLC2A9b differing only at the N-terminal intracytoplasmic tail (Keembiyehetty *et al.* 2006). We have shown that SLC2A9a and SLC2A9b are expressed in the mouse testis including the Leydig cells (Kim & Moley 2007). SLC2A9a strictly localizes in the midpiece, but SLC2A9b localizes in the acrosome, midpiece, and principal piece of the mouse sperm (Kim & Moley 2007). We have shown that surface SLC2A8 expression is regulated by the insulin/insulin-like growth factor-1 (IGF1) signaling pathways through the IGF1 receptor in the mouse blastocyst (Carayannopoulos *et al.* 2000, Pinto *et al.* 2002). In different diabetic models, hepatic SLC2A8 has shown altered expression patterns (Gorovits *et al.* 2003). We have also reported that SLC2A9 protein expression was significantly increased in the kidney and liver from STZ-induced diabetic mice compared with non-diabetic mice (Keembiyehetty *et al.* 2006). Although SLC2A8 (Ibberson *et al.* 2002, Schurmann *et al.* 2002, Chen *et al.* 2003, Gomez *et al.* 2006, Kim & Moley 2007) and SLC2A9 (Kim & Moley 2007) are expressed in the testis and sperm, it is not known whether their expression or localization is altered in the diabetic male reproductive system or not. The objective of this study was to determine whether sperm quality, fertilization capacity, and subsequent embryo development are altered in diabetic male mice and whether differences in facilitative glucose transporter (SLC2A) expression in the testis and sperm exist.

Results

Expression of SLC2A8, SLC2A9a, and SLC2A9b is altered in diabetic testis and sperm

In order to detect changes in the expression and localization of SLC2A1, SLC2A3, SLC2A5, SLC2A8, SLC2A9a, and SLC2A9b in the diabetic testis, RT-PCR as well as immunofluorescence staining and western blot analysis were performed. No changes in SLC2A1, SLC2A3, or SLC2A5 expression were detected (data not shown), and these findings were consistent with previous reports in diabetic rat (Burant & Davidson 1994). SLC2A8 mainly localized in a punctated intracellular compartment of the normal testis (Fig. 1A, panel a). However, the overall expression of SLC2A8 protein was slightly decreased in the STZ-injected testis (Fig. 1A, panel b) and markedly decreased in the Akita testis (see box in Fig. 1A, panel c). SLC2A9a protein was similarly expressed in the normal and STZ-injected testis (Fig. 1A, panels d–e). However, SLC2A9a was not expressed in the Akita testis (Fig. 1A,

panel f). As shown in Fig. 1B and C, we confirmed that SLC2A9a protein was not expressed in the Akita testis, although *Slc2a9a* mRNA was expressed. SLC2A9b was less expressed in both of the diabetic testes when compared with normal testes (Fig. 1A, panels g–i and 1C).

Next, we examined SLC2A localization in the diabetic sperm by indirect immunofluorescence staining. SLC2A8 similarly localized in midpiece and principal piece as well as the acrosomal region of the normal and both of the diabetic sperm (Fig. 2A, panels a–c). SLC2A9a localized in the midpiece of the normal and STZ-injected sperm (Fig. 2A, panels d–e), but SLC2A9a was not detected in the Akita sperm (Fig. 2A, panel f). As shown in Fig. 2B, we confirmed that SLC2A9a protein was not expressed in the Akita sperm similar to the lack of expression in the testis. SLC2A9b similarly localized in the acrosomal region, midpiece, and principal piece of the normal and diabetic sperm (Fig. 2A, panels g–i). SLC2A9b expression in the acrosome and principal piece was significantly lower in the Akita sperm than in the normal and STZ-injected sperm (see the arrows in Fig. 2A, panel i). We confirmed SLC2A protein expression in the sperm by western blot analysis (Fig. 2B).

Insulin protein is unchanged in location but decreased in expression in diabetic sperm

Insulin in the normal and STZ-injected sperm was similarly expressed and localized in the acrosomal region, midpiece, and principal piece (Fig. 3A and B), but insulin was significantly decreased in the Akita sperm (Fig. 3C). Because STZ injection destroys only the pancreas, STZ-injected sperm should still express insulin as much as normal sperm. Since the Akita mouse has a single amino acid substitution of the insulin 2 gene, Akita sperm might show faint staining with insulin.

SLC2A8, SLC2A9a, and HSD3B expression are decreased in isolated diabetic Leydig cells

As shown in Fig. 4, SLC2A8 protein was slightly lower in both of the diabetic Leydig cells than in the normal Leydig cells. SLC2A9a was also significantly decreased in STZ-injected Leydig cells and was not detected in the Akita Leydig cells. 3 β -hydroxysteroid dehydrogenase (HSD3B), which catalyzes the conversion of 3- β -hydroxy-5-ene steroids to 3-oxo-4-ene steroids (progesterone and androstenedione), was significantly decreased in both of the diabetic Leydig cells. This result provides supporting evidence as to why diabetic males' testosterone levels are lower than normal males' levels (Scarano *et al.* 2006).

Sperm concentration, motility, and fertility were compromised in diabetic male mice

To examine the quality and fertility of the diabetic sperm, computer-assisted sperm analysis (CASA) and *in vitro*

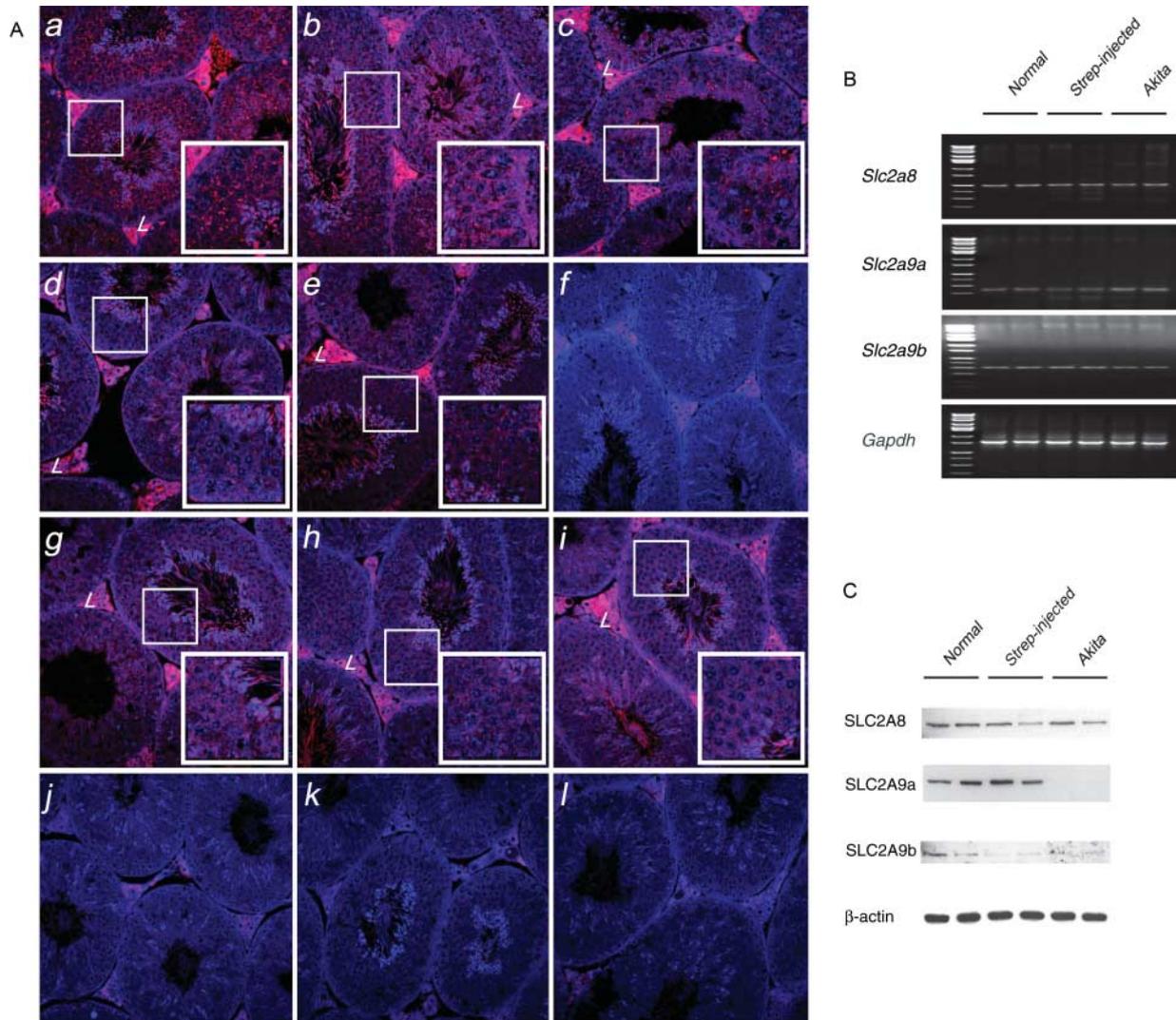


Figure 1 (A) Immunofluorescence of SLC2A8 (a–c), SLC2A9a (d–f), and SLC2A9b (g–i) in the normal (a, d, g, and j), STZ-injected (b, e, h, and k), and Akita (c, f, i, and l) testis. (j, k, and l) Negative controls. Bouin's fixed paraffin-embedded testicular sections were incubated with anti-SLC2A8, anti-SLC2A9a, or anti-SLC2A9b antibodies. The sections were then incubated with a secondary antibody, Alexa Fluor 546 goat anti-rabbit immunoglobulin G (red fluorescence). To-Pro-3 iodide was used to stain the nuclei (blue fluorescence). This experiment was performed over three times per group with at least five animals in each group for each experiment. Comparative RT-PCR (B) and western blot analysis (C) of SLC2A8, SLC2A9a, and SLC2A9b in the normal, STZ-injected, and Akita testis. *Gapdh* (B) and β -actin (C) were used as internal control for RT-PCR and western blot analysis respectively.

fertilization (IVF) were performed. Cauda epididymal sperm concentration was significantly lower in both types of the diabetic male mice than in normal mice (Fig. 5A, panel a). In the diabetic male mice, the percentage of motile sperm was significantly decreased (Fig. 5A, panel b) and the percentage of motile sperm with progressive motility was also decreased by 50% (Fig. 5A, panel c). Path velocity, progressive velocity, curvilinear velocity, and lateral amplitude were all significantly reduced in the diabetic mice (data not shown). Beat frequency of both types of the diabetic males was same as that of the normal males. Straightness and linearity of Akita mice were slightly lower than those of STZ-injected and normal mice. The percentage of rapidly moving sperm was lower,

and the percentage of static sperm was higher in both types of the diabetic males than in the normal males (Fig. 5A, panels d and e). Next, we performed IVF with normal cumulus–oocyte complex (COC) and diabetic sperm.

As shown in Fig. 5B and C, the normal sperm group exhibited 88.8% of the fertilization rate, with 71.7% of fertilized embryos developing to the blastocyst stage. On the other hand, sperm isolated from STZ-injected mice showed a markedly decreased fertilization rate (43.6%) with 50% of the fertilized embryos developing to the blastocyst stage. Sperm isolated from Akita mice also showed a significantly decreased fertilization rate (17.9%) with 7.1% of the fertilized embryos developing to the blastocyst stage. In fact, control oocytes fertilized

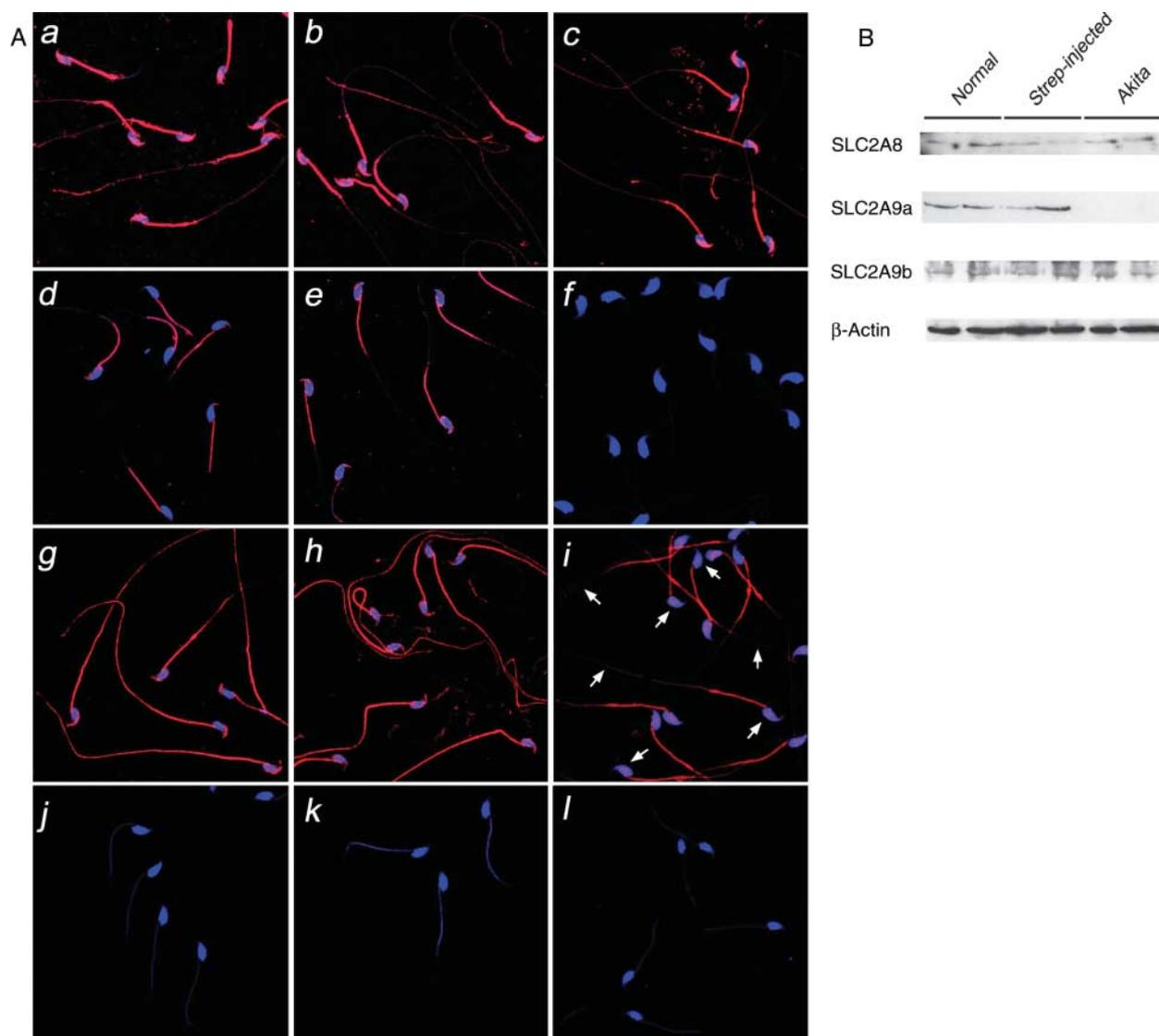


Figure 2 (A) Immunofluorescence of SLC2A8 (a–c), SLC2A9a (d–f), and SLC2A9b (g–i) in the normal (a, d, g, and j), STZ-injected (b, e, h, and k), and Akita (c, f, i, and l) sperm. (j, k, and l) Negative controls. Paraformaldehyde-fixed sperm were incubated with anti-SLC2A8, anti-SLC2A9a, or anti-SLC2A9b antibodies. The sperm were then incubated with a secondary antibody, Alexa Fluor 546 goat anti-rabbit immunoglobulin G (red fluorescence). To-Pro-3 iodide was used to stain the nuclei (blue fluorescence). This experiment was performed over three times per group with at least five animals in each group for each experiment. Arrows are directed at SLC2A9b expression at the acrosome and principal piece in the Akita sperm. (B) Western blot analysis of SLC2A8, SLC2A9a, and SLC2A9b in the normal, STZ-injected, and Akita sperm. β -actin was used as internal control.

with STZ-injected and Akita sperm showed increased fragmentation during cleavage stages and poor embryo developmental quality.

Insulin treatment of Akita mice improves sperm motility and concentration

Insulin- or placebo-containing pellets were placed in non-diabetic and diabetic Akita mice. Insulin pellet therapy for 7 days significantly increased the sperm total and progressive motility (51 and 103% increase respectively) as well as increasing total sperm concentration (51%).

Discussion

It is well known that the fertility of germ cells is directly linked with glucose metabolism. Glucose is required specifically for sperm fusion to zona-free murine oocytes. Studies by Urner & Sakkas (1996) have established that glucose must be transported into and metabolized by the male gamete in order for fertilization to occur. Moreover, glucose, not fructose or any other hexose sugars, is required in the medium during fusion for successful fertilization as well as to assure maintenance of viability of the embryo throughout the preimplantation period (Sakkas *et al.* 1993). In addition, spermatogenesis is

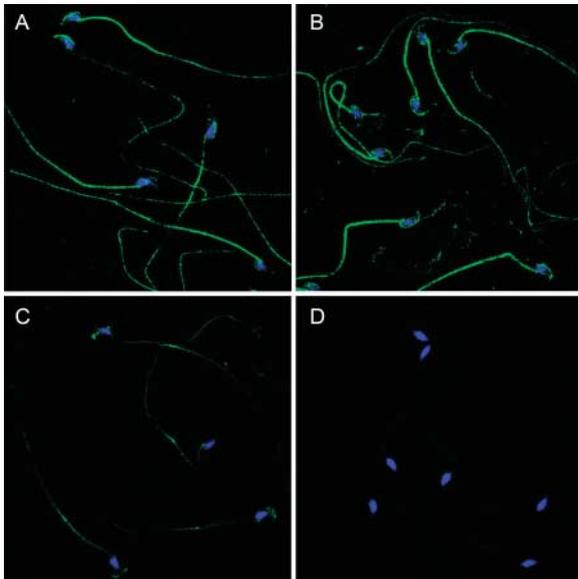


Figure 3 (A) Immunofluorescence of insulin in the normal, (B) STZ-injected, and (C) Akita sperm. (D) Negative control. Paraformaldehyde-fixed sperm were incubated with anti-insulin antibody. The sperm were then incubated with a secondary antibody, Alexa Fluor 488 goat anti-guinea pig immunoglobulin G (green fluorescence). To-Pro-3 iodide was used to stain the nuclei (blue fluorescence). This experiment was performed over three times per group with at least five mice per group for each experiment.

disturbed in diabetes and responsible in part for infertility (Sexton & Jarow 1997, Glenn *et al.* 2003). However, factors involved in the development of male infertility with insulin-dependent diabetes are poorly understood. SLC2A8 is highly expressed in the testis

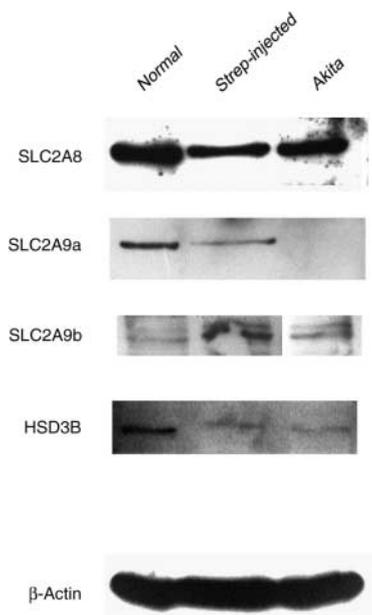


Figure 4 Western blot analysis of SLC2A8, SLC2A9a, SLC2A9b, and HSD3B in the Leydig cells from the normal, STZ-injected, and Akita testis. β -actin was used as internal control.

(Carayannopoulos *et al.* 2000, Doege *et al.* 2000, Gomez *et al.* 2006, Kim & Moley 2007) and as such may play a major role in substrate delivery to develop sperm. These observations led us to focus on SLC2A expression in diabetic male mice. The most commonly used animal models of diabetes include rodents, dogs, and primates induced by chemical toxins such as STZ or alloxan. However, toxin-induced diabetes in mice has been less successful because of strain-dependent resistance to STZ (Rossini *et al.* 1977). We have used two different kinds of type 1 diabetic models, STZ-induced and Akita mice. By western immunoblotting and immunofluorescent microscopy, the total amount of SLC2A8 protein was decreased in the testis, Leydig cells, and sperm of both diabetic models. Furthermore, the plasma membrane localization of SLC2A8 was slightly decreased in the STZ-injected testis and markedly decreased in the Akita testis. Recently, we have shown that SLC2A9a and SLC2A9b are expressed in the mouse testis and differentially localize in the sperm (Kim & Moley 2007). As shown in Figs 1 and 2, SLC2A9a protein was not expressed in the Akita testis and sperm, although *Slc2a* mRNA was expressed. This absence of SLC2A9a protein expression in the Akita mice may result from some post-transcriptional events regulated by insulin signaling, which have been described previously (Freeman & Wolf 1994, Han *et al.* 1995). SLC2A9b expression in the acrosome and principal piece was significantly lower in the Akita sperm than in the normal and STZ-injected sperm. The present study indicates that expression of SLC2A9 as well as SLC2A8 may be regulated by insulin signaling and/or hyperglycemia and may play an important role in sperm maturation and fertilization. Overall, diabetes might lead to the alteration of SLC2A expression in the sperm and testis.

These conclusions are consistent with our prior studies in rodent preimplantation embryos as well as other studies in human, rodent, and ovine placental tissue showing decreased SLC2A expression in models of maternal diabetes (Das *et al.* 1998, Hahn *et al.* 1998, Moley *et al.* 1998b, Chi *et al.* 2000a, Eng *et al.* 2007). Maternal high glucose concentrations lead to subsequent down-regulation of SLC2A expression and result in decreased intracellular glucose, triggering a cell death pathway which we have shown is dependent on BAX and p53 expression (Moley *et al.* 1998a, Keim *et al.* 2001). Recent work has shown that exposing preimplantation embryos to type 2 diabetic conditions *in vitro*, specifically hyperinsulinemia and hyperglycemia, leads to apoptosis, decreased glucose uptake and decreased expression of SLC2A8 protein in the blastocyst stage (Chi *et al.* 2000b, Eng *et al.* 2007). This work now suggests that male gametes respond in a similar fashion with a change in the expression of SLC2As in response to paternal diabetes.

According to recent reports, insulin is expressed in human ejaculated sperm (Aquila *et al.* 2005) and epididymal mouse sperm (Kim & Moley 2007), and it

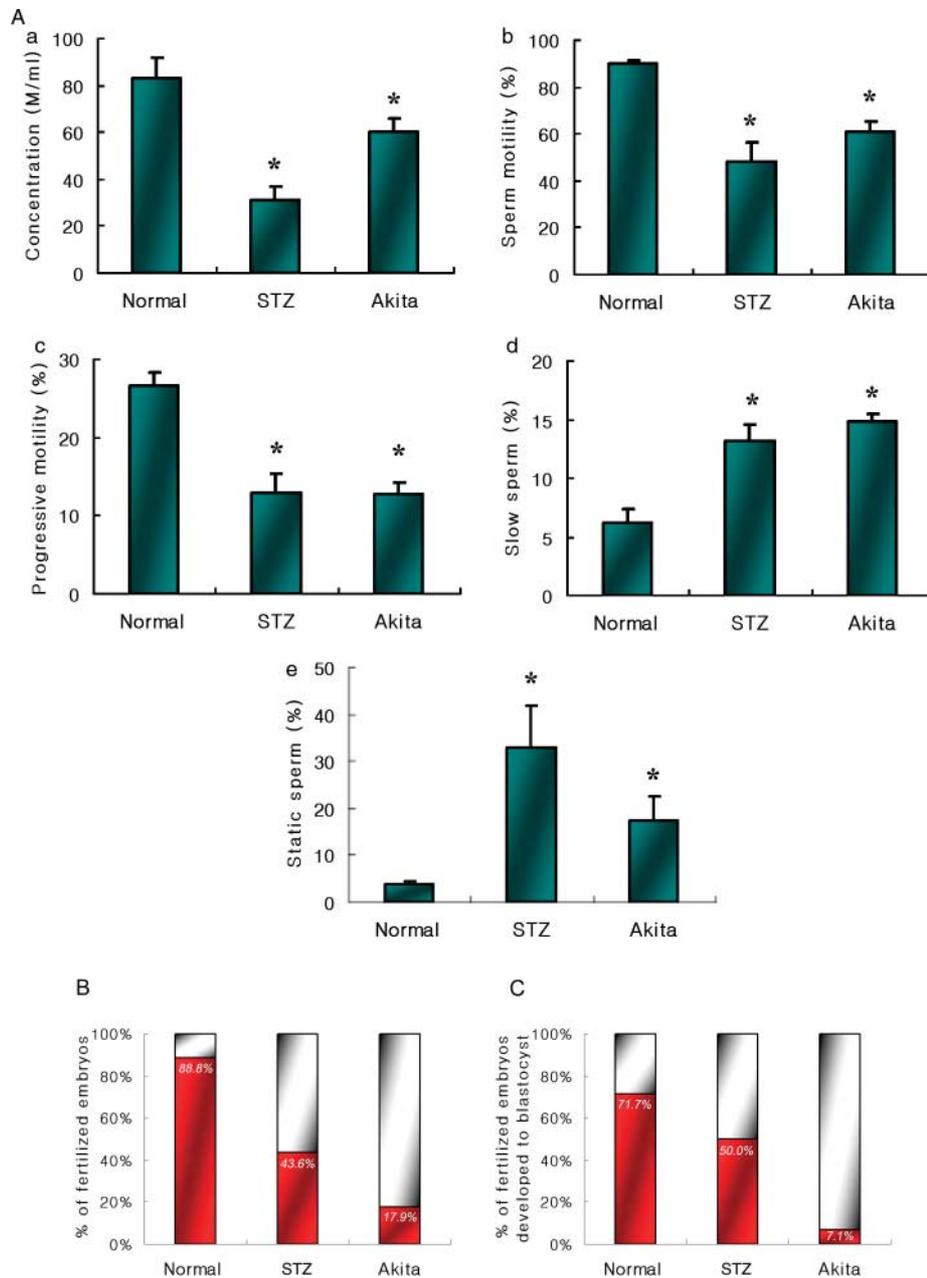


Figure 5 Computer-assisted sperm analysis (CASA) and *in vitro* fertilization (IVF). (A) Mean sperm concentration: (a) motility, (b) progressive motility, (c) slow, (d) and static (e) motility in normal, STZ-injected, and Akita males. Asterisks indicate significant differences in normal group. (B) Percentage of fertilized embryos with normal oocytes. (C) Percentage of fertilized embryos developed to blastocyst.

might provide an autocrine regulation of glucose metabolism by sperm (Aquila *et al.* 2005). In this study, we confirmed these findings by detecting insulin in control and STZ-injected diabetic mouse as shown in Fig. 3A and B. But insulin detection in the sperm from Akita diabetic mice is fainter than that of the normal and STZ-injected sperm. These mice have a single amino acid substitution in the insulin 2 gene causing misfolding of the insulin protein (Barber *et al.* 2005). Insulin is detected in secretory granules in Akita mice; however, it is profoundly decreased in concentration, as we showed here (Wang *et al.* 1999). Interestingly, treating the Akita mice with insulin normalized the sperm concentration,

and total and progressive motility of the mice, suggesting that insulin signaling and increased SLC2A9 expression improves sperm quality in diabetic mice.

To determine sperm quality and fertilization capacity of sperm from diabetic versus control mice, we performed CASA and IVF with normal COC and cauda epididymal sperm. The epididymis imparts sperm maturation by providing a specific fluid environment of secretory products from its epithelium and serves as a site for the sperm to acquire motility and fertilizing potential (Amann *et al.* 1993, Cooper 1998). In addition to sperm maturation, the epididymis plays a significant role in the transport, concentration, protection, and

storage of the sperm. Thus, the inevitable role of the epididymis in sperm maturation and the adverse effects of diabetes on testicular functions are well established. However, there is not enough information on the impact of diabetes on the epididymis. As shown in Fig. 5, this study supports, in part, that diabetes could lead to reduced sperm motility and fertility as well as compromised epididymal functions for sperm maturation. The sperm isolated from STZ-injected and Akita males showed markedly low rates of fertilization and development to the blastocyst stage. Although litter size of STZ-injected and Akita male groups through the natural mating with normal females was significantly lower than that of normal male group, *in vivo* fertilization rate and embryo development were higher than that of *in vitro* (Table 1). These differences probably reflect the different origin of the sperm. For IVF, we used cauda epididymal sperm that contains a much higher percentage of non-motile sperm. In natural mating, motile and good quality sperm are naturally selected from the vas deferens and female reproductive tract during ejaculation and before fertilization.

Previous studies have also demonstrated paternal effects on cell division in human preimplantation embryos obtained by assisted reproductive technologies (Menezo 2006). It was first proposed by Renard & Babinet (1986) that some factor in the male gamete acted at the pronucleus stage to affect later steps in embryonic development. They demonstrated that proper nucleoplasmic interaction was necessary to allow normal embryo development. The exact factor, however, was not clear. In the early 1990s, this paternal effect idea was brought into the clinical realm when Janny & Menezo (1994) reported that the quality of sperm did determine developmental potential of IVF embryos, contrary to popular view. It has been speculated that embryo quality may be negatively affected by deficiencies in the sperm nuclear genome or sperm-derived cytoplasmic factors, specifically the centrosome as well as oocyte-activating factor, responsible for calcium homeostasis in the oocyte (Tesarik 2005). Early paternal effects can be detected as early as the one-cell zygote and are associated with poor zygote and embryo morphology and low cleavage speed. This pathology is not thought to be associated with increased sperm DNA damage, but more likely this is due to as of yet unidentified cytoplasmic factors. Centrioles and oocyte activation factors have been raised, but it is still speculative. By contrast, one study

disputes this hypothesis and reports lower blastocyst formation rates in human ICSI procedures with DNA damaged sperm (Nasr-Esfahani *et al.* 2005). This study showed that DNA fragmentation as detected by the Comet assay does not preclude fertilization; however, the embryos derived from these sperm have a lower potential to reach the blastocyst stage. All these studies are done in ICSI male factor patients whose sperm parameters fall below normal World Health Organization values.

In this study, the diabetic mouse sperm parameters were also abnormal. Although the exact etiology of these abnormalities is not known, the growing evidence indicates that oxidative stress is increased in diabetes, due to the overproduction of reactive oxygen species (ROS) and decreased efficiency of antioxidant defenses (Giron *et al.* 1999, Wiernsperger 2003). Oxidation of lipids, proteins, and other macromolecules such as DNA occurs during the development of diabetes (Ohkawa *et al.* 1979), and mitochondrial DNA mutations have also been reported in diabetic tissues, suggesting oxidative stress-related mitochondrial damage (Lee *et al.* 1997). Mammalian sperm cells present a specific lipid composition with a high content of polyunsaturated fatty acids (plasmalogens and sphingomyelins). The lipids in spermatozoa are the main substrates for peroxidation and excess amounts of ROS and free radicals have adverse effects on sperm motility and fertility (Aitken *et al.* 1989). Furthermore, oxidative damage to lipids and DNA of spermatozoa is associated with declining motility and diminished fertility of human sperm (Chen *et al.* 1997, Kao *et al.* 1998). In this regard, decreased IVF rate in STZ-injected and Akita males might result from oxidative stress from dead sperm, but this stress should be more or less overcome through *in vivo* selection procedures during ejaculation and before fertilization. In fact, oocytes fertilized with STZ-injected and Akita sperm showed fragmentation and poor embryo development, which may be also affected by oxidative stress from sperm. The molecular mechanisms of reduced spermatogenesis, motility, and fertilization capacity in diabetes, however, remain to be elucidated.

In addition insulin treatment of the Akita mice for 7 days changed the sperm motility and concentration. It has been reported that IGF1 and insulin as well as IGF1 promote spermatogonial differentiation into primary spermatocytes by binding to the IGF1 receptor (Nakayama *et al.* 1999). It has been also shown that both the sperm plasma membrane and the acrosome represent cytological targets for insulin (Silvestroni *et al.* 1992, Kim & Moley 2007). We confirm here that insulin is present in mature sperm from control and STZ-treated mice but not Akita males. All reports suggest that insulin signaling may be important for spermatogenesis, sperm maturation, and capacitation, and support our results suggesting that insulin-deficient diabetic mice show

Table 1 Comparison of litter size.

Male	Female	Litter size
Normal	Normal	9.3
STZ injected		2.3*
Akita		5.8*

*Significantly differ from the normal group ($P < 0.01$).

decreased sperm quality and fertilization rate. Interestingly, SLC2A9b expression was increased in the insulin-treated Akita mice and coincided with improved sperm parameters.

In conclusion, diabetes in two different mouse models results in male subfertility by altering spermatogenesis, steroidogenesis, and sperm maturation. Insulin signaling might play an important role in SLC2A8 and SLC2A9 expression during spermatogenesis, steroidogenesis, and sperm maturation. The distribution of SLC2As suggests that they may play different roles not only in internal glucose movement, but also in the traffic of sugars for the maturation and motility maintenance of the sperm during the capacitation and fertilization processes. The present study should provide a clue for the treatment of male infertility in patients with diabetes.

Materials and Methods

Animals

Mice were housed according to Institutional Animal Care and Use Committee and National Institutes of Health guidelines. The 8-week-old B6XSJL F1 and type 1 diabetic Akita mice, which have significant hyperglycemia resulting from a single amino acid substitution in the insulin 2 gene and are on a C57 black background, were purchased from Jackson Laboratories, Bar Harbor, ME, USA. To generate a chemically induced diabetic model, B6XSJL male mice received four to five injections of STZ (Sigma Chemical Co. Ltd) at a dose of 100 mg/kg (dissolved in sodium citrate buffer pH 4.4). Seven-day post-injection, a tail blood sample was measured for glucose concentrations via a Hemocue B glucose analyzer (Stockholm, Sweden). Mice with blood glucose levels ≥ 300 mg/dl were selected. After 1 month, the testes and sperm were isolated. The STZ-induced B6XSJL mice, the Akita mice, and the non-injected B6XSJL mice were all age matched. The Akita mouse is on a C57 black background and in order to use the B6XSJL as a control, we compared C57 black age-matched males to B6XSJL males and found no differences in the sperm or testes SLC2A expression (data not shown). There were also no differences in sperm parameters using CASA (data not shown). For this reason, we used age-matched B6XSJL non-diabetic mice as the control for both diabetic groups.

Total RNA extraction and RT-PCR

Total RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's instruction. RT with oligo(dT) priming was performed to generate cDNAs from 1 μ g total RNA using Superscript II (Invitrogen) following the instruction provided by the manufacturer. DNA amplification was carried out with Taq DNA polymerase (Invitrogen) using the primers in Table 1. Amplified fragments were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

Immunohistochemistry and western immunoblot analysis of testes

Immunohistochemistry of SLC2As in the testes was performed in Bouin's fixed paraffin-embedded or frozen sections as described previously (Keembiyehetty *et al.* 2006, Kim & Moley 2007). In brief, the testes were removed, fixed in Bouin's Solution overnight, and embedded in paraffin. Testicular sections were incubated with anti-SLC2A8, anti-SLC2A9a, or anti-SLC2A9b antibodies. The sections were then incubated with a secondary antibody, Alexa Fluor 546 goat anti-rabbit immunoglobulin G. To-Pro-3 iodide was used to stain the nuclei. Western blot analysis was performed on the testes as described previously (Kim & Moley 2007).

Immunofluorescence and western immunoblot analysis of sperm

Indirect immunofluorescence of sperm was performed as described previously (Kim & Moley 2007). In brief, paraformaldehyde-fixed sperm were incubated with anti-SLC2A8, anti-SLC2A9a, or anti-SLC2A9b antibodies. Sperm were then incubated with a secondary antibody, Alexa Fluor 546 goat anti-rabbit immunoglobulin G. To-Pro-3 iodide was used to stain the nuclei. Western blot analysis was performed on the sperm as described previously (Kim & Moley 2007).

IVF

Preparation of spermatozoa

The cauda epididymis from male was removed at 12.0–13.0 h post-hCG injection and quickly transferred to a culture dish containing 500 μ l HTF medium (Specialty Media, Phillipsburg, NJ, USA), then transferred to another dish with 500 μ l HTF medium. Some incisions were made in the cauda epididymis, and gentle squeezing using fine forceps allowed the spermatozoa to swim out. Capacitation was allowed to proceed for 1.5 h at 37 °C in a 5% CO₂ incubator. The concentration of the spermatozoa was determined, and if necessary, adjusted to obtain similar sperm concentrations from normal, STZ-injected, and Akita males.

Preparation of oocytes

Adult non-diabetic B6 female mice that were 7- to 8-weeks old were induced to superovulate by i.p. injection of 5 IU pregnant mare's serum gonadotropin followed by 5 IU hCG 48 h later. Animals were killed 14.0–15.0 h post-hCG injection. Oviducts were collected in a 35 mm dish containing 2 ml HTF medium. The COCs were recovered by gentle dissection of the oviducts. COCs were transferred to a 200 μ l droplet of HTF medium covered with mineral oil.

Insemination and fertilization

Capacitated spermatozoa in the range 1×10^6 to 2×10^6 /ml were added to each insemination droplet with the COCs and placed in an incubator for 4–6 h. Oocytes then were washed

two or three times with 100 µl potassium simplex optimized medium (Specialty Media) under oil and finally transferred to 50 µl droplets of the same medium. The dishes were incubated at 37 °C in 5% CO₂ in a humidified incubator. Fertilization was assessed by recording the number of pronucleus and 2-cell embryos 9–10 h and 24 h after fertilization respectively. Embryos were observed at 200× magnification under the inverted microscope. To assess the level of parthenogenesis, eggs were incubated under the same conditions but without sperm addition. This level was consistently 0%.

CASA

The functional analysis of male reproduction was determined by measuring sperm motility of mice using the CASA system (Hamilton-Thorne Research, Beverly, MA, USA). The cauda epididymis was diced, and the sperm were allowed to disperse into the medium for 10 min at 37 °C. Among the motility parameters measured were the following: percent motility – the percent of motile sperm within the analysis field divided by the sum of the motile plus immotile sperm within the analysis field; path velocity (VAP) – the average velocity of the smoothed cell path, expressed in microns per second; progressive velocity (VSL) – the average velocity measured in a straight line from the beginning to the end of the track; curvilinear velocity (VCL) – the sum of the incremental distances moved in each frame along the sampled path divided by the time taken for the sperm to cover the track; amplitude of lateral head (ALH) – the ALH displacement corresponds to the mean width of the head oscillation as the sperm swims; beat cross frequency – the frequency with which the sperm track crosses the sperm path; straightness (STR) – the departure of the cell path from a straight line; and linearity (LIN) – the departure of the cell track from a straight line.

Placement of insulin pellets

LinBit™ insulin implants (LinShin Canada Inc., Scarborough, Ontario, Canada) were subcutaneously inserted under the mid-dorsal skin of Akita male mice during anesthesia. According to the manufacturer's instructions (<http://www.linshincanada.com>), we added two implants for the first 20 g in body weight and another implant for each additional 5 g. The same number of blanks was placed for use as controls. At 2 days post-surgery, a tail blood sample was measured for glucose concentrations via a Hemocue B glucose analyzer. Blood glucose levels ≥ 100 mg/dl were selected. After 1 week, the testes were isolated.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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