

Pendrin and sodium/iodide symporter protein expression in the testicular tissue of normal and diabetic rats in prepubertal and post pubertal stages

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Summary

Pendrin (PDS) and sodium/iodide symporter (NIS) are transmembrane proteins that are located in numerous tissue types, particularly thyroid follicular epithelial cells, where they are entrusted with the regulation of iodine molecules. In the present study, we aimed to clarify changes in PDS and NIS protein expression, in the testicular tissue of prepubertal and post pubertal rats at normal or diabetic conditions. Forty Wistar albino male rats (20 prepubertal and 20 post pubertal) were divided into four groups, as follows: group I was prepubertal control, group II was prepubertal diabetic (60 mg/kg intraperitoneal [ip] streptozotocin [STZ]), group III was post pubertal control, and group IV was post pubertal diabetic (60 mg/kg ip STZ). Ki67 immunoreactivity decreased in testicular tissue of both the prepubertal and post pubertal diabetic groups; the apoptotic tubule index and apoptotic cell number increased in the diabetic groups as compared to the control groups. Pendrin immunoreactivity was detected in seminiferous tubules and Leydig cells; and was significantly reduced in the diabetic groups (P<0.05). The number of cells positive for NIS was significantly decreased in prepubertal and post pubertal rats with diabetes, compared to the controls. Enzyme-linked immunosorbent assay (ELISA) analysis showed that PDS and NIS values were significantly reduced in the prepubertal and post pubertal diabetic groups as compared to the control groups. Our results indicate a potential relationship between puberty and PDS and NIS expression in rat testicular tissue and showed the decreasing effects of diabetes on PDS and NIS expression in testicular tissues in rats.

Key words: Diabetes, Pendrin, Rat, Sodium/iodide symporter, Testis

Introduction

Diabetes mellitus is a chronic metabolic disease and its incidence and prevalence is increasing worldwide, as a result of the increase in life span, decreases in physical activities, changes in working conditions, and the spread of obesity. The World Health Organization has estimated that approximately 438 million people worldwide will be affected by diabetes in 2030; according to data from the International Diabetes Federation (IDF, 2013), this number will be 592 million in 2035.

In addition to the well-known complications of diabetes, such as retinopathy, nephropathy, it has more recently been shown that decreased libido, retrograde ejaculation, erectile dysfunction, and sexual functional disorders are among the most important complications of diabetes in men.

The considerably widespread solute carrier (SLC) group of carrier membrane proteins consists of approximately 300 members, which are classified into 52 different families (Ramachandran et al., 2006). These proteins are anion exchangers that transport monovalent and/or divalent anions, including sulfate (SO4 2-), chloride (Cl-), iodide (I-), bicarbonate (HCO3-), and oxalate (Ox2-) ions (Mount and Romero, 2004). Thus, “loss-of-function” mutations in SLC26 genes cause a number of diseases in humans, including Pendred syndrome, deafness, diastrophic dysplasia, and congenital chloride diarrhea (Rode et al., 2012).

Pendrin (PDS), a member of the SLC26 family, is a transmembrane protein expressed by the Pendred syndrome (SLC26A4 PDS) gene. It includes 780 amino acids, has a molecular weight of approximately 86 kDa, and is entrusted with the transport of iodine (I-) located on the apical surface of thyroid follicular cells. Pendrin is described as a heterogeneous transporter transmembrane protein because it transports anions such as Na+ independent-Cl/HCO3- and iodines (Scott et al., 1999). Pendrin gene expression is not limited only to thyroid follicular cells; it is also found in tissues such as the inner ear, the kidney (Everett et al., 1999), the testis (Russo et al., 2011; Micali et al., 2013), and the uterus (Suzuki et al., 2002).

The sodium/iodide symporter (NIS), having 13 domains, is another transmembrane protein. It is expressed by the SLC5A5 gene in humans and is entrusted with Na+ and I- transport (Micali et al., 2014). The NIS has 643 amino acid residues and its molecular weight is approximately 70-90 kDa depending on the degree of glycosylation (Smanik et al., 1997). It is known that the amino-terminal and middle areas in humans and rats share a high homology (a 93%
similarly) of NIS sequences, and contain an additional 20 amino acids in the carboxy-terminal of NIS in humans (hNIS) alone (Smanik et al., 1996).

The aims of this study were to localize and quantify PDS and NIS in testicular tissue by immuno-histochemical and enzyme-linked immunosorbent assay (ELISA) methods in normal and diabetic rats.

Materials and Methods

Animals

Four experimental groups (n=10 each) were created from 40 Wistar albino male rats, 20 prepubertal (30-day) animals and 20 post pubertal (8-10 weeks), which were obtained from the Experimental Animals Research Unit of Trakya University, Edirne, Turkey, and kept in standard laboratory conditions (22 ± 1°C, in a 12-h light/dark cycle; relative humidity 55%), and which had the same biological and physiological characteristics; and received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institute of Health. The groups were established as follows:

Group I: Prepubertal control (aged 30 days)
Group II: Prepubertal diabetes (aged 30 days), which received 60 mg/kg intraperitoneal [ip] streptozotocin [STZ], (Sigma-Aldrich, Germany)
Group III: Post pubertal control
Group IV: Post pubertal diabetes, which received 60 mg/kg ip STZ

The blood-glucose levels of the rats were measured from blood samples taken from the tail veins of experimental animals at the beginning of the experiment, 48 h after STZ administration, and at the end of the experiment, using a glucometer (IME-DC, Germany). The animals receiving STZ with blood glucose levels higher than 250 mg/dl at the end of 48 h were accepted as having diabetes and were included in the study (Karaca et al., 2015; Balali Dehkordi et al., 2017). The post pubertal diabetic group started with 10 animals, 3 rats died in the experimental period and the experiment was completed with 7 animals, and there was no death in other groups.

Histopathological analysis

All rats were sacrificed under anesthesia with xylazine (10 mg/kg/bw) and ketamine (90 mg/kg/bw) IP on the 15th day following STZ injection; cardiac blood and testis tissue samples were obtained. Half of the testes samples were stored at -80°C for ELISA analysis, while the other half were maintained in 10% buffered formaldehyde solution for 24 h for histopathologic and immunohistochemical examinations, after which they were processed by routine histological operations and paraffin blocks were obtained. The testis samples were cut into 6 μm-thick sections, stained with haematoxylin and eosin, and examined under a microscope (Olympus BX51, Tokyo, Japan). The blood samples were centrifuged, and the sera were stored at -80°C until analysis.

PDS, NIS and Ki67 immunohistochemical evaluations

Testis were removed and fixed in 10% neutral buffered formalin solution, and following the routine laboratory methods, blocked in paraffin. Immunocytochemical stains were performed based on the avidin-biotin complex (ABC) technique described by Oguz et al. (2015). The samples were incubated with specific rabbit polyclonal anti-PDS (AntiSLC26A4 antibody, ab98091, 1:100; Abcam), rabbit anti-mouse NIS monoclonal antibody (1/50; anti-sodium iodide symporter; ab83816; Abcam), and rabbit anti-mouse Ki67 monoclonal antibody (1:200; AB9260; Millipore) at room temperature for 60 min, followed by detection with 3-amino-9-ethylcarbazole, a chromogen. The slides were counterstained with hematoxylin and mounted in xamount aqueous mounting medium. Anti-PDS, anti-NIS and anti-Ki67-positive cells were counted in random 100 high-power fields using a light micro-scope for each sample and are given as positive cell count per mm². Thyroid tissue sections were used as positive controls.

TUNEL assay

A terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) assay was used to assess testicular cell apoptosis, using an apoptosis detection kit (Millipore, ApopTag) according to the manufacturer’s instructions. For each slide, 20 fields were randomly chosen, with TUNEL-positive cells staining dark brown within the nucleus of apoptotic cells. The TUNEL-positive cells were quantified under high-power magnification by a blinded researcher, and the apoptotic index was calculated (the number of TUNEL-positive cells/total number of cells counted ×100).

Chemical immunoassay of testosterone levels

The serum testosterone levels were measured in triplicate using 100 μL samples of each serum and commercially available chemical immunoassay kits (Siemens, Immulite 2000; Siemens Healthcare Diagnostics Inc., USA).

PDS and NIS ELISA evaluations

The testis tissue samples were weighed and then homogenized in PBS (10 mg/100 μL) with a glass homogenizer on ice. The homogenate was then centrifuged at 5000 × g for 5 min, and the supernatant was collected for ELISA assay. All reagents, working standards, and samples were prepared according to the kit procedure. The samples were diluted at 1:10 for assay. Pendrin and NIS were measured using a rat SLC26A4/Pendrin ELISA kit and a rat sodium/iodide symporter (SLC5A5) ELISA kit (Sandwich ELISA) (LifeSpan BioSciences Inc., Seattle, Washington, USA), according to the manufacturer’s instructions. All samples were run in duplicate, and the optical density value of each well was determined immediately, using a
A statistical comparison of differences between groups was performed using analysis of variance and Fisher’s multiple comparison tests. Differences were considered significant at \( P<0.05 \). The statistical analyses of histological scores were performed via the Kruskal-Wallis test and the Mann-Whitney U test with Bonferroni correction.

**Results**

**Blood glucose and testosterone measurements**

The blood glucose levels of the rats were measured at the beginning of the experiment, 48 h after STZ administration (blood glucose levels higher than 250 mg/dl at the end of 48 h were accepted as having diabetes), and at the end of the experiment. Results were summarized in Table 1.

Although total serum testosterone levels measured in blood samples obtained with cardiac puncture were not detected on the measurement scale in the prepubertal groups; a statistically significant decrease was observed in group IV when compared with group III (\( P<0.05 \); \( P=0.044 \)).

**Body weight variations**

The rats in all experimental groups were weighed before and after the experiment; these values are given in Table 2 and weight variations are shown in Fig. 1. In group II and group IV body weight was significantly lower compared to group I and group III, according to the measurements taken at the end of the experiment, respectively (\( P<0.05 \); Table 2 and Fig. 1).

**Histopathological and TUNEL assay**

Testis tissue showed a normal histological structure in the control groups. However, seminiferous tubule wall and the spermatogenic cells (spermatogonia, spermatocytes, of the diabetic rats exhibited significant damage (Figs. 2a-d).

The TUNEL method was applied to cross sections obtained from all groups, and the apoptosis values were scored using the “apoptotic cell index” and the “apoptotic tubule index”. These index scores were statistically significantly increased in group II compared to group I (\( P<0.05 \); Table 3). Similarly, index values in group IV were significantly increased compared to group III. TUNEL (+) cells were found in high numbers in spermatogonia in the control groups - group I and group III. The number of TUNEL (+) cells was observed in the other cells of spermatogenic series, as well as spermatogonia in the groups with diabetes (Figs. 3a-d).

![Fig. 1: Body weight changes (g). * P<0.05, Compared with control prepubertal group, and ** P<0.05, Compared with control post pubertal group](image)

![Fig. 2: Testis H&E staining. (a) Control prepubertal group, (b) Diabetic prepubertal group, (c) Control post pubertal group, and (d) Diabetic post pubertal group, (magnifications: a, c, d: x200; b: x400)](image)
Table 3: Apoptotic tubule and apoptotic cells index

<table>
<thead>
<tr>
<th>Apoptotic changes</th>
<th>Control prepubertal</th>
<th>Diabetic prepubertal</th>
<th>Control post pubertal</th>
<th>Diabetic post pubertal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptotic tubule</td>
<td>10.75 ± 3.8</td>
<td>30.7 ± 9.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.6 ± 2.8</td>
<td>26.4 ± 7.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P-value</td>
<td>0.004&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>0.004&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Apoptotic cell</td>
<td>6.1 ± 1.1</td>
<td>104.60 ± 10.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.3 ± 0.7</td>
<td>98.2 ± 5.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P-value</td>
<td>0.007&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>0.03</td>
<td>0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

P<0.05, <sup>a</sup> Compared with control prepubertal group, and <sup>b</sup> Compared with control post pubertal group

Fig. 3: Testis TUNEL staining micrographs. (a) Control prepubertal group, (b) Diabetic prepubertal group, (c) Control post pubertal group, and (d) Diabetic post pubertal group, (arrows: TUNEL positive cells), (magnifications: a, b: ×200; c, d: ×400)

Fig. 4: Immunopositive Ki67 cell counts of testicular tissue. * P<0.05, Compared with control prepubertal group, and ** P<0.05, Compared with control post pubertal group

Ki67 immunoreactivity

Although the number of Ki67 immune-positive cells counted in 10 different areas, 100 areas in total, for each experimental animal belonging to every group decreased significantly in groups II and IV compared to group I (P<0.05), it was found that the decrease in group IV was at a similarly significant level to that in group III (P<0.05; Figs. 4 and 5a-e). It was observed that the difference in values between group II and group IV was not statistically significant (P>0.05).

PDS immunoreactivity

In the PDS immune staining performed in the testis, Sertoli cells were observed in seminiferous tubules of

NIS immunoreactivity

With regard to the NIS immune staining, no immune-positive cells were found in testicular seminiferous tubules in all groups. The numbers of NIS-positive cells were summarized (Fig. 8). In contrast, NIS-positive cells were observed in interstitial Leydig cells in all groups at varying rates (Figs. 9a-e).

ELISA PDS findings

The ELISA evaluations performed in the supernatants obtained by homogenizing the samples of testicular tissues showed that the highest value (810.9 ± 34.2 pg/ml) was obtained in the post pubertal control group (group III); the lowest value (364.2 ± 21.3 pg/ml) was observed in the prepubertal diabetes group (group II).
Fig. 6: PDS immunohistochemical staining micrographs. (a) Control prepubertal group, (b) Diabetic prepubertal group, (c) Control post pubertal group, (d) Diabetic post pubertal group, and (e) Prepubertal control group; Negative section, (thick arrows: spermatogium and Sertoli cells; medium thick arrow: Leydig cell; thin arrows: PDS immune positive cells in the developmental stage), (magnifications, ×400)

Fig. 7: PDS immunopositive cells number/(mm)$^2$. * P<0.05, Compared with control prepubertal group, and ** P<0.05, Compared with control post pubertal group

Fig. 8: NIS immunopositive cells number/(mm)$^2$. * P<0.05, Compared with control prepubertal group, and ** P<0.05, Compared with control post pubertal group

Fig. 9: NIS immunohistochemical staining micrographs. (a) Control prepubertal group, (b) Diabetic prepubertal group, (c) Control post pubertal group, (d) Diabetic post pubertal group, and (e) Prepubertal control group; Negative section, (arrows: NIS positive cells), (magnifications, ×400)

Fig. 10: PDS ELISA results in testicular samples. * P<0.05, Compared with control prepubertal group, ** P<0.05, Compared with control post pubertal groups

The PDS values decreased considerably in the prepubertal diabetes group compared to the prepubertal control group (Fig. 10). The ELISA PDS value was higher in the post pubertal control group compared to the prepubertal control, prepubertal diabetic, and post pubertal diabetic groups (P<0.05).

**ELISA NIS findings**

The ELISA analyses showed the presence of the NIS protein in testicular tissue; significant decreases were observed in the post pubertal diabetic group compared to the prepubertal control group; and were decreased in the prepubertal diabetic group compared to the prepubertal control group (P<0.05). Group III had the highest level of NIS expression in testicular tissue, while
the lowest level of NIS expression was found in the prepubertal diabetes group (Fig. 11).

![NIS ELISA results in testicular samples.](image)

**Fig. 11:** NIS ELISA results in testicular samples. * P<0.05, Compared with control prepubertal group; and ** P<0.05, Compared with control post pubertal groups

**Discussion**

Pendred syndrome is a disease transmitted by congenital recessive autosomal heredity and is accompanied by goiter and a loss of hearing (Suzuki et al., 2002); inner ear anomalies are observed in many affected individuals (Johnsen et al., 1987; Yoshida et al., 2002). The pendrin gene is also expressed in kidney, testis, lung, and female genital tissues, as well as the thyroid; it is considered that the PDS protein has an important role in iodine transport within tissue and in setting homeostasis.

Sexual function disorders are often observed in men with diabetes; of these, a decreased libido, erectile dysfunction, hypogonadism, and retrograde ejaculation are among the most commonly observed (Scott et al., 1999). In the present study, an increase in body weight occurred at a considerably slow rate in prepubertal rats with diabetes, statistically significant weight loss was found in the post pubertal diabetes group.

In the present study, it was observed that PDS was expressed in testicular seminiferous tubules and Leydig cells. In addition, significant decreases were found in the testicular tissue of both the prepubertal and post pubertal diabetic groups compared to the control groups. It was observed that decreases of PDS immune reactivity in particular were more specific in germinal series cells in the different developmental stages of seminiferous tubules.

The ELISA analyses showed that PDS and NIS protein expression was significantly decreased in the diabetic groups (prepubertal and post pubertal) compared to the control groups at the tissue level, similar to what was found the immunohistochemical parameters. In a study conducted in mice, Ramachandran et al. (2006) reported that in the organs examined at postnatal days 21 and 45, such as the thyroid, lung, female genital organs, kidneys, brain, adrenal gland, etc; the highest level of PDS mRNA expression was found in the testis. In the same study, it was shown that PDS mRNA expression was higher on postnatal day 45 than on postnatal day 21. The authors observed that in the testis and kidneys, PDS mRNA expression was respectively 7 and 5 times higher on postnatal day 45 than on postnatal day 21. In the present study, it was found that the number of PDS immune-positive cells was approximately 1.5 times higher in the post pubertal control group compared to the prepubertal control group - 52.4 ± 9.7 and 35.6 ± 7.6, respectively. Similarly, a higher number of PDS-positive cells were observed in post pubertal rats with diabetes compared to prepubertal rats.

Lacroix et al. (2001) reported that the NIS was stained pale in human testis; in addition, PDS showed a very weak immune positivity in Sertoli cells. NIS mRNA and protein levels are fairly variable in non-thyroid tissues. Wapnir et al. (2004) histochemically revealed NIS proteins in healthy human bladder, endometrium, kidney, prostate, and pancreas, while in the present study, NIS immunoreactivity was observed only in Leydig cells, and the reaction was largely found in the plasma membrane.

Russo et al. (2011) showed NIS expression in fetal and post pubertal testicular tissue by reverse transcriptase polymerase chain reaction (RT-PCR). In the same study, immune positive cells were found in seminiferous tubule and Leydig cells, both human and rat, and the results of our study show similarities and differences with regard to the results observed in seminiferous tubule cells. Russo et al. (2011) claimed that NIS immunoreactivity was increased with increasing age in rats and mice. The previous studies suggested a weak NIS expression in testicular tissue (Wapnir et al., 2003). In the testicular tissue NIS expression was localized in the Leydig cells and lumen side of the seminiferous ducts.

In the present study, no NIS immune positive reaction was observed in the Sertoli cells of seminiferous tubules. These results show a resemblance with those obtained by Russo et al. (2011). Lacroix et al. (2001) reported that the PDS and NIS genes are not expressed in adrenal gland, skin, and muscle cells, but that they are expressed in lung tissue at a low level compared to normal thyroid. A study conducted using the Northern blotting technique suggested that there is no PDS expression in testicular tissue (Suzuki et al., 2002). In the present study, there was a decrease in cell proliferation in testicular tissue in the prepubertal and post pubertal diabetes groups compared to the control groups. Krause et al. (2007) showed an increase of PDS and NIS expression in the cell proliferation of cold nodules and the thyroglobulin synthesis stage compared to normal thyroid parenchyma. The results of this study suggest that there may be a relationship between decreased Ki67 immunoreactivity in seminiferous tubule epithelial cells and decreased PDS and NIS expression in diabetic groups. That is, impairments in ion transport proteins may initiate apoptosis in seminiferous tubule cells.

In conclusion, our results show that PDS and NIS were expressed at different levels in the testicular tissue.
of both prepubertal and post pubertal rats. Diabetes could inhibit the expression of the PDS and NIS proteins in rat testicular tissue. Moreover, expression of PDS and NIS proteins in rat testicular tissue also increased after pubertal maturation. Our results indicate a potential relationship between puberty and PDS and NIS expression in rat testicular tissue and showed the decreasing effects of diabetes on PDS and NIS expression in testicular tissues in rats.

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