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Ghrelin and Jacalin Localization in Developing Rat Kidney

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ABSTRACT

Introduction: Lectins are hormone consists of carbohydrate binding proteins that perceive explicit epitopes present on certain glycoproteins and play roles in metabolism.

Materials and Methods: Here we examined the localization of two different lectins, ghrelin and jacalin, in rat kidney of both sexes at neonatal 20 days and postnatal 1, 2, 4, 6, 8 and 12 weeks by immunohistochemistry.

Results: Ghrelin and jacalin were found in the intrarenal kidney from neonatal day 20. Ghrelin immunoreactivity was mild in the proximal convoluted tubule (PCT), slightly stronger in the distal convoluted tubule (DCT), and absent in the renal corpuscle (RC). In contrast, jacalin immunoreactivity was intense in the RC but absent in the PCT and DCT. At postnatal one week, the ghrelin intensity increased and started to appear in the glomerular capillary of the RC and collecting duct (CD), while the jacalin immunoreactivity was intense in the RC, collecting tubules (CT), and loop of Henle. At postnatal two weeks, ghrelin immunoreactivity was intense in the DCT, CT, and loop of Henle, while jacalin immunoreactivity was intense in the RC, CD, and CT. It was also high in the DCT but mild in the PCT. From four weeks until 12 weeks, the localization of the two lectins was the same, and the immunoreactivity increased with age.

Conlusions: Our research elucidates the neonatal and postnatal intra-renal localization of ghrelin and jacalin.

Keywords: Immunohistochemistry; Ghrelin; Jacalin; Kidney; Rat.

Introduction

Ghrelin is a lectin hormone which was found firstly in stomach of rat and human (Kojima *et al*., 1999) as an endogenous ligand for an orphan receptor, growth hormone secretagogue receptor, from the pituitary gland (Howard *et al*.,1996 & McKee, 1997). Gherlin consists of 28-amino acid peptide has critical roles in growth hormone (GH) which discharge from the pituitary gland and in stimulation of appetite in rats and humans (Kojima *et al*.,1999, Takaya *et al*., 2000; Kojima *et al*., 2001; Nakazato *et al*., 2001 and Kojima and Kangawa, 2002).

The declaration of the ghrelin hormone has been found in the stomach as well as different organs (Gnanapavan *et al*., 2002). In pancreas of human and rat, ghrelin cells were distinguished as novel endocrine cells in the islets of pancreas (Wierup *et al*., 2002, 2004; Wierup and Sundler, 2005) and glucagon-secreting A cells (Date *et al*., 2002; Kageyama *et al*., 2005). These ghrelin cells in pancreas assume significant roles in the control of secretions of insulin and glucagon from pancreatic islets (Salehi *et al*., 2004; Iwakura *et al*., 2005; Qader *et al*., 2005). In human salivary organs, immunoreactions of ghrelin were seen in the parotid sublingual and submandibular organs, and the expansion of oral keratinocytes was exhibited as an important role of salivary ghrelin (Groschl *et al*., 2005).

In the kidney, Mori *et al*. (2000) exhibited the expression of mRNA of prepro-ghrelin in kidneys of the mouse and proposed paracrine or endocrine roles for ghrelin in kidneys. They also showed that ghrelin is also involved in normal kidney development and also in the renal hypertrophy detected in nephrectomy and diabetes. Aydin *et al*. (2008) revealed that the ghrelin urine level was higher than the blood level, proposing that the kidney might secrete more ghrelin than the stomach. Finally, the ghrelin expression has been distinguished in the distal convoluted tubules (DCT) and collecting tubules (CT) (Gnanapavan *et al*.,2002 Yabuki *et al*., 2006; Venables *et al*., 2011 and Yildirim *et al*., 2016).

Jacalin is a lectin from the seeds of jackfruit Artocarpus integrifolia with unknown function and present on the luminal border of the DCT as well as cells of the CT in developing human kidney (Engel *et al*., 1997). Yabuki *et al*. (2002) studied 21 types of lectins in 3-4-month-old female DBA/2Cr mice and found that jacalin was located on the basement membrane of the proximal CT (PCT), proximal straight tubules (PST), DCT, loop of Henle, thick ascending limb, and the collecting ducts (CD).

However, the above studies are based on adult rodent kidney, and very little is known about ghrelin or jacalin in postnatal kidney development. Thus, the aim of the present study was to examine the functional role

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of ghrelin and jacalin by observing their distribution in rat kidney at several neonatal and postnatal developmental stages by immunohistochemistry (IHC).

Materials and methods

Animals and tissue preparation

All experiments were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and the protocols accepted by the Ethics Animal Care Committee of Damanhour University, Egypt (Approval No. 24102020).

28 male Sprague-Dawley rats (250–300 gm) of different ages (20 days prenatal, postnatal one week, two weeks, three weeks, four weeks, six weeks, and three months; four rats for each age) were used in this study. Following ether inhalation, the animals were euthanized by cervical dislocation. Then the kidneys were immediately isolated and preserved in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) overnight at 4°C for IHC.

Antibodies

The following antibodies were used: normal rabbit IgG (1:500; Santa Cruz, sc-2027) as a negative control, polyclonal goat anti-rat ghrelin antibody (1:250; Abcam, catalog No. ab104307, Cambridge, UK), and jacalin, which specifically binds to GalNAc-O- (1:150; Vector Laboratories, Cat: B-1155-5, Inc., USA).

The fixed specimens were processed by the conventional paraffin embedding technique including dehydration through ascending grades of ethanol, clearing with three changes of xylene, and embedding in paraffin wax at 65°C. Four-µm thick sections were stained by Hematoxylin and Eosin (H&E) and Periodic acid Schiff (PAS) as previously described by Bancroft and Layton (2013).

Immunohistochemistry

The serial paraffin sections were cut and deparaffinized by xylene and rehydrated in graded alcohols for the immunostaining of ghrelin. For the antigen retrieval of ghrelin, the sections were heated in 10 mM citrate buffer (pH 6.0) for 20 min at 105°C. Briefly, the sections were covered with 0.5% TritonX-100 (Nacalai, Kyoto, Japan) in PB saline (PBS) for 20 min. Endogenous peroxidase signals were blocked with 3% hydrogen peroxide in methanol for 5 min at room temperature. Non-specific background staining was blocked with 5% bovine serum albumin (BSA: Sigma-Aldrich, Cat: A9647) diluted in 0.1 M PBS (pH 7.2) for 1 h and then incubated with anti-ghrelin antibodies overnight at 4°C. For the negative control sections, PBS was substituted for the primary antibody-containing solution. Next, the sections were rinsed in PBS and incubated with biotinylated goat anti-rabbit IgG antiserum (Histofine kit, Cat: 424032, Nichirei, Tokyo, Japan) with anti-ghrelin for 30 min at room temperature. After rinsing with PBS, the sections were incubated with streptavidin-peroxidase conjugate for 30 min at room temperature. Antibody binding with the streptavidin-biotin complex was detected by peroxidase/3,3-diaminobenzidine (DAB) (peroxidase/DAB ChemMate Detection Kit; Dako, Cat: K5007, CA, USA). Lastly, the nuclei were counterstained lightly with Mayer's hematoxylin.

For the jacalin staining, antigen retrieval was performed using 0.05% trypsin at 37°C for 2 min, followed by another washing with distilled water. Deactivation of endogenous peroxidase was conducted using 0.3% H_2O_2 / methanol for 20 min. After washing with PBS, the nonspecific reaction was blocked with 1% BSA (Avidin-biotin Blocking Kit, Vector Laboratories, Inc., USA)/PBS for 60 min at room temperature. The biotinylated jacalin was incubated at 4°C overnight. For the negative controls, the biotinylated jacalin was soaked in a solution containing 800 mM galactose (Vector Laboratories, Cat: S-9003, Inc., USA). Binding was done by leaving these mixtures at room temperature for 30 to 60 min. After that, the mixture was substituted in place of the unabsorbed jacalin and incubated for 60 min. Using peroxidase and the DAB system without jacalin, a second negative control experiment was conducted to measure the endogenous peroxidase activity in the tissue. After washing with PBS, the VECTASTAIN® Elite ABC-HRP Kit (Vector Laboratories, Cat: PK-6100, Inc., USA) was applied to the negative controls at room temperature for 30 min. DAB solution was applied to the samples after washing with PBS for 4 min. The reaction was stopped by applying distilled water. Mayer's hematoxylin was used as a counterstain.

Micrographs of the sections were taken with a digital camera (Leica EC3, Leica, Germany) connected to a microscope (Leica DM500).

Results

Rat kidney development was incomplete at birth and continued in the early postnatal life to reach maturity. We divided the development into the several stages described below.

Neonatal stage (20 days)

Ghrelin immunoreactivity appeared mild in the PCT, moderate in the DCT, and nowhere in the renal corpuscle (RC) (Fig. 1A). Jacalin immunoreactivity was intense in the RC and moderate in the renal capsule (Fig. 1B). It also appeared in the glomerular capillary of the RC and apical surface of the DCT (Fig. 1C). The medulla showed mild jacalin immunoreactivity in undifferentiated mesenchymal tissues but intense reactivity in the apical cytoplasmic surface of the early formed CT and loop of Henle (Fig. 1D).

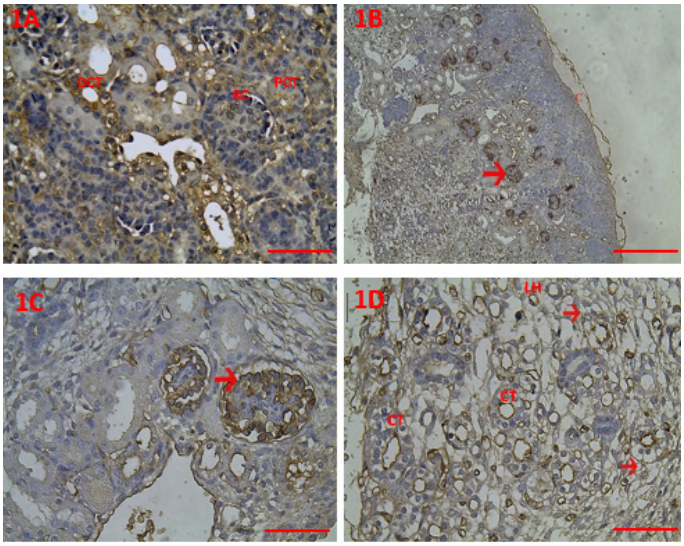

Figure 1. Photomicrographs showing the kidney from 20-day-old neonate. 1A: Ghrelin immunoreactivity was mild in the PCT, moderate in the DCT, and absent in the RC. Magnification, X40. 1B: Jacalin immunoreactivity was intense in the RC (arrow) and moderate in the renal capsule. Magnification, X10. 1C: Jacalin immunoreactivity was intense in the glomerular capillaries only. Magnification, X40. 1D: Jacalin immunoreactivity was mild in undifferentiated mesenchymal tissues (arrows) and intense in early CT and loop of Henle (LH). Magnification, X40. Scale bars=400 µm (A) and 40 µm (B–F).

Post-natal Stage
(1 week)

In rat, nephrogenesis occurs at a rapid rate between birth and 8 days and is completed by 11 days of age. At this age, the development continues with immature nephrons located in the subcapular kidney and with mature nephrons located deeply near the medulla kidney. In 1-week-old mice, undifferentiated mesenchymal tissues appeared prominent in the medulla and showed no PAS staining (Fig. 2A).

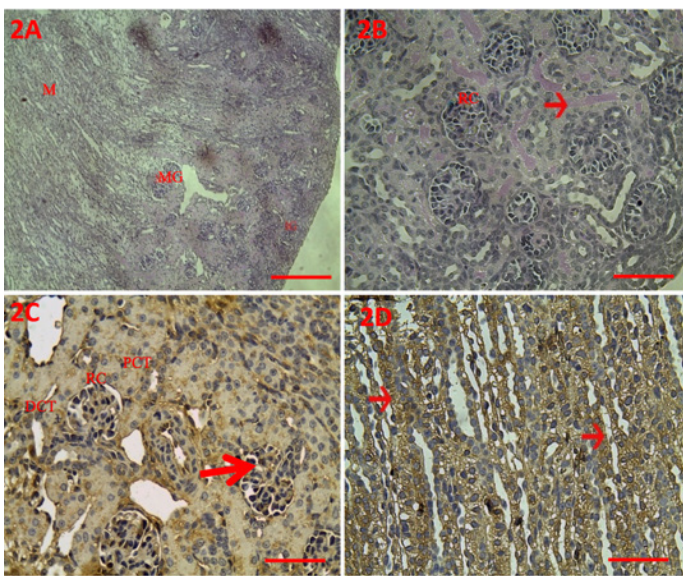

Figure 2. Photomicrographs showing the kidney from one-week-old rat. 2A: The subcapusular cortex with immature glomeruli (IG), mature glomeruli (MG) located near the medulla, and medulla (M) with undifferentiated mesenchymal tissues. PAS. Magnification, X10. 2B: The renal cortex with the RC glomerular basement membrane, the PCT with a well-developed brush border (arrow), and DCT with a undeveloped brush border. PAS. Magnification, X40. 2C: Ghrelin immunoreactivity was mild in the PCT, DCT, and glomerular capillary (arrow) of the RC. Magnification, X40. 2D: Ghrelin immunoreactivity was moderate in the CD of the medulla (arrows). Scale bars=400 µm (A) and 40 µm (B–F).

Additionally, a mature RC appeared with a thick basement membrane, and the PCT was lined by a high cuboidal epithelium with narrow lumen and had a well-developed brush border that showed strong PAS staining. The DCT were lined with a low cuboidal epithelium and wide lumen and showed PAS staining apically on the surface of the lining epithelium (Fig. 2B). Moderate ghrelin immunoreactivity was observed in the PCT, DCT, and glomerular capillary of the RC (Fig. 2C). Moderate ghrelin immunoreactivity was also observed in the CD (Fig. 2D). Jacalin immunoreactivity was strong in the glomerular capillaries of the RC, CT, and loop of Henle, but no reactivity was observed in the PCT or DCT (Fig. 3).

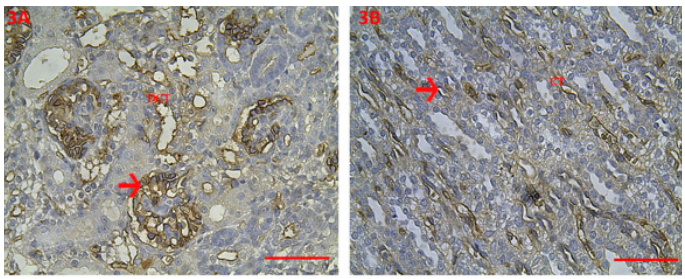

Figure 3. Photomicrographs showing jacalin immunoreactivity of the kidney from one-week-old rat. 3A: The immunoreactivity was intense in the glomerular capillary (arrow) and apical surface of the DCT. Magnification, X40. 3B: It was also intense in the collecting tubules (CT) and loop of Henle (arrow). Magnification, X40. Scale bars=40 µm.

Mature Stage
(2 weeks)

In 2-weeks-old rat, the kidney showed clear signs of maturity. The RC was formed from Bowman's capsule of the parietal and visceral layers, which were separated from each other by typical Bowman's space surrounding the glomeruli and well–developed basement membrane. Proximal tubule differentiation was evident by the characteristic prominent brush border, and the DCT was lined by a low cuboidal epithelium with wide lumen. PAS staining indicating the brush border had not fully formed (Fig. 4A).

Ghrelin immunoreactivity was intense in the DCT, mild in the PCT, and nowhere in the RC (Fig. 4B). The medulla showed moderate and strong ghrelin immunoreactivity in the CT and CD, respectively (Fig. 4C). Jacalin immunoreactivity was strong in the RC (Fig. 5A) and the glomerular basement membrane and apical cytoplasmic surface of the DCT but mild in the PCT (Fig. 5B). In the medulla, the immunoreactivity was moderate in the apical cytoplasmic surface of the large CD and CT (Fig. 5C).

(4 weeks)

Based on the PAS staining, the glomerular basement membrane was well developed, and the PCT had a well-developed brush border (Fig. 6A). The CD of the medulla also showed a well-developed basement membrane (Fig. 6B). Ghrelin immunoreactivity was mild in the glomerular capillaries of the RC and PCT but strong in the DCT (Fig. 6C). The medulla showed

strong immunoreactivity in the CT and loop of Henle (Fig. 6D).

Jacalin immunoreactivity was strong in the glomerular capillary and basement membrane of the RC but moderate in the apical surface of the DCT and PCT (Fig. 7A). Jacalin immunoreactivity was strong in the apical cytoplasm of the CD (Fig. 7B).

(6 weeks)

Ghrelin immunoreactivity was strong in the DCT and mild in the glomerular capillary of the RC and in the PCT (Fig. 8A). The medulla showed strong immunoreactivity in the CD (Fig. 8B). Jacalin immunoreactivity was strong in the glomerular capillary and basement membrane of the RC and the apical surface of the DCT (Fig. 8C). The

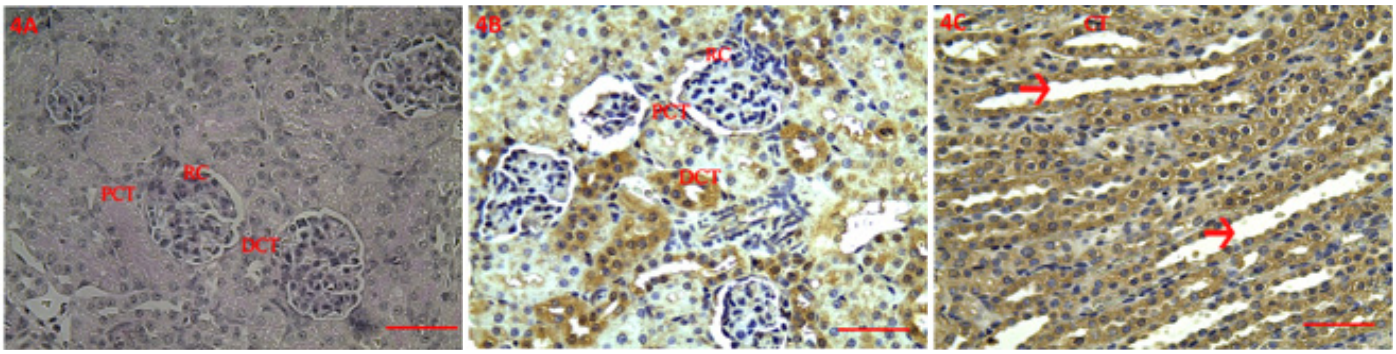

Figure 4. Photomicrographs showing the kidney from two-week-old rat. 4A: The glomerular basement membrane of the RC, well-developed brush border of the PCT, and undeveloped brush border of the DCT. PAS. 4B: Ghrelin immunoreactivity was mild in the PCT, intense in the DCT, and absent in the RC. 4C: Ghrelin immunoreactivity was moderate in the CT and large CD (arrows). Magnification, X40. Scale bars=40 µm.

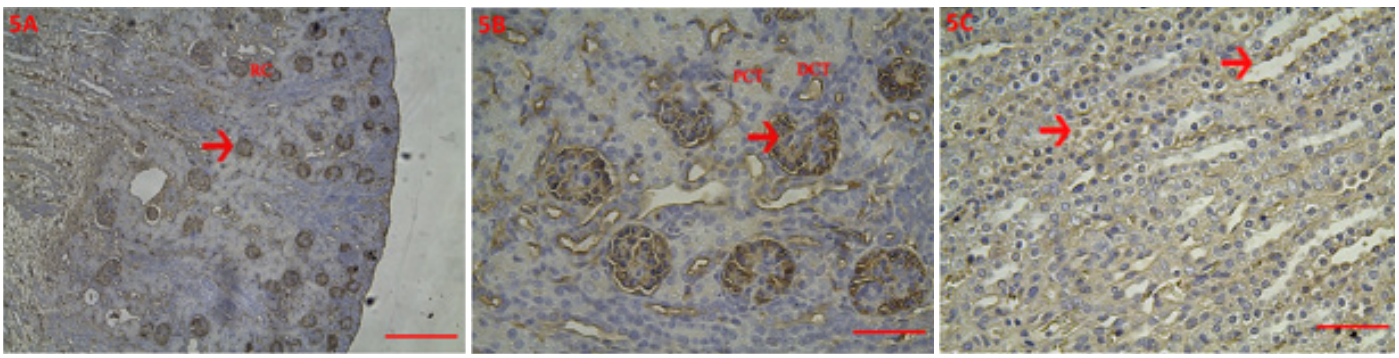

Figure 5. Photomicrographs showing jacalin immunoreactivity of the kidney from two-week old rat. 5A: The immunoreactivity was intense in the renal corpuscle (RC). Magnification, X10. 5B: It was also intense in the glomerular basement membrane (arrow) and apical surface of the DCT but mild in the PCT. Magnification, X40. 3C: It was mild in the apical surface of the CD (arrows). Magnification, X40. Scale bars=400 µm (A) and 40 µm (B, C).

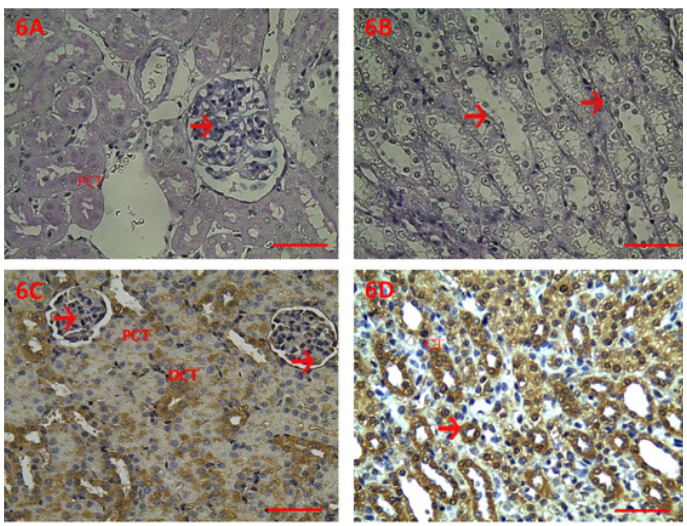

Figure 6. Photomicrographs showing the kidney from four-week-old rat.6A: The glomerular basement membrane (arrow) and well-developed brush border of the PCT. PAS. 6B: The basement membrane of the CD (arrows). PAS. 6C: Ghrelin immunoreactivity was mild in the glomerular capillaries (arrows) and PCT and intense in the DCT. 6D: Ghrelin immunoreactivity was intense in the CT and loop of Henle (arrow). Magnification, X40. Scale bars=40 µm.

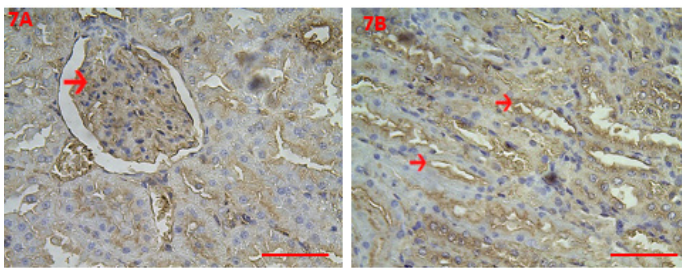

Figure 7. Photomicrographs showing Jacalin immunoreactivity in the kidney of four-week-old rat.7A: The immunoreactivity was intense in the glomerular capillary and basement membrane (arrow) and in the apical surface of the DCT and mild in the PCT. Magnification, X40. 7B: It was also intense in the apical surface of the CD (arrows). X40. Scale bars=40 µm.

immunoreactivity in the medulla showed no change from four weeks.

(12 weeks)

The renal cortex showed a uniform adult appearance, and the RC were more prominent and increased in size with wide Bowman's space and well-developed basement membranes of the glomerular capillaries and parietal epithelium. The PCT increased in number,

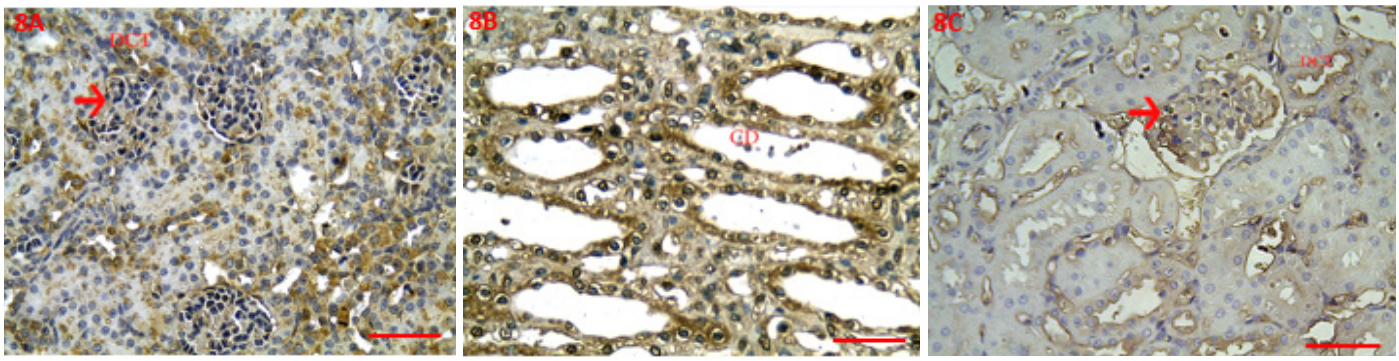

Figure 8. Photomicrographs showing the kidney from six-week-old rat. 8A: Ghrelin immunoreactivity was intense in the DCT and mild in the glomerular capillary (arrow). Magnification, X40. 8B: It was also intense in the CD. X40.8C: Jacilin immunoreactivity was intense in the glomerular capillary and basement membrane (arrow) as well as the apical surface of the DCT. Magnification, X40. Scale bars=40 µm.

showed a well-developed brush border and basement membrane with narrow lumen, and were mostly concentrated at the urinary pole. The DCT showed no PAS staining, were few in number, had a wide lumen and were abundant at the vascular pole of each RC (Fig. 9A).

Ghrelin immunoreactivity was intense in the DCT and CD and moderate in the PCT and glomerular capillaries (Fig. 9B and 9C).

Jacalin immunoreactivity was stronger than at six week and intense in the glomerular basement membrane of the RC and apical surface of DCT (Fig. 10A). The medulla showed intense jacalin immunoreactivity in the apical cytoplasmic surface of the large CD and CT (Fig. 10B).

Discussion

Ghrelin is a unique peptide hormone initially recognized in rat and human stomach and assumes different significant roles in metabolism. The ghrelin cells localization has been investigated in the stomach (Date *et al*., 2000; Rindi *et al*., 2002; Yabuki *et al*., 2004) and pancreas (Date *et al*., 2002; Wierup *et al*., 2002, 2004; Kageyama *et al*., 2005; Wierup and Sundler, 2005). Several studies have suggested that the kidney is a significant site for the secretion, clearance and degradation of ghrelin. Yoshimoto *et al*. (2002) revealed that ghrelin is secreted by nephrons and filtered through the glomeruli.

We analyzed the neonatal and postnatal localization of ghrelin by immunohistochemistry (Table 1), finding signals in different locations across different ages,

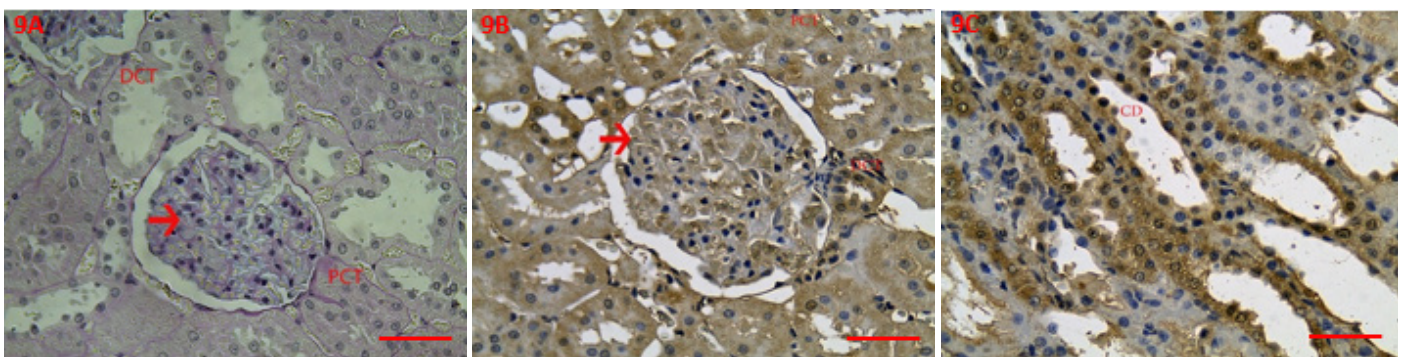

Figure 9. Photomicrographs showing the kidney of 12-week-old rat. 9A: A well-developed glomerular basement membrane (arrow), PCT, and DCT. PAS. 9B: Ghrelin immunoreactivity was intense in the DCT and moderate in the PCT and glomerular capillaries (arrow). Magnification, X40. 9C: Ghrelin immunoreactivity was intense in the CD. Magnification, X40. Scale bars=40 µm.

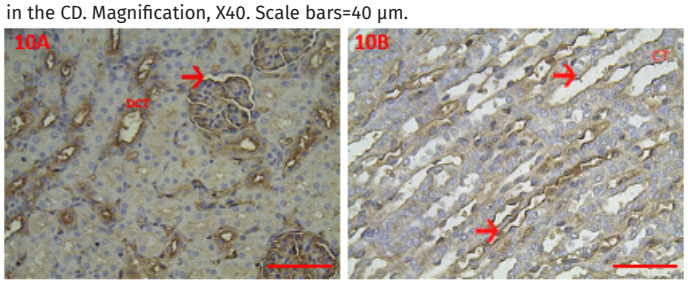

Figure 10. Photomicrographs showing jacalin immunoreactivity in the kidney of 12-week-old rat. 10A: The immunoreactivity was intense in the glomerular basement membrane (arrow) and apical surface of the DCT. Magnification, X40. 10B: It was also intense in the apical surface of the large CD (arrows) and CT. Scale bars=40 µm.

with increasing intensity in the DCT, PCT, RC, CD, and loop of Henle. On the other hand, Yabuki *et al*. (2006) reported the intrarenal localization of ghrelin in 3-month-old rat and observed its expression only on the basolateral membrane of the distal tubules and no expression in other segments of the nephron and interstitial cells, including juxta-glomerular cells.

Kuloglu and Dabak (2009) observed ghrelin in both the distal tubules and CD of diabetic rats but only in the distal tubules of healthy controls. Ghrelin levels increased with age at 4 and 6 weeks in the diabetic group, suggesting ghrelin may has roles

Table1. Ghrelin staining in the renal tubular system

	PCT	DCT	RC	CT
20 days neonatal	mild	moderate	none	none
1 week	mild	moderate	mild in glomerular capillaries of RC	none
2 weeks	mild	intense	mild in glomerular capillaries of RC	moderate
4 weeks	mild	intense	mild in capillaries	intense
6 weeks	moderate	intense	mild	intense
12 weeks	moderate	intense	moderate	intense

in the pathophysiological mechanism of diabetic nephropathy. However, they made no observations of the ghrelin levels over time in the control group. We found ghrelin levels also increased in normal rat developing kidneys and in many different locations.

Ghrelin was identified to reduce kidney excretion of sodium. However, the function of ghrelin on the kidney propose actions in the distal nephron, the ghrelin receptors sites have not been distinguished. Venables *et al*. (2011) found receptor expression in the straight parts of the distal tubules (thick limbs of Henle) and thin limbs of the loops of Henle, which is consistent with our data and with ghrelin promoting sodium retention. However, no expression was detected in other structures, including the proximal tubules, glomeruli, and CD which is inconsistent with our findings, likely because we observed many more stages of development. They also reported no ghrelin receptors in the intra-renal or extra-renal arteries, despite investigations that ghrelin has a vasodilator action.

Yildirim *et al*. (2016) found ghrelin levels increased at the beginning of diabetes in rat models. However, after maturity, the levels were the same between diabetic and healthy rats. They concluded that the ghrelin level decreased with development in diabetic rat, but our observations suggest the ghrelin level increased with normal kidney development.

High jacalin levels were found in the glomerular capillary and basement membrane of the RC, the apical surface of the DCT and PCT, and the apical cytoplasm of the CD (Table 2). Our observations are consistent with Yabuki *et al*. (2002), who reported jacalin in the basement membrane of the PCT, PST, and DCT, loop of Henle, thick ascending limb, and CD of 3-month-old female mice. In human, Engel *et al*. (1997) reported jacalin in only the luminal border of the distal tubules and the CD.

In conclusion, the increasing ghrelin and jacalin levels in developing rat kidney tissues suggest that both lectins have important roles in the physiological function of the organ.

Table 2. Jacalin staining in the renal tubular system

	PCT	DCT	RC	CT
One day	none	none	intense and moderate in renal capsule	intense
1 week	none	intense at apical surface	intense	intense
2 weeks	mild	intense	intense	intense
4 weeks	mild	intense	intense	intense
6 weeks	mild	intense	intense	intense
12 weeks	mild	intense	intense	intense

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