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INSULIN GENE EXPRESSION AND ITS DETECTION IN THE KIDNEYS OF RATS WITH IMPAIRED GLUCOSE TOLERANCE

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Abstract- ***In impairedglucosetolerance (IGT) –a major risk factor for the development of type 2 diabetes mellitus (DM)–reportedcomplicationsincluderetinopathy, renal disease, polyneuropathy and dyslipidemia. Additionally, impaired β cell function has been demonstrated. Renal diseasemay be related to local insulinaction, as itstimulatessodiumreabsorption and nitric oxide production. Recently, we found that rat kidneys are able to produce insulin as well as the Pdx-1 and Nkx6.1 transcription factors, which play a key role in differentiation of β-cells and insulin expression, under standard physiological conditions. In this work, we investigated the expression of insulin and its related transcription factors in the kidneys of rats which were administered a 30% sucrose solution for six months, to induce an IGT model. Theseanimalsshowed a significantdecrease in the renal genicexpression of insulin and itsrelatedtranscriptionfactors and consequently, a decline in itstissuedetectionthroughimmunohistochemistry. Also, IGT animals showed renal tissue damage at the cortex and medulla. In addition to IGT, sucrosetreatedratspresentedhyperinsulinemia, increasedtryglicerides and a decrement in HDL blood levels. We suggest that as in the pancreas, IGT leads to a decline in local insulinproduction in thekidney and thereforeanimpairment in sodiumreabsorption and nitric oxide production. This may contribute to kidney tissue damage.***

Keywords - kidney, insulin, pancreatic transcription factors, Pdx-1, Nkx6.1, impaired glucose tolerance

1. INTRODUCTION

IGT is one of the highest risk factors for type 2 diabetes mellitus development and is often associated with metabolic syndrome [1]. IGT also serves as a marker for the state of insulin resistance and predicts both large and small-vessel vascular complications. IGT is also independently associated with microvascular complications of diabetes, including retinopathy, renal disease, and polyneuropathy [2].There is evidence that IGT is also associated with both renal and retinal injury at the time of diabetes diagnosis suggesting that these complications occur early in glucose dysregulation [3, 4].The development of postchallenge hyperglycemia mainly seems to be caused by low whole-body insulin sensitivity followed by a progressive decline in β-cell function, indicating a loss of β-cell compensation [5]. Studies that have assessed insulin secretion in IGT, have demonstrated impaired β-cell function [6, 7]. These observations suggest that impaired insulin secretion in IGT is primary and precedes this metabolic alteration [8, 9]. Impaired insulin secretion may affect renal functioning which may lead to renal disease.Insulin's key kidney functions as anantinatriureticand its participation in blood pressure have been well documented[10]. Insulin's stimulation of sodium reabsorptionis carried out upon the whole nephron stimulating different mechanisms[11-14]while playing a key role in the regulation of body fluid volume. Also, insulin stimulates the production of nitric oxide (NO), from endothelial nitric oxide synthase (eNOS) [15], which has important vasodilator actions.Both actions are carried out through the activation of insulin receptors which are located at the basolateral side along the entire length of the renal tubulesby the PI 3-kinase pathway[16-19].We recently reported insulin genic expression as well as the transcription factors Pdx-1 and Nkx6.1 which play a key role in the differentiation of insulin producing cells and insulin expressionin normal rat kidneys as well as its tissular detection by immunohistochemistry, at tubular level only[20]. In this work, we investigated the kidney expression of insulin, Pdx1 and Nkx6.1 transcription factors, its renal tissue immunodetection and its relation to kidney tissue damage in an IGT model in rats.

2.METHODS

All reagents used were analytical grade. They were obtained from Sigma Chemical Co. (St. Louis, MO., USA), Promega (Madison, WI), Qiagen (Germany), or from local suppliers (Merck and J.T. Baker, Mexico).
The experiments in this study were performed following the guidelines stated in "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985) and Mexican regulations "Norma Oficial Mexicana NOM-062-ZOO-1999, Especificacionestécnicas para la producción, cuidado y uso de animales de laboratorio" (published in December, 1999). The studies were approved by the Ethical Committee of the Biological Sciences Division Council of our University.

2.1Experimental Animals

Male Wistar rats (200-220 g of b. wt.) were obtained from our animal facility, fed with Purina standard chow, and maintained at 20-22º C with 12-hr. light /dark cycles. Three experimental groups were formed. 1) Sucrose treated group. Rats received 30% sucrose solution in purified water, as drinking solution, *ad libitum* for six months, n=5. 2) Control group (6 months). These animals received purified water as drinking solution for the same time, n=6. 3) Initial control group. No treatment was administered to these animals, n=6.

2.2Glucose and weight monitoring

Each month, animals were fasted for 7 hours. Blood glucose was measured with an automatic glucometer (Abbot Diabetes Care Ltd. U.K.) in tail vein and their weight was registered.

2.3Glucose tolerance test

Animals from each group were fasted for 7 hours. One dose of glucose (3 g/Kg b.w.) was administered intraperitoneally. Blood glucose was measured in tail vein with an automatic glucometer at 0, 15, 30, 60 and 120 min. after glucose administration[21].

2.4Insulin tolerance test

One dose of insulin (1U/Kg) was administered intraperitoneally to each animal. Glucose was measured in tail veinat 0, 15, 30, 60 and 120 min after insulin administration.

2.5Tissue and blood collection

For sacrifice, animals were anaesthetized with a dose of sodium pentobarbital (50 mg/Kg b.w., i.p.). Blood was collected by cardiac puncture. The kidneys and pancreas were dissected through an incision in the abdominal cavity.

2.6Serum glucose measurement

Serum glucose was measured in 50 µl of serum rat using the orto-toluidine method [22].

2.7 Serum insulin concentration

The serum insulin concentration for each animal, at sacrifice time, was measured with High Rate Insulin ELISA (Mercodia, Uppsala,Sweden) according to the manufacturer's protocol.

2.8 Serum triglycerides, cholesterol, HDL, LDL and VLDL concentration

These serum parameters were measured in each animal at sacrifice time according to the manufacturer's protocol (Randox Laboratories Ltd. U.K).

2.9 Gene expression analyses

2.9.1 RNA isolation

RNA was isolated from a fragment of kidney or pancreatic tissue (50 mg) from each animal and prepared using the column method according to the manufacturer's protocol PROMEGA (Madison, WI). Briefly, the tissue was homogenized in 100 µL of lysis buffer. 350 µL of dilution buffer was added to 175 µL of lysate. The sample was heated at 70ºC for 3 minutes and then centrifuged for 10 minutes. 250 µL 95% ethanol was added to cleared lysate and mixed. The lysate was transferred to the column and centrifuged for 1 minute. 600 µL of wash solution was added and centrifuged for another minute. 50 µL of DNase was applied to thecolumn and incubated for 15 minutes at room temperature. After this time, 200 µL of DNase stop solution was added and centrifuged for 1 minute and washed two times. To eluate the RNA, 100 µL of nuclease free water was added to the membrane column.

2.9.2 RT-PCR

Total RNA was quantified spectrophotometrically at 260/280 nm. The integrity of RNA samples were evaluated in a 1% agarose gel. The cDNA of each sample was synthesized from 2 µg of total RNA in a total volume of 25 µL using oligo-$(dT)_{20}$primers and the PROMEGA kit (Madison, WI) at 37 ºC for 60 min according to the manufacturer's protocol. The PCR mixture contained 0.5 µL (5 µM) of each primer, 2.5 µL of $MgCl_2$, 0.3 µL of Go-Taq polymerase, 0.25 µL of dNTPs mixture, 5.0 µL of buffer and 15.95 µL of nuclease-free water, 1.0 µL cDNA sample. A parallel reaction was made with ribosomal 18s primer as an internal control for the PCR analysis. Primer sequences and the size of each amplified PCR product are shown in Table 1. All oligonucleotide primers were synthesized and used to prime the amplification of the cDNA template, based on the previous published sequences (NCBI) of insulin (C07149), Pdx1 (NM_022852), Nkx6.1 (NM_031737) and ribosomal 18s. Amplification started with 5 min at 94 ºC, followed by 35 cycles of 95 ºC, 63 ºC for ribosomal 18s and Pdx-1, 64.2 °C for insulin, 63.8 °C for NKx-6.1; and 72 ºC, 30 sec. for each step. A final extension step of 5 min. at 72°C, and the reaction products were sequence-verified. PCRproducts were separated and detected on a 1.7% agarose gelfollowed by ethidium bromide staining and densitometry analysis using a Kodak EDAS 290 (Kodak, USA).

PCR products were confirmed by direct sequencing. A BLAST search against the rat genome using the electronic PCR program from the NCBI Genome Database of the primer pairs confirmed no pseudogenes.

2.9.3 Sequentiation of PCR products

The Automated DNA sequencing of each PCR product was carried out on capillary-based electrophoresis sequencers. The unit utilizes an ABI Prism 310 (1-capillar) and an ABI Prism 3100 (16-capillary). Genetic Analyzers from Applied Biosystems, with Big Dye Terminator Cycle Sequencing chemistry.

2.9.4 Insulin, Pdx-1 and Nkx6.1 immunodetec-Tionperoxidaseimmunohistochemistry

Fragments of kidney and pancreatic tissue were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. After deparaffination, 5 µm thick tissue sections were treated with boiling 0.01 M citrate buffer (pH 6.0) for 20 min. for antigen retrieval. Then, these tissue sections were incubated in 0.2% IgG-free albumin in phosphate buffered saline (PBS) for 30 min. Sections were then labeled with mAbs anti-insulin (Zymed Labs, Inc, San Francisco, CA) (1:100), mAbs anti-Pdx-1 (Millipore Corp. Temecula, CA) (1:500) and mAbs

anti Nkx6.1 (Santa Cruz Biotechnology Inc. CA) (1:250), respectively, for 2 hours at room temperature. Secondary antibodies conjugated with peroxidase were used for detection.HRP-rabbit anti-guinea pig IgG (1:100), HRP-goat anti-rabbit IgG (1:100), HRP-rabbit anti-goat IgG (1:100) (Zymed Labs, Inc, San Francisco) respectively for 2 hours at room temperature. 3,3'-diaminobenzidine (DAB) 0.06% in PBS was used as the staining solution. These sections were stained with hematoxylin. Then, they were mounted in Entellan (Merck, Darmstadt, Germany) and analyzed with a 100x objective on a DM-1000 Leica microscope.

2.10 Statistical Methods.

ANOVA and Tukey test were used to compare the parameters values of different experimental groups (SPSS, Chicago, Ill. USA). A difference was considered significant when $p<0.05$(Fig. 2).

3. RESULTS

3.1 Serum glucose concentration and body weight

These parameters were measured monthly for six months. There were no changes in serum glucose values in the control group during the time of the experiment (six months). The sucrose treated group was not different from the control group. Only in the last month, was glucose significantly increased (control group, six months) = 4.17 ± 0.25mM; sucrose treated group = 7.32 ± 0.4mM, $^{+}p< 0.05$ (Table 2). Body weight increased monthly in both

Table 1. Primer sequences and product size of genes that were further tested with RT- PCR.

Gene	**Insulin**
ProductLenght	232 bp
Forward Primer	CCAGTTGG*T*AGAGGGAGCAG
Reverse Primer	CACCTTTGTGGTCCTCACCT
Gene	**Pdx-1**
ProductLenght	247 bp
Forward Primer	GGGACCGCTCAAGTTTGTAA
Reverse Primer	GGCTTAACCTAAACGCCACA
Gene	**NKx6.1**
ProductLenght	209 bp
Forward Primer	GGGCTTGTTGTAATCGTCGT
Reverse Primer	ACTTGGCAGGACCAGAGAGA
Gene	**18s RNAr**
ProductLenght	140 bp
Forward Primer	GTAACCCGTTGAACCCCATT
Reverse Primer	CCATCCAATCGGTAGTAGCG

groups and there was no difference among them at the sixth month (control group,six months) = 565.06 ± 29.48 g; sucrose treated group = 635.75 ± 29.11 g) (Table 2).

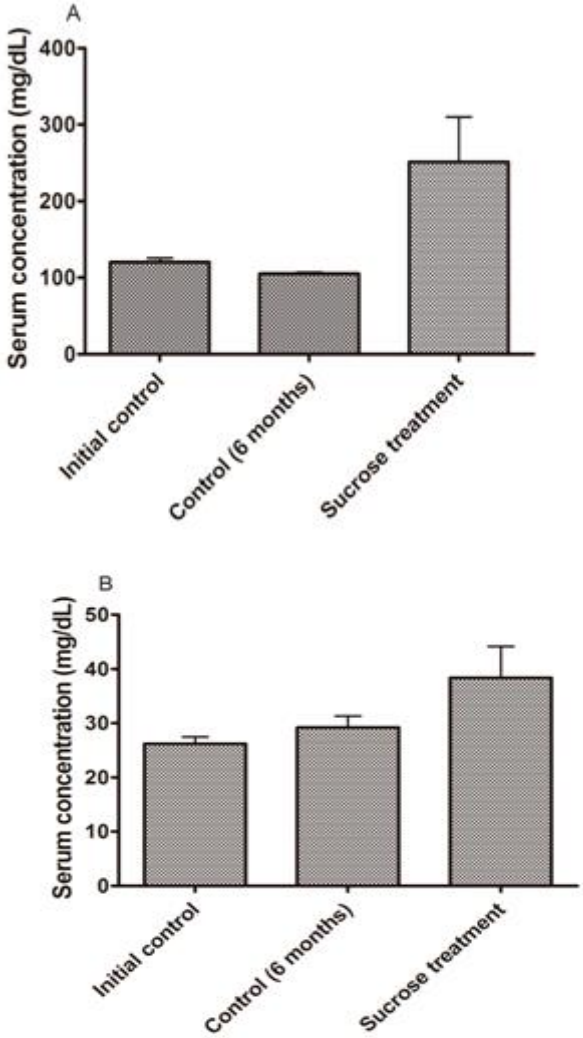
3.2 Triglycerides, LDL and HDL serum concentration

Serum triglycerides concentration increased notably in the sucrose treated group. There was no difference between the two control groups (sucrose treated= 251.18 ± 59.09 mg/dL; control (six months) = 105.23 ± 1.9mg/dL initial control group = 120.23 ± 5.49 mg/dL) (Fig. 1A). LDL levels of experimental groups did not show difference between them(Fig. 1B). On the contrary, HDL serum concentration decreased significantly in the sucrose treated group with respect to the control groups while a decrease was observed in the six months control group (sucrose treated= 10.056 ± 2.35; control (six months) = 27.66 ± 5.09; initial control = 46.89 ± 3.53), $^{*}p < 0.05$ respect to initial control. $^{+}p< 0.05$, respect to control six months). (Fig. 1C).

There were no significant changes between the experimental groups regarding total cholesterol serum concentrations (data not shown).

3.3 Serum insulin concentration

This parameter was measured at the end of the treatments. The sucrose treated group showed significantly higher levels than both control groups, which, in turn showed no difference among them (initial control = 0.250± 0.126ng/mL; controlsix months = 0.467 ± 0.155ng/mL; sucrose treated= 3.188 ± 0.932ng/mL ; $^{*}p<0.05$respect to initial control group and $^{+}p<0.05$ respect to six months control group) (Fig. 2).



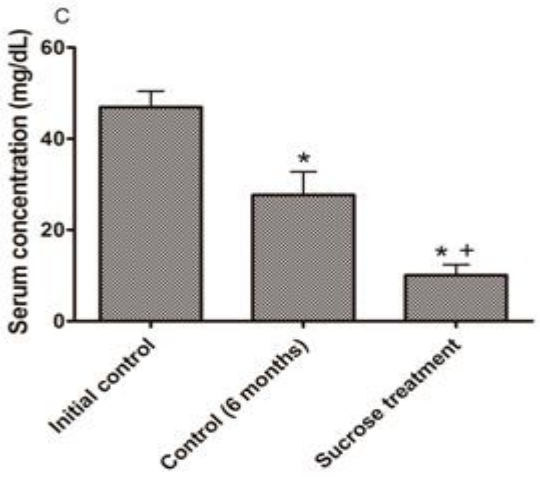


Figure 1. (A) Triglycerides, (B) LDL and (C) HDL serum levels. Each bar represents the mean ± S.E.M. n=5. *p < 0.05 respect to initial control. +p< 0.05, respect to control (six months).

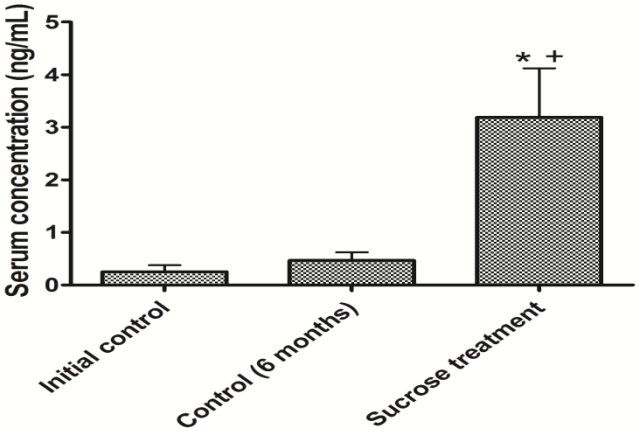


Figure 2. Serum insulin levels. Each bar represents the mean ± S.E.M. n=5. p< 0.05.

3.4 Glucose tolerance test

This test was performed for measuring insulin-stimulated glucose clearance in the three experimental groups of animals that had fasted for 7 hrs.(Fig. 3). Fifteen minutes after glucose administration we observed a sharp increase in serum glucose in the control (six months) and sucrose treated group (17.24 ± 1.22mM; 18.202 ± 3.008mM, respectively) compared to the initial control group (7.93 ± 0.25mM). After some time, serum glucose levels showed a decrease, reaching normal levels only in the initial control group. After 120 minutes, serum glucose values for the sucrose treated (8.14 ± 0.77mM) and the six months control (5.32 ± 0.23mM) groups were significantly higher than the initial control group (3.015 ± 0.23mM). This value was even greater in the first group. There was a significant difference between the two control groups (initial and six months).

3.5 Insulin tolerance test

Insulin tolerance test was performedto evaluate insulin sensitivity. At fifteen and thirty minutes we observed a similar decrease in plasmatic glucose. After this point, values increased for both control groups but this was not the case for the sucrose treated group (Fig. 4).

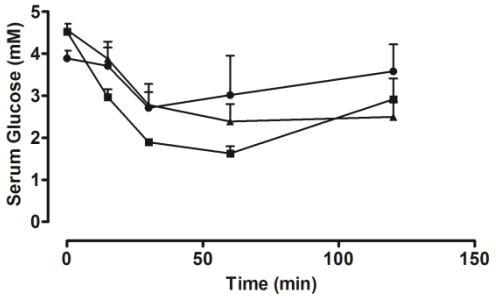


Figure 3. Glucose tolerance test. (●) Initial control group,(■) Control (six months group), (▲) Sucrose treated group. Each point represents the mean ± S.E.M. n=5. *p < 0.05 respect to initial control. +p< 0.05, respect to control (six months).

Table 2. Serum glucose levels and weight in experimental groups.

Month	Serum glucose (mM) Un-treated group	Serum glucose (mM) Treated group	Weight (g) Un-treated group	Weight (g) Treated group
Initial Control	3.91 ±0.85	3.91 ±0.85	223.83 ± 1.61*+	223.83 ± 1.61
1	3.88 ± 1.49	4.19 ± 0.15	358.25 ± 27.19 *	340.16 ± 8.25*
2	3.57 ± 0.37	3.50 ± 0.20	439.7 ± 19.58	441.83 ± 15.61
3	3.38 ± 0.35	3.65 ± 0.37	473.41 ± 19.82	530.83 ± 15.41
4	3.77 ± 0.36	3.49 ± 0.19	570.16 ± 11.94	567.33 ± 15.86
5	4.10 ± 0.18	3.23 ± 0.20	586.24 ± 5.19	573.5 ± 25.97
6	3.14 ± 0.26	7.33 ± 0.70*+	565.06 ± 29.48	635.75 ± 29.11*+

Mean values ± SEM. n=5. *p < 0.05 respect to all times of untreated group (control six months). +p< 0.05 respect to all times of saccharose treated groups.

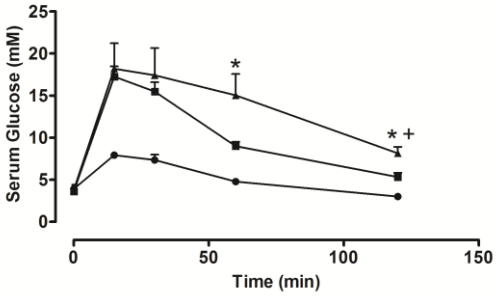


Figure 4. Insulin tolerance test. (●) Initial control group, (■) Control (six months group), (▲) Sucrose treated group. Each point represents the mean ± S.E.M. n=5.

3.6 Insulin, Pdx-1 and Nkx6.1 gene expression

The RNA samples were used for RT-PCR amplification to look for insulin, Pdx-1 and Nkx6.1 genes, in the kidneys of the different experimental groups. RT-PCR products of kidney RNA, for insulin, showed the same band size that was found in pancreatic RNA (232 bp) for the initial control group (Fig. 5). Transcription factors, Pdx-1 and Nkx6.1, revealed the same fragment length, as corresponding pancreatic transcription factors, 247 bp for Pdx-1 and 209 bp for Nkx6.1 (Fig. 5). RT-PCR products of these genes were sequenced to determine the percentage of homology with pancreatic insulin, Pdx1, and Nkx6.1 genes. A BLAST search against the rat genome, using the electronic PCR program from the NCBI Genome Database, showed that RT-PCR products of RNA rat kidney presented a homology with respect to the same pancreatic rat genes of 100% for insulin (195/195); 97% for Pdx-1 (119/123) and 98% (178/181) for Nkx.6 (Table 3).

The relative mRNA level was determined through a densitometric relation between each gene and the ribosomal 18s gene (Fig 6). Thedensitometric relation results showed that for insulin, there was a significant difference among the control groups (initial and 6 months) and a significant decrease in the sucrose treated group (initialcontrol= 3.38 ± 0.36; six months control= 2.59 ± 0.38; sucrose treated= 0.76 ± 0.38). For the transcription factor Pdx-1 the initial control group densitometric relation was significantly greater than the value for the 6 months control and sucrose treated groups. No significant difference was shown among the last two groups, (initialcontrol= 12.35 ± 1.45; six months control= 1.49 ± 0.44; sucrose treated= 2.28 ± 0.75). Nkx6.1 transcription factor, relative mRNA level, showed a pattern similar to the above insulin results (control= 7.73 ± 0.57; six months control= 2.12 ± 0.18; sucrose treated= 0.33 ± 0.08).

3.7 Insulin, Pdx1 and Nkx6.1 immunodetection

The initial control group kidney tissueshowed a positive immunoreactivity for insulin (Fig. 7), Pdx-1 (Fig. 8) and Nkx6.1 (Fig. 9) in the cortex and medulla zones in proximal, distal and Henle's loop tubular cells, respectively at the apical side zone. Noimmunolabelling in the glomeruli cells was observed. The six months control group also showed immunoreactivity for insulin and for the two transcription factors in the tubular cells but not in the glomeruli cells. Compared to the initial control, the six months group showed a lower immunoreactivity, in both medulla and cortex, for insulin, Pdx-1 and Nkx6.1, at the apical side of the cells (Fig.7,8&9). The sucrose treated group showed light immunolabelingfor insulin in medulla and cortex tubular cellscompared to the initial control group as well asscatteredimmunolabels located out of the apical side in this zone (Fig. 7). Pdx-1 and Nkx6.1 showed very light immunolabels at cortex and medulla zones (Fig. 8,9). It is important to remark that both cortex and medulla tissue for sucrose treated animals showed morphological alterations such as cellular disorganization, loss of cell borders, tubular lumen obstruction by hyaline matter, heterogeneous chromatin nuclei, cellular lysis, hypertrophic cells and hemorrhage zones (Fig. 7-9). The cortex zone presented the most evident damage. Glomeruli of sucrose treated animals presentedan increase in the thickness of both basement membrane and Bowman's capsule (Fig. 10)

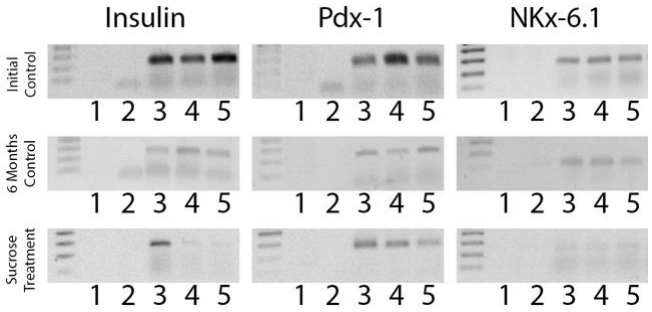


Figure 5. Insulin, Pdx-1 and Nkx6.1 RT-PCR products. Samples from three animals (lanes 3,4,5) representing insulin, Pdx-1 and Nkx6.1 expression are shown. Lane 1 is the negative control (free nucleases water) and lane 2 is a total RNA control.

Table 3.Sequence results of PCR products for kidney genes of insulin, Pdx-1 and Nkx6.1

% IDENTITY	GENEBANK ACCESSION NUMBER	BLAST RESULT
100 **195/195**	NM_019129.3	*Rattusnorvegicus*Insulin 1 mRNA.
97 **119/123**	NM_022852.3	*Rattusnorvegicus* Pancreatic and duodenal homeobox 1 (Pdx1), mRNA
98 **178/181**	NM_031737.1	*Rattusnorvegicus* NKx6.1 homeobox 1 (Nkx6-1), mRNA

4. DISCUSSION

It has been shown that insulin stimulates kidney sodium reabsorption at different tubular segments through the activation of its receptors which are located at the basolateral side along the entire length of the renal tubules, with the greatest densityfound in the thick ascending limb of Henle's loop and in the distal convoluted tubule[23].

Recently, we showed the gene expression of insulin in rat kidneysand its renal tissulardetection with an immunoperoxidase assay at the apical side of the different tubular segments which are involved in sodium reabsorption[20]. We suggested a local insulin genic expression by the kidney, specifically by tubular (proximal, distal and Henle's loop) cells.

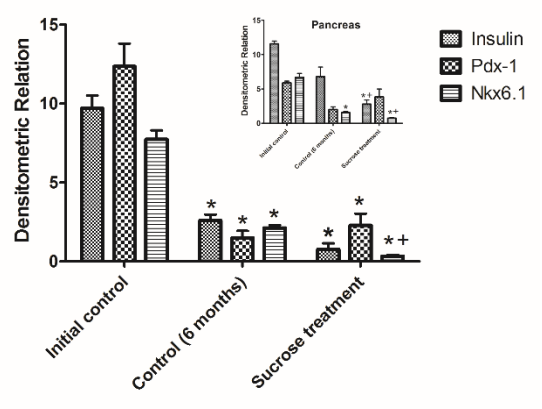


Figure 6. Relative insulin, Pdx-1 and Nkx6.1 mRNA levels in kidney and pancreas. Each bar represents the mean ± S.E.M. n=5. *p < 0.05 respect to initial control. +p< 0.05, respect to control (six months).

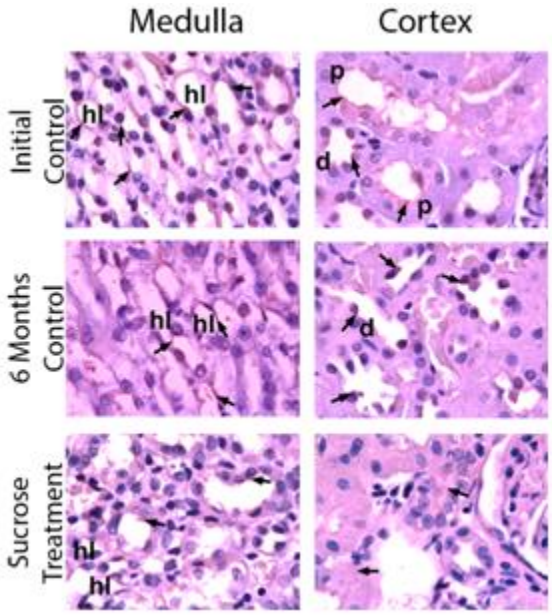


Figure 7. Immunoperoxidase labeling of rat renal medulla and cortex (X 1000 magnification). Staining (arrows) for Insulin. (p) proximal, (d) distal tubules and (hl) Henle' s loop is shown.

This could also play a local role in regulating ion-water homeostasis. In support of these findings, kidneys also expressed the transcription factors necessary forthe development and function of β-cells: Pdx-1 and Nkx6.1 which were also detected through animmunoperoxidase assay at the apical side of the different tubule segments.

In this work we investigated the expression of insulin and its transcription factors, Pdx-1 and Nkx6.1, in a model of IGTas a risk factor for DM 2 and often associated with MetS. Sucrose treated animals presented IGT, hyperinsulinemia, hypertriglyceridemia and low levels of HDL. These last two lipid abnormalities have been pointed out as risk factors for renal disease and have been associated with renal tissue injury[24, 25].The gene expression of insulin, Pdx-1 and Nkx6.1 in these animals was significantly lower than in the initial control group. A similar behavior pattern of these genic expressions was observed in the pancreatic tissue (Fig 6). Immunoperoxidase assays in kidney tissue showed a very lightimmunoreactivity for insulin and its relatedtranscription factors. In addition, we observed tubular tissue damage (Fig.7,8,9).This may be related with a kidney function impairment which progresses to chronic kidney disease. A decline in insulin expression may affect its action in kidney tissue, the stimulation of sodium transport mechanisms and nitric oxide (NO) production.

An increase of expression and activity of Na^+-H^+ exchanger type 3 was demonstrated by insulin incubation of proximal rat tubules, and in a cell line from opossum kidney [12, 26]. It has been shown that Na^+-K^+-ATPase was activated by insulin in isolated proximal tubules [11].

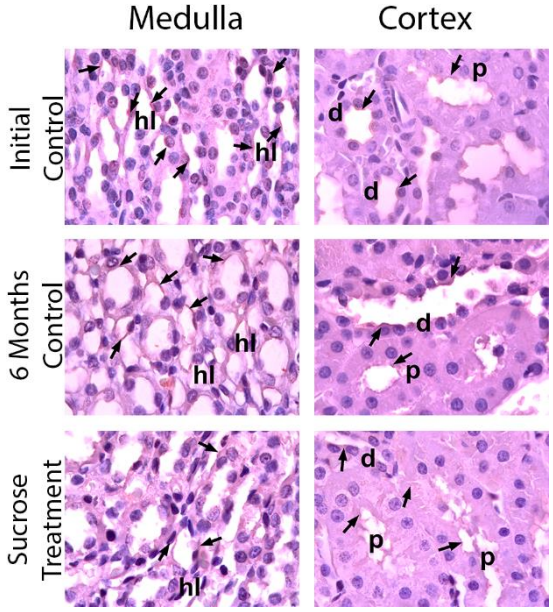


Figure 8. Immunoperoxidase labeling of rat renal medulla and cortex (X 1000 magnification). Staining (arrows) for Pdx-1. (p) proximal, (d) distal tubules and (hl) Henle' s loop is shown.

It has been demonstrated in isolated thick ascending limbs of Henle's loop microperfused with insulin an increased activity of both Na^+-K^+-$2Cl^-$cotransporter (NKCC2) and Na^+-K^+-ATPase [10, 27]. Song et al. [10] showed that chronic infusion of insulin to distal collecting tubules of Sprague–Dawley rats increased Na-Cl (NCC) cotransporter activity without change in the protein expression of this. At the collecting tubule, insulin has been shown to increase the activity of Na^+-K^+-ATPase [28]and the epithelial sodium channel (ENaC) in isolated tubules and A6 cell lines in which the activity is dependent of PI3K[16, 29, 30, 31].

It has been reported that in a hyperinsulinemia state (resultant of insulin resistance) there is an alteration of the main renal sodium transporters in the kidney and this may affect sodium balance and blood pressure[32]. In obese hyperinsulinemicZucker rats (with impaired glucose tolerance) protein abundances for both insulin receptor subunits, IR-α and IR-β, in all three regions of the kidney are significantly reduced with the inner medulla seeming to be the most sensitive[33]. This reduction of receptor protein may

lead to decreased IR signaling for insulin-related cellular events for the mechanisms involved in renal sodium reabsorption. In renal tubules of obese Zucker rats with hyperinsulinemia (6 to 13 fold higher insulin circulating levels), the abundances of α subunit of Na^+-K^+-ATPase, NCC cotransporter and the β subunit of ENaC were significantly increased in spite of an abnormal tissue response to insulin [34].

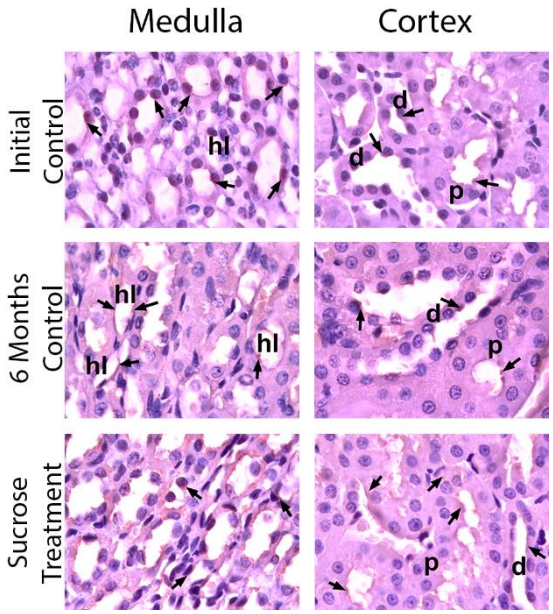


Figure 9. Immunoperoxidase labeling of rat renal medulla and cortex (X 1000 magnification). Staining (arrows) for Nkx6.1. (p) proximal, (d) distal tubules and (hl) Henle' s loop is shown.

Our results showed a significant decrease in expression and immunodetection (at the different zones of renal tubules) of insulin, Pdx-1 and Nkx6.1 in sucrose treated animals which maycauses a decreased interaction of the hormone with its receptor. These results may be related with increased activity of sodium transporters reported in hyperinsulinemia. We suggest that insulin expression may participates in the stimulation of sodium reabsorption mechanisms in the renal tubular cells. The decline of insulin expression at this level, in addition to reduced insulin receptor subunits in hyperinsulinemia might have a compensatory upregulation in reducing volume depletion and sodium loses, as proposed by Tiwari et al.,[33]in STZ treated rats.

Insulin has important vascular actions that involve the stimulation of the production ofNO from endothelial nitric oxide synthase (eNOS) by the PI 3-kinase pathway, leading to vasodilation, increased blood flow, and the augmentation of glucose disposal in skeletal muscle [35]. Insulin plays a homeostatic role in normal kidney functioning and has direct dose-dependent dilator effects on the renal microvasculature [36]. NO is one of the important modulators of renal blood flow, renovascular resistance and glomerular filtration rates[15]. Reduction of epithelial NO synthesis by the inhibition of NO synthase induces the alteration of these parameters and renal morphological changes [37]. These alterations may be determinant of renal injury development and cardiovascular diseases[38]. In insulin-resistant, hyperinsulinemic states, as found in obese Zucker rats, a greatly diminished endothelial NO production and a reduction in insulin dependent vasodilation of the renal microvessels in renal cortex and medulla were found which favor renal vasoconstriction[39]. This condition may lead to renal injury. In addition, renal morphological changes have been observed. Kim et al., [40] reported that a chronic NO blockade induces a significant increase in the expression of α_1 subunit of Na^+-K^+-ATPase and also in Na^+-H^+ exchanger and Na+-K^+-$2Cl^-$ and Na^+-Cl^-cotransporters (which are blocked by NO) and hypertension in male Sprague-Dawly rats. In addition to microvascular alterations, these conditions may contribute to renal damage.

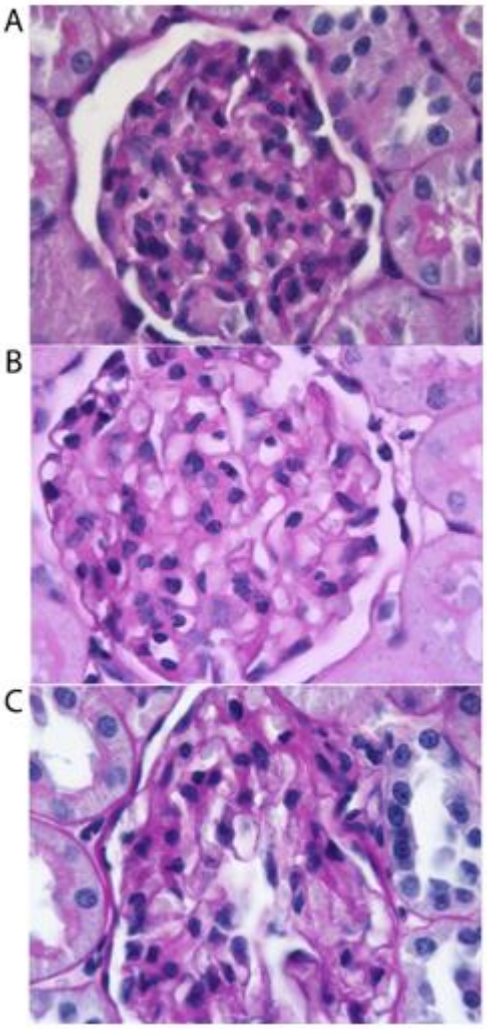


Figure 10. Renal tissue glomerules of A) initial control group, B) control six months group, C) sucrose treated group. Staining with PAS tinction (X 1000 magnification) is shown.

Thus, it is possible that in our model, the decline in kidney tubules insulinexpression could affect NO production and consequently impair vasodilation, leading to renal hypertension with the accompanying observed renal tissular damage.

We found a significant decrease in the expression of insulin and related factors in kidneys associated to age. An

additional decline due to sucrose treatment was observed for insulin, Pdx-1 and Nkx6.1. Genic expression factors in kidneys behaved the same way as in the pancreas. There is no previous report on the expression of insulin or its related factors in kidneys; however, there are some data in relation to what occurs in the pancreas. It is known that during normal aging, the pancreatic islet undergoes morphological and functional decline [41]. In a study of beta cell function-related genes performed by Ihm et al. [42], a decreased expression of insulin and Pdx-1 was found in older rats compared to younger ones. These authors proposed that the age-dependent decline in glucose-stimulated insulin release is mostly due to an impaired beta cell function rather than to a decrease in beta cell mass associated with aging, and that the reduced expression of Pdx-1 may be one of the causes for insulin secretory defectsassociated with aging. Since Pdx-1 expression was reduced in the six months control group, it is possible that kidney insulin-producing cells are affected by a similar mechanism. A minor reduction in Pdx-1, although not causing diabetes, may increase the susceptibility to diabetes when coupled with insulin resistance or other genetic and/or environmental factors that impair glucose tolerance [42]. Because Pdx-1 is a redox-sensitive transcription factor, it is believed that aging related increases in oxidative stress in beta cells may cause decreased Pdx-1 expression in islets [43] and it could be possible that insulin-producing cells in kidneys are affected in a similar fashion.

Regarding Nkx6.1 expression, although it decreased in the six months control group there was no significant difference between this group and the sucrose treated rats in both the pancreas and kidneys. In atype 2 DM it was found that NKX6.1 expression in islets was more severely affected than other transcription factors, Pdx-1 among them [44]. The reasonfor this Nkx6.1 particular susceptibility is still unclear.
The six months control also exhibited an altered response in the glucose tolerance test. It has been reported that glucose-stimulated insulin secretion per given unit of β-cell mass decreases in a linear fashion with age at a rate of ~ 0.7% per year [45]. This decline is linked to a progressive decrease in glucose tolerance [42]and a defect of insulin secretion that results in a high incidence of type 2 DM associated with aging [46]. In humans, the decline in glucose tolerance is known to begin in the third or fourth decade of life and is progressive throughout the life span [47]. As expected, sucrose treated rats displayed even further altered glucose tolerance tests, showing diabetic features.

In regard to serum insulin levels, it has been reported that serum insulin levels increase with age[48]. We only observed a slight trend toward increased insulin levels in the six months control group but it was not statistically significant. There is an important difference in terms of age between the rats in our study and those in the study of the above mentioned authors; our rats after six months of treatment were approximately 8 months old, compared to the other study's 24 month old rats.

As we proposed insulin expressed in kidney tubules may participate in the stimulation of renal sodium absorption, a decline in its production could induce similar effects as thoseobserved in sucrose treated animals, with IGT and hyperinsulinemia where there is a decrease of insulin and its receptor interaction. In addition, *in vitro* studies of a model of proximal tubular cellular aging demonstrated an increase in transepithelial Na^+ transport [49]. They found an increase in the number of Na^+/K^+- ATPase α_1- and β_1 subunits, in the membrane. This may be caused by a reduction in the abundance of eNOSin aged subjects consequently impairing NO production and leading to endothelial dysfunction[50].
NO has been shown to inhibit both Na^+/K^+- ATPase and Na/H^+ exchange activities in proximal tubule cells[51, 52]. As we mentioned, a decrement in NO production along with a decrease in insulin levelsmay increase the activity of other sodium reabsorption mechanisms such as Na^+-K^+-$2Cl^-$ cotransporter[40].

In summary, we found that in sucrose treated animals with impaired glucose tolerance there was a significant decrease in insulin expression and its immunodetection in kidney tissue which also showed damage. These results may be relatedwith the effects observed in conditions with a reduced insulin action at the renal level, such as hyperinsulinemia or DM 1. We suggest thatinsulin reduced expression in the kidneys may contribute to the tissue damage reported in IGT and in our model. For the six months control group a similar conclusion may be suggested, however this group presented a smaller decrease in the gene expression of insulin and its transcription factors, as with itstissularimmuno detection. Further more hyper insulinemia and hyper trygliciridemia were not detected as in the case of the sucrose treated group, which may contribute to an increased risk of kidney damage.

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REFERENCES

[1] Tabák AG, Herder C,Rathmann W, Brunner EJ,Kivimäki M.Prediabetes: a high-risk state for diabetes development. Lancet, 2012; 379: 2279–90.

[2] Singleton JR, Smith AG, Russell JW, Feldman EL.Microvascular Complications of Impaired Glucose Tolerance. Diabetes, 2012; 52: 2867-73

[3] Harris MI, Klein R, Welborn TA, Knuiman MW. Onset of NIDDM occurs at least 4–7 years before clinical diagnosis. Diabetes Care, 1992;15: 815–19.

[4] Mykkanen L, Haffner SM, Kuusisto J, Pyorala K, Laakso M. Microalbuminuria precedes the development of NIDDM. Diabetes, 1994;43:552–7.

[5] Faerch K, Vaag A., Holst JJ, Hansen T, Jørgensen T, Borch-Johnsen K. Natural history of insulin sensitivity and insulin secretion in the progression from normal glucose tolerance to impaired fasting glycemia and impaired glucose tolerance: the inter99 study.Diabetes Care, 2009; 32:439-44.

[6] Pratley RE, Weyer C. The role of impaired early insulin secretion in the pathogenesis of type II diabetes mellitus. Diabetologia, 2001;44: 929–45.

[7] Ferrannini E, Gastaldelli A, Miyazaki Y, Matsuda M, Pettiti M,Natali A, Mari A, De Fronzo RA. Predominant role of reduced beta-cell sensitivity to glucose over insulin resistance in impaired glucose tolerance. Diabetologia,2003; 46: 1211–19.

[8] Hanefeld M, Koehler C, Fuecker K, Henkel E, Schaper F, Temelkova-Kurktschiev T. Insulin secretion and insulin sensitivity pattern is different in isolated impaired glucose tolerance and impaired fasting glucose. Diabetes Care, 2003; 26:868-74.

[9] Abdul-Ghani MA, Tripathy D, DeFronzo RA. Contributions of β-Cell Dysfunction and Insulin Resistance to the Pathogenesis of Impaired Glucose Tolerance and Impaired Fasting Glucose.Diabetes Care, 2006;29: 1130–39.

[10] Song J, Hu X, Riazi S, Tiwari S, Wade JB, Ecelbarguer CA. Regulation of blood pressure, the epithelial sodium channel (ENaC), and other key renal sodium transporters by chronic insulin infusion in rats. Am JPhysiol Renal Physiol, 2006; 290: F1055-64.

[11] Féraille E, Carranza ML, Rousselot M, Favre H. Insulinenhancessodiumsensitivity of Na-K-ATPase in isolated rat proximal convoluted tubule. Am J Physiol, 1994; 267: F55–62.

[12] Gesek F, Schoolwerth A. Insulin increases Na(+)-H+ exchange activity in proximal tubules from normotensive and hypertensive rats. Am J Physiol, 1991; 260: F695–703.

[13] Ito O, Kondo Y, Takahashi N, Kudo K, Igarashi Y, Omata K, Imai Y, Abe K. InsulinstimulatesNaCltransport in isolatedperfused MTAL of Henle's loop of rabbit kidney. Am J Physiol, 1994; 267: F265–70

[14] Blazer-Yost BL, Liu X, Helman SI. Hormonal regulation of ENaCs: insulin and aldosterone. Am J PhysiolCellPhysiol, 1998; 274: C1373–79.

[15] Raij L.Nitric oxide and thekidney.Circulation, 1993; 87Suppl V: V26-29.

[16] Blazer-Yost BL, Esterman MA, Vlahos CJ. Insulin-stimulated trafficking of ENaC in renal cells requires PI 3-kinase activity. Am J PhysiolCellPhysiol, 2003; 284: C1645–53.

[17] Fuster DG, Bobulescu IA, Zhang J, Wade J, Moe OW. Characterization of theregulation of renal Na+/H+ exchanger NHE3 by insulin. Am J Physiol Renal Physiol, 1997; 292: F577-F585.

[18] Ito O, Kondo Y, Oba M, Takahashi N, Omata K, Abe K. Tyrosinekinase, phosphatidylinositol 3-kinase, and proteinkinase C regulateinsulin-stimulatedNaClabsorption in thethickascendinglimb. KidneyInt, 1997; 51: 1037-41

[19] Zeng G,Nystrom FH, Ravichandran LV, Cong LN, Kirby M, Mostowsky H, Quon MJ. Roles forinsulin receptor, PI3-kinase and Akt in insulin signaling pathways related to production of nitric oxide in human vascular endothelial cells. Circulation, 2000; 101: 1539-45.

[20] Soto C, Juárez J, Pérez J, González I, Esquivel A, Uria E, Pérez S,Raya L. Insulin gene expression and its detection in the rat kidney. Int. Jour. Sci. Res. Tech, 2014; 3: 194-200.

[21] Goren H, Kulkarni R, Kahn C. Glucose homeostasis and tissue transcript content of insulin signaling intermediates in four inbred strains of mice: C57BL/6, C57BLKS/6, DBA/2, and 129X1. Endocrinology, 2004; 145: 3307-23.

[22] Baner C. Chemical Laboratory Methods. New York 7 Academic Press, 1985.

[23] Nakamura R, Emmanouel DS, Katz AI. Insulin binding sites in various segments of the rabbit nephron. J ClinInvest, 1983; 72: 388–92.

[24] Vaziri ND. Dyslipidemia of chronic renal failure: the nature, mechanisms, and potential consequences. Am J Physiol Renal Physio, 2006; 290: F262-72.

[25] Locatelli F., Pozzoni P., Del Vecchio L. 2006. Renal manifestations in the metabolic syndrome. J Am SocNephrol. 17: S81–S85.

[26] Klisic J, Hu MC, Nief V, Reyes L, Fuster D, Moe OW, Ambul PM. Insulin activates Na+/H+ exchanger 3: biphasic response and glucocorticoid dependence. Am J Physiol. Renal Physiol,2002; 283: F532-9.

[27] Takahashi N, Ito O, Abe K. Tubular effects of insulin. Hypertens Res,1996; 19: S41-5

[28] Féraille E., Rousselot M., Rajerison R., Favre H. Effect of insulin on Na+,K+-ATPase in rat collecting duct. JPhysiol, 1995; 488: 171-80.

[29] Blazer-Yost BL, Esterman MA, Vlahos CJ. Insulin-stimulated trafficking of ENaC in renal cells requires PI 3-kinase activity Am J PhysiolCellPhysiol, 2003; 284: C1645-53.

[30] Pearce D. The role of SGK1 in hormone-regulated sodium transport. TrendsEndocrinolMetab, 2001; 12: 341-7.

[31] Zhang YH, de la Rosa DA, Canessa CM, Hayslett JP. Insulin-induced phosphorylation of ENaC correlates with increased sodium channel function in A6 cells. Am J Physiol Cell Physiol, 2005; 288: C141-7.

[32] Tiwari S, Halagappa VK, Riazi S, Hu X, Ecelbarguer CA. Reduced expression of insulin receptors in the kidneys on insulin-resistant rats. J AmSocNephrol, 2007; 18: 2661-71

[33] Tiwari S, Riazi S, Ecelbarguer CA. Insulin's impact on renal sodium transport and blood pressure in health, obesity, and diabetes. Am JPhysiol Renal Physiol, 2007; 293: F974-84

[34] Bickel CA, Knepper MA, VerbalisJG, Ecelbarger CA. Dysregulation of renal salt and water transport proteins in diabetic Zucker rats. Kidney Int, 2002; 61: 2099-110.

[35] Montagnani M, Ravichandran LV, Chen H, Esposito DL, Quon MJ. Insulin receptor substrate-1 and phosphoinositide-dependent kinase-1 are required for insulin-stimulated production of nitric oxide in endothelial cells. MolEndocrinol,2002; 16: 1931-42.

[36] Knight SF, Imig JD.Obesity, insulin resistance, and renal function. Microcirculation, 2007; 14: 349-62.

[37] Mimram J. Nitric oxide inhibition and renal alterations. J. Cardiovasc. Pharmacol,2001;38 (Suppl 2): S65-S70.

[38] Fujiwara H, Narimatsu Y, Hashimoto S, Hiramatsu K.Angiography of occlusive vascular disease.NihonRinsho, 1999; 57:1526-30.

[39] Hayashi K, Kanda T, Homma K, Tokuyama H, Okubo K, Takamatsu I, Tatematsu S, Kumagai H, Saruta T. Altered renal microvascular response in Zucker obese rats. MetabClinExp, 2002; 51: 1553-61.

[40] Kim JS, Choi KC, Jeong MH, Kim SW, Oh YW, Lee JU. Increased expression of sodium transporters in rats chronically inhibited of nitric oxide synthesis. J Korean Med Sci,2006; 21: 1-4.

[41] Zhu M, Hu J, Perez E, Phillips D, Kim W, Ghaedian R, Napora JK, Zou S. Effects of long-term cranberry supplementation on endocrine pancreas in aging rats. J. Gerontol. A BiolSci MedSci, 2011; 66: 1139–51.

[42] Ihm SH, Moon HJ, Kang JG, Park CY, Oh KW, Jeong IK Oh YS, and Park SW. Effect of aging on insulin secretory function and expression of beta cell function-related genes of islets. Diabetes Res ClinPract, 2007;77 (Suppl 1): S150–4.

[43] Kaneto H, Matsuoka TA, Nakatani Y, Kawamori D, Miyatsuka T, Matsuhisa M., Yamasaki, Y. Oxidative stress, ER stress, and the JNK pathway in type 2 diabetes. J MolMed,2005; 83: 429–39.

[44] Kjørholt C, Akerfeldt MC, BidenTJ, Laybutt DR. Chronic hyperglycemia, independent of plasma lipid levels, is sufficient for the loss of beta-cell differentiation and secretory function in the db/db mouse model of diabetes. Diabetes, 2005; 54: 2755–63.

[45] Szoke E, Shrayyef MZ, Messing S, Woerle HJ, Van Haeften TW, Meyer C, Mitrakou A, Pimenta W, Gerich, JE. Effect of agingon glucose homeostasis: accelerated deterioration of beta-cell function in individuals with impaired glucose tolerance. Diabetes Care,2008; 31: 539–43.

[46] Chang AM and Halter JB. Ageing and insulinsecretion. Am J PhysiolEndocrinolMetab, 2003; 284: E7–12.

[47] DeFronzo RA. Glucose intolerance and aging. Diabetes Care, 1981; 4: 493–501.

[48] Kim DH, KimJM, Lee EK, Choi YJ, Kim CH, Choi JS, Deuk NK, Yu BP, Chung HJ. Modulation of Foxo1 phosphorylation/acetylationbybaicalinduringaging. J. NutrBiochem, 2012; 23: 1277–84.

[49] Silva E, Gomes P, Soares-da-Silva P. Overexpression of Na+/K+-ATPaseparallels the increase in sodium transport and potassium recycling in an in vitro model of proximal tubular celular ageing. JMembraneBiol,2006; 212: 163-75.

[50] Baylis C.Sexualdimorphism in theaging kidney: differences en the nitric oxide system. NatRevNephrol, 2009; 5:384-96.

[51] Roczniakand A, Burns KD. Nitric oxide stimulatesguanylatecyclase and regulatessodium transport in rabbit proximal tubule. Am JPhysiol, 1996; 270: F106-15.

[52] McKee M, Scavone C, Nathanson JA. Nitric oxide, cGMP, and hormone regulation of active sodium transport. ProcNatlAcadSci,1994; 91: 12056-60.