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Saudi Journal of Biological Sciences

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ORIGINAL ARTICLE

Changes in the NFκB and E-cadherin expression are associated to diabetic nephropathy in *Psammomys obesus*



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Received 28 December 2015; revised 29 April 2016; accepted 10 May 2016
Available online 18 May 2016

KEYWORDS

Diabetic nephropathy;
Psammomys obesus;
Nuclear factor κB (NFκB);
E-cadherin;
Inflammation;
High energy diet

Abstract Diabetes mellitus is a major leading cause of end-stage renal failure, characterized by kidney inflammation and glomerular dysfunction, in worldwide. Kidney inflammation is associated to modifications in the expression levels of pro-inflammatory molecules, such as nuclear factor-κB (NFκB) and adhesion molecules, such as E-cadherin, leading to glomerular dysfunction. However, the relationships between these two processes in human diabetic nephropathy remain an open question. Since *Psammomys obesus* is an ideal animal model to study diabetes mellitus temporal evolution, we have used this model to study the correlation between kidney structural changes and modification on the expression levels of NFκB and E-cadherin over time. We have demonstrated that, after induction of diabetes mellitus with a high energy diet (HED), *P. obesus* develops the characteristic symptoms of human disease. In detail, at the third month nuclear factor NFκB is expressed in the kidney of diabetic *P. obesus* and structural renal changes, such as mesangial expansion or interstitial fibrosis, are detectable; at 6 months, thickening of glomerular basement membrane, glomerular sclerosis, and tubular atrophy occurs; at 9 months, symptoms of the final

Abbreviations: DN, diabetic nephropathy; ECM, extra cellular matrix; HED, high energy diet; ND, natural diet; NFκB, nuclear factor kappa B

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Peer review under responsibility of King Saud University.



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<http://dx.doi.org/10.1016/j.sjbs.2016.05.009>

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stages of the disease, such as down expression of E-cadherin, happens. As a result of these observations we proposed that NF κ B activation and E-cadherin down-expression are interlinked on diabetic kidney disease (DKD).

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1. Introduction

Diabetes is an important problem of public health (Centers for Disease Control and Prevention, 2007) characterized by levels of plasma glucose concentration consequence of the defect of insulin secretion or insulin action or both (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). The latest stage of this disease usually evolves into diabetic nephropathy (DN) leading to end-stage renal insufficiency worldwide (Lozano et al., 2012). It is characterized by inflammation and specific renal morphological alterations, such as, mesangial proliferation, capillary basement membrane thickening, interstitial fibrosis, and tubular atrophy (Lane et al., 1993). Hyperglycemia, induces overexpression of the nuclear factor kappa B (NF κ B) on several organs (Chen et al. (2001) and, among them, kidney (Guijarro and Egido, 2001; Xiao et al., 2001). NF κ B, which is implicated in tumoral processes (Disis, 2010; Basseres and Baldwin, 2006), is a proinflammatory factor and plays an important role in the expression of other proinflammatory genes including cytokines, chemokines, and adhesion molecules such as cadherin genes (Kuphal et al., 2004). Indeed, recent studies have suggested that NF- κ B activation reduces E-cadherin expression (Kuphal et al., 2004). E-cadherin is a transmembrane glycoprotein localized on the surfaces of epithelial cells in regions of cell-cell contact known as adherent-junctions (Gumbiner, 1996) playing a key role in the maintenance of normal structure and function in normal epithelial tissues. Thus, down expression of E-cadherin has been correlated with tumor cell and metastasis (Vlaminckx et al., 1991). However, the precise relationships between NF κ B and E-cadherin expression in human diabetic nephropathy and tumors are not known. Therefore, study of kidney structural changes associated with alterations on the expression levels of NF κ B and E-cadherin over time can help in the prognosis of the evolution from diabetic nephropathy toward cancer. Our hypothesis is that DN, manifested as modifications on kidney structure, are related with anti-correlated changes on NF κ B and E-cadherin expression. To validate it, we have performed histological analysis and NF κ B and E-cadherin immuno-labeling on *Psammomys obesus*, which has been proposed as an ideal animal model to study diabetes mellitus and its complications (Kalman et al., 1993, 1996; Scherzer et al., 2011). We have confirmed that, after induction of diabetes mellitus with a high energy diet (HED), *P. obesus* develops the same characteristic of human DN. This nephropathy is associated, at early stages with an inflammatory state and an overexpression of NF κ B and, at final stages, with a down expression of E-cadherin. Therefore we proposed that NF κ B activation and E-cadherin down-expression are interlinked on diabetic nephropathy.

2. Materials and methods

2.1. Animals and experimental protocol

2.1.1. Animals

In the present study, we have used 27 mature *P. obesus* Cretzschmar (Morsy et al. 1996), of both sexes, aged from 3 to 5 months (90–150 g weigh), trapped in the semi-desert region of Beni-Abbes-Abadla south-west of Algiers (wilaya of Béchar 30° 7' North latitude and 2° 10' West longitude). *P. obesus* were housed and adapted to laboratory conditions

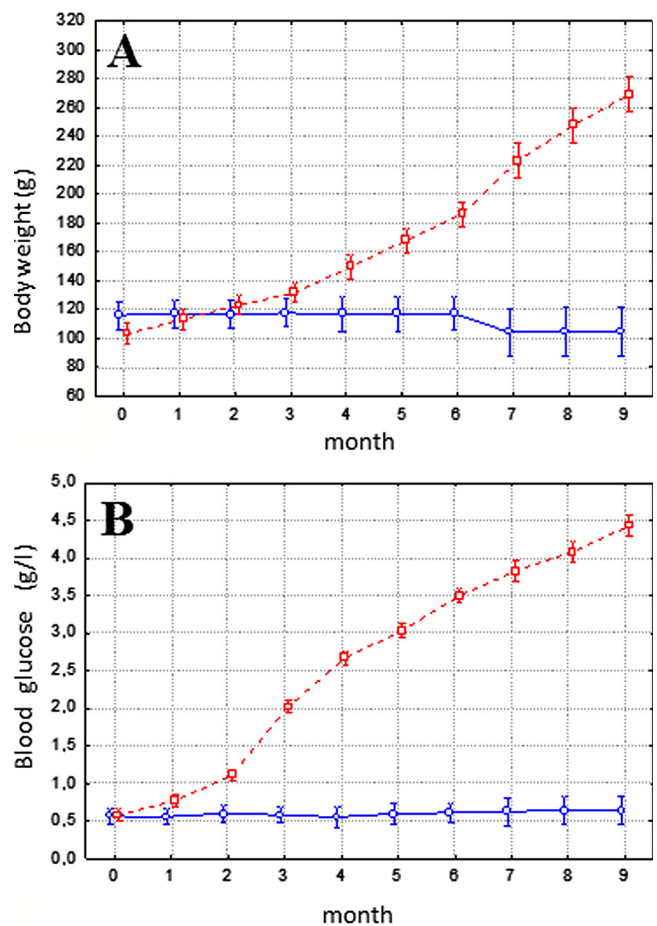


Figure 1 Body weight (A) and blood glucose evolution (B) in *P. obesus* ($n = 27$). Although animals ($n = 9$) that received natural halophilic plants remained stable throughout the entire period (continuous line), 18 animals filled on high energy diets (discontinuous line) showed an important increase in body weight and blood glucose concentration. Data are expressed as mean \pm standard deviation.

(25 °C, 70% hygrometry, and a 12-h light–dark cycle). Upon their arrival in the laboratory, the animals were numbered, weighed, and transferred to separate suitable cages. After a two-week acclimation period, during which they were raised on a natural diet (20 kcal/day) and were fed only halophilic plants rich in water and mineral salts (*Salsolafoetida*), they were randomly distributed into two groups:

- 9 animals, divided into 3 subgroups ($n = 3$), were raised with natural diet (50 g/day/animal, equivalent to 20 kcal/day).
- 18 animals, divided into 3 subgroups ($n = 6$), fed a standard laboratory high energy diet (32 kcal/day/animal) and salty water (0.9% NaCl) *ad libitum*.

2.1.2. Biochemical analysis

Both groups were followed up for 3, 6 and 9 months, blood glucose and weight were measured every month. Blood was collected from retro orbital venous plexus in EDTA tubes. Plasma glucose measurements were done with the enzymatic method using test kits from BioSystems. During all experimental procedures animals were maintained in an Animal

Research Facility according with institutional guidelines for animal's care (Directive 2010/63/EU).

2.1.3. Histological study

Kidneys from animals fed with natural diet or with HED were resected, immersed in 10% formaldehyde for 48 h and then were paraffin embedded. Sections of 5 μm, obtained on a microtome, were stained with hematoxylin–eosin, Trichrome's Masson or Periodic acid–Schiff. They were observed using a light microscope (Motic SFC-18, Hong Kong) for general histologic analysis.

2.1.4. Immunohistochemical staining

2.1.4.1. E-cadherin labeling. Kidneys were labeled with E-cadherin with purified mouse monoclonal antibody (BD Transduction Laboratories purified mouse anti-E-cadherin, n°610182, BD Biosciences). Sections 5 μm thick of the selected paraffin blocks were dewaxed in xylene for 20 min, rehydrated in graded alcohol, permeabilized in PBS/0.25% Tween® 20 and incubated in 3% hydrogen peroxide/PBS for 10 min to block endogenous peroxidase activity. The antigens were retrieved by boiling for 20 min in citrate buffer (tri-sodium

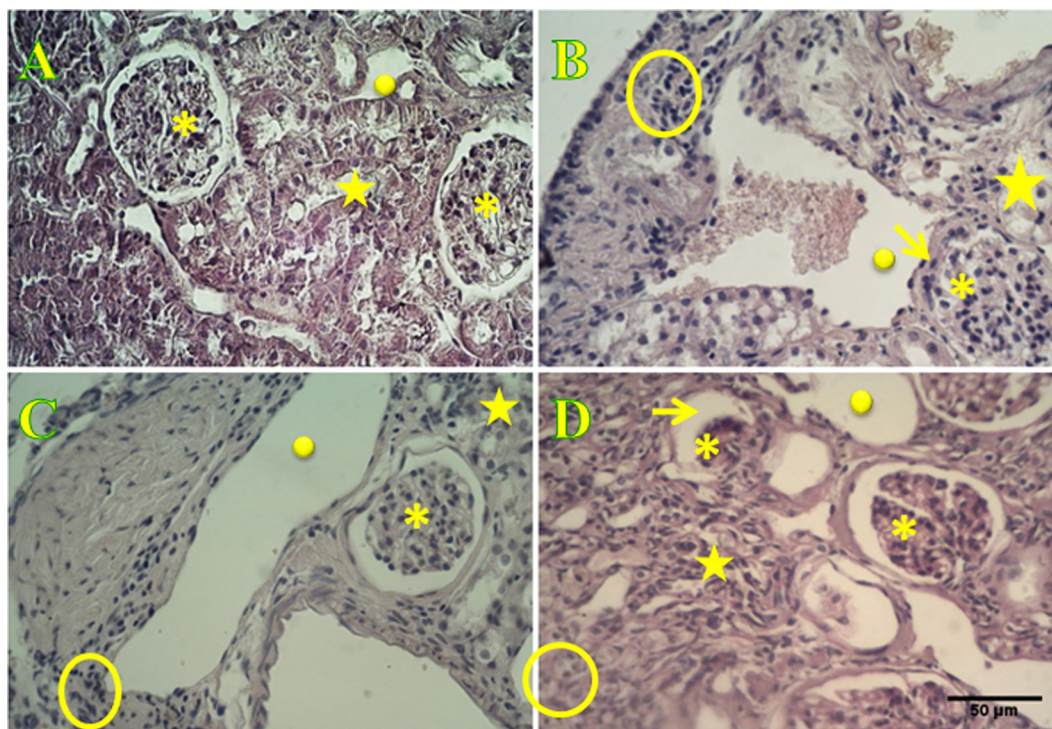


Figure 2 Histological characterization of *P. obesus* kidney using hematoxylin and eosin staining. Histological section of non-diabetic *P. obesus* kidney, fed with ND, evidence normal glomerulus encircled by a Bowman's space and blood capillary have standard diameters. Absence of cell accumulation at the interstitial space denotes that inflammatory process does not occur. Proximal and distal tubes are well organized. Sections *P. obesus* kidney observed in 3, 6 and 9 months after animals were fed with an HED (B, C and D respectively shown an increment on the blood capillary diameters and blood flow, and incipient inflammation which is evidenced by cell accumulation at the interstitium appearing between tubules (focal interstitial inflammation) leading to their disorganization and denaturation. Glomerular hypertrophy characterized by the absence of the Bowman's space is observed at the 3rd month and evolves toward atrophy at the 6th month leading to the total degeneration of the glomerulus at the 9th month. Asterisks correspond to glomerulus, filled-circles to blood capillary, empty-circles to the interstitium between tubules, stars to proximal and distal tubes and arrows points to the Bowman's space. Scale bar is 50 μm.

dehydrate, MERCK Millipore) pH 6.0, using a microwave, followed by cooling to room temperature for 20 min. After washing in phosphate buffered saline, different sections were incubated with mouse monoclonal anti-E-cadherin diluted in blocking solution (5% Normal Goat Serum in 3% BSA/PBT).

Sections were washed in phosphate buffered saline before incubation with Anti-Mouse biotinylated secondary antibody (BA9200, anti-mouse biotinylated, Vector Laboratories) for 25 min at room temperature. The sections were then washed in phosphate buffered saline, incubated with avidin/biotin technology system allowing more accessibility for binding to a biotinylated target (Vectastain Elite ABC Peroxydase kits universal, Vector, PK-6100) for 30 min at room temperature, washed and revealed with 3,30-diaminobenzidine peroxydase substrate (kit DAB Vector, SK-Vector Laboratories) solution to yield an insoluble brown deposit.

2.1.4.2. NF κ B immunolabeling. Antibody against NF- κ B p50 (Abcam, Ab32360) was associated with the secondary donkey anti-rabbit antibody conjugated with an Alexa Fluor 594 from Life Technologies (A21207 Thermo Fisher).

The sections 5 μ m thick were dewaxed in xylene for 20 min, rehydrated in graded alcohol and permeabilized by incubation in PBS/0.25% Tween $\text{\textcircled{R}}$ 20. Heat mediated antigen retrieval was performed using citrate buffer pH 6.0 and sections were incubated with the primary rabbit anti-NF- κ B p50 antibody diluted in blocking solution at 1:200. Slides were washed three times with PBS and incubated for 25 min with secondary donkey anti-rabbit antibody conjugated with Alexa Fluor 594 in a dark wet chamber. After final washing, the slides were cover slipped. To determine localization of NF- κ B, the nuclei were

counter-stained with Fluoro shield DAPI histology mounting medium (Sigma–Aldrich).

Pictures were taken with a digital camera unit (Canon Powershot A640, Japan) connected to a fluorescent microscope (Zeiss Axioskop 2, Germany).

2.2. Statistical analysis

All results were expressed as mean \pm standard deviation. Statistical differences were assessed using (ANOVA) followed by post hoc Tukey honest significant difference test. Difference is considered statistically significant for p values $<$ 0.05.

3. Results

3.1. Body weight and blood glucose evolution

We have observed that the body weight in *P. obesus* fed with natural diet remains stable during the nine months of the study whereas in the case of rats nourished with high energy diet the body weight significantly increases since the third month (Fig. 1A). This weight gain is preceded by a significant increment in the levels of blood glucose, starting at the second month, (Fig. 1B) in animals fed with HED while in animals following ND this level is constant all along the experience. The plasma glucose concentration was raised to 2 g/l, at the third month of our study, which is higher to the glycemic level required to consider *P. obesus* as diabetic animals, level that has been established as 1.4 g/l–8.3 mmol/l (Leibowitz et al., 2001).

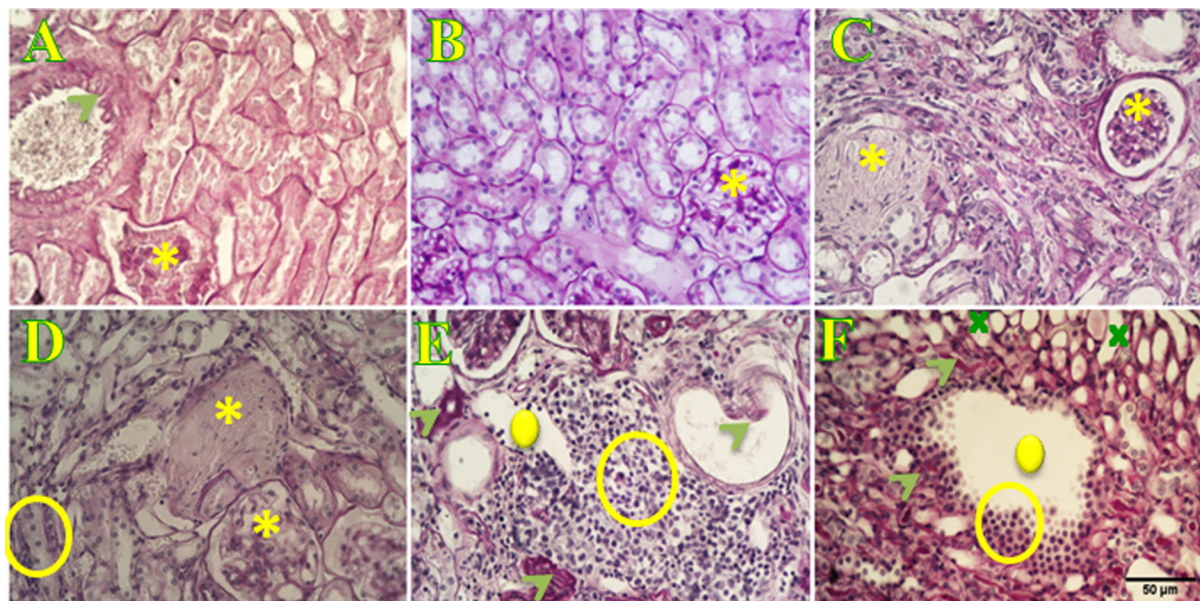


Figure 3 Histological characterization of *P. obesus* kidney using PAS staining. Normal kidney (A) of animals following a ND, as well as kidney of animals fed with an HED for three months (B) do not show any symptoms of atherosclerosis, glomerulosclerosis, glomerular endothelial interstitial fenestration and focal interstitial inflammation. On the contrary, animals fed with an HED after 6 (C and D) and 9 months (E and F) depict global glomerular sclerosis (asterisk in C and D). Atherosclerosis, evidenced by the presence of hyaline deposits (arrow-heads in E) leading to arterial intima thickening (arrow in E), and glomerular and interstitial fenestration (cross) occurs at nine months after initiating a high energy diet (F). Scale bar is 50 μ m.

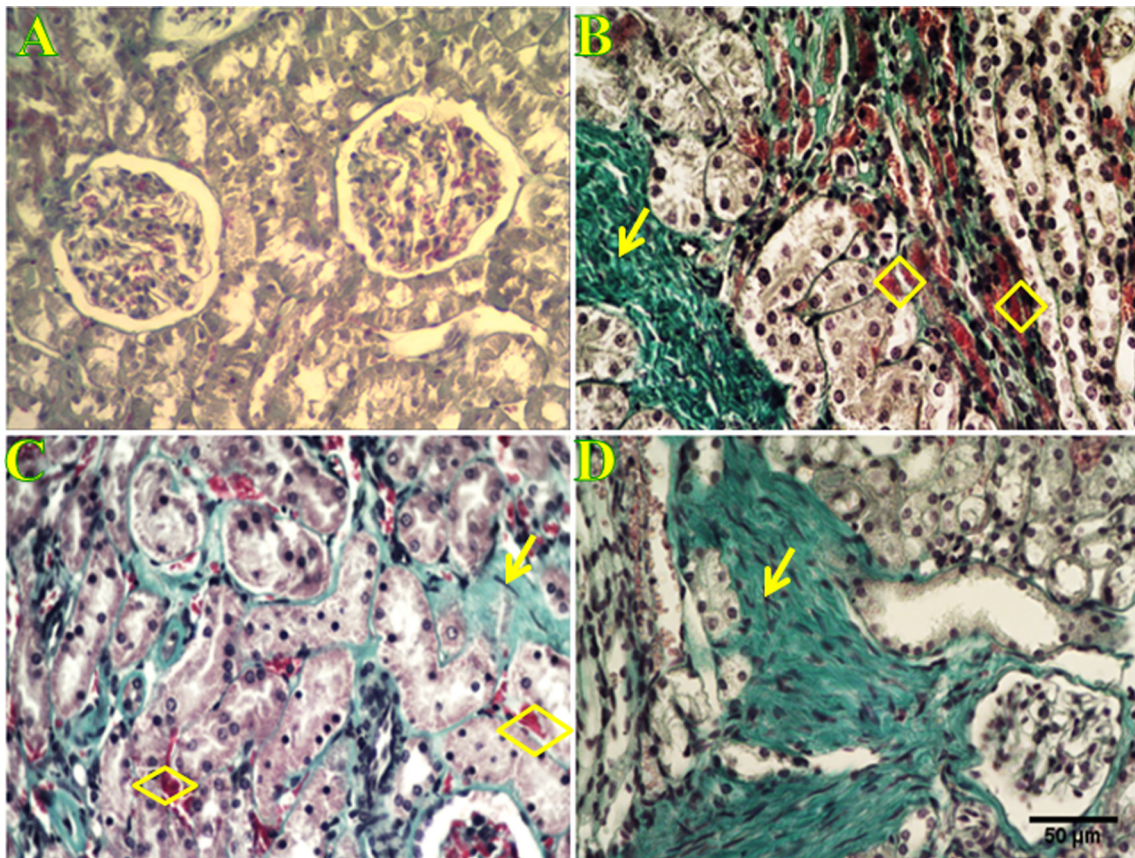


Figure 4 Histological characterization of *P. obesus* kidney using Masson's Trichrom staining. Histological section stained with Masson's Trichrom in normal kidney (A) of *P. obesus* fed with a ND does not show any deposit of extracellular matrix evidencing the absence of interstitium fibrosis. On the contrary, sections from kidney of *P. obesus* fed with an HED for 3, 6 and 9 months (B, C and D respectively) show interstitial fibrosis, characterized by a loss of renal parenchyma and the occurrence of collagen fibers accumulation (arrows), and an increment of the number of vessels (rhombus). Scale bar is 50 μ m.

3.2. Histological results

The histological analysis performed on animals fed with ND and HED depict clear differences on kidney observable since the third month of HED. A mesangial expansion occurs at the third month on HED rats leading to the shrinkage of the capsular space of the Bowman's capsule (Figs. 2B and 3B). This expansion is associated with an increment on the number of vessels (Fig. 4B) and interstitial fibrosis (Fig. 4B). Mesangial atrophy becomes apparent at the six month (Fig. 2D and C) and it is associated with glomerulus sclerosis (Fig. 2C) and inflammation (Fig. 3C). This mesangial expansion decreases at the nine month, leading to a glomerular atrophy (Fig. 2D), and an increment of the inflammation is observable (Figs. 2D, 3D and F). Simultaneously, hyaline deposits appear (Fig. 3E and F) and the inner layer of arterioles(intima) grows (Fig. 3E). These phenomena are accompanied by an interstitial fenestration (Fig. 3F).

3.3. NFκB and E-cadherin immuno-labeling

We have observed that in *P. obesus* fed with ND the nuclear factor κB is not over-expressed (Fig. 5A), whereas its expression increases in animals following an HED, becoming detect-

able by immuno-labeling since three months (Fig. 5B–D). On the contrary, E-cadherin is high-expressed in the kidney of *P. obesus* fed with ND (Fig. 6A) and in animals following an HED until the third month (Fig. 6B) but its expression levels decrease at the sixth month (Fig. 6C) becoming almost undetectable at 9 months (Fig. 6D) after starting the HED.

4. Discussion

Despite that it is known that NFκB and E-cadherin are implicated on diabetes mellitus prognosis, the relationships between the expressions of these two proteins are not well described in human diabetic nephropathy. We have studied the possible correlation existing between the expressions of both proteins by using the animal model *P. obesus* which it is known to reproduce all the characteristics of human diabetes (Kalderon et al., 1986). Our work shows that the sand rat *P. obesus*, maintained on high energy diet, develops hyperglycemia and obesity at three months after HED initiation. This is in agreement with previous works (Kahn, 2000; Donath et al., 1999) showing that, when the gerbil *P. obesus* is fed a HED, the animals rapidly develop hyperglycemia associated with hyperinsulinemia leading to absolute insulin deficiency. We also show that after the same period of time,

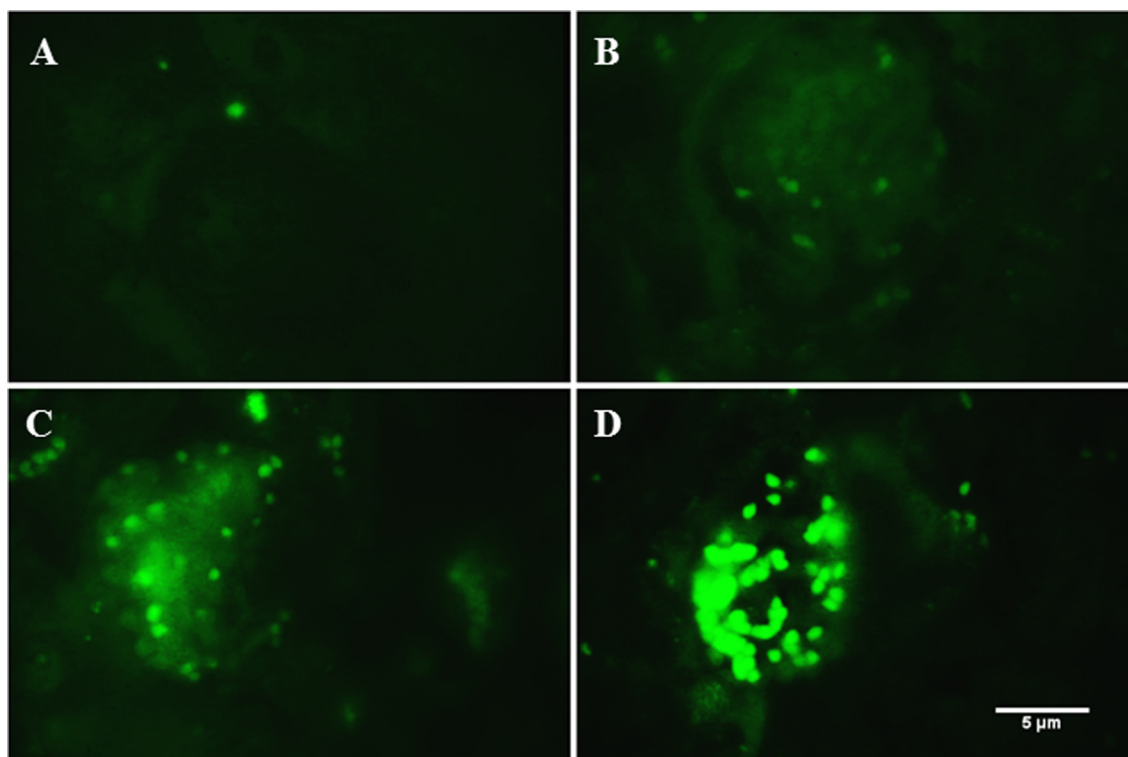


Figure 5 Immuno-labeling of NF κ B within *P. obesus* kidney. The nuclear factor κ B is not expressed on control kidney (A) of animals been fed by ND, whereas it is induced at the first 3 months (B) after starting the HED. NF κ B expression levels increase at later stages of diabetic nephropathy as observed at 6 (C) and 9 (D) months. Scale bar is 5 μ m.

P. obesus shows kidney pathologic lesions. This can be explained by an adaptation of new glucose metabolism leading to a remodeling of kidney parenchyma, associated with a decrease on matrix-degrading metalloproteinase activity (Kanwar et al., 2011), and characterized by the accumulation of collagen and fibronectin at the extracellular matrix (ECM) (Wang et al., 2011). It has also been described that ECM is accumulated in the glomeruli, tubule-interstitial regions and in kidney arteriolar, leading to glomerular hypertrophy, interstitial fibrosis and atherosclerosis (Scherzer et al., 2000). We observe, in *P. obesus* kidney, the occurrence of these phenomena in the third month following HED. Moreover, our histological study shows vacuolar changes associated with glycogen deposition at the nine month from HED. Since glycogenic vacuolization of cells cytoplasm at the renal tubules is related with the initiation of Armani–Ebstein lesions (Lau et al., 2013) we suggest that, like humans, *P. obesus* develops this lesion at the end-stage of diabetic nephropathy. In addition, vascular hypertrophy observed in this study, characterized by thickening of renal arterial wall, is similar to what has been observed in humans whom arteriosclerosis leads to hypertension and ischemic nephropathy as a consequence of the increase of angiotensin II blood levels (Remuzzi et al., 1998). We observe that these structural changes of renal arteries are already prominent before glomerular alterations and advanced kidney disorders as previously described (Sato and Yoshinaga, 1987).

Taking together our results, and considering that diabetic nephropathy in humans became observable 10 years after diabetes mellitus initiation (Melmed et al., 2011) *P. obesus*

can reproduce its symptoms in only nine months, this gerbil becomes the ideal model to study kidney pathologies and consequences of diabetes. Among them inflammatory processes are one of the first results of hyperglycemia. Indeed, hyper caloric diet and diabetes are the origin of inflammation and lifestyle diseases such as obesity, insulin resistance, coronary heart disease, and atherosclerosis (Smith et al., 2009). This inflammation process involves the expression of pro-inflammatory molecules, such as nuclear factor- κ B (Medzhitov and Janeway, 2000), which together orchestrate the activation of other genes including those of proteins implicated on cell adhesion (Kuphal et al., 2004), such as E-cadherin (Chua et al., 2007). Our results demonstrate that higher expression of NF- κ B in glomerulus and interstitium in *P. obesus* is correlated, at end-stage of DN, with an increment on the macrophage infiltrations. This is in accordance with the relationship between NF- κ B expressions, tubule-interstitial injury and interstitial infiltration of macrophages in human diabetic nephropathy (Sanz et al., 2010). Associated to these observations we also demonstrate that expression of the intercellular adhesion protein E-cadherin is reduced at the end-stage of DN. Therefore, our results show that the anti-correlation on the expression levels of NF κ B and E-cadherin, already demonstrated biochemically (Kim et al., 2000), leads to injuries in the kidney which are associated with diabetes nephropathies. Since down expression of E-cadherin is associated with tumor progression and metastasis (Jeanes et al., 2008) and over-expression of NF- κ B is related with the increment of the expression levers of pro-metastasis proteins (Nozaki et al., 2005) we suggest the use of NF- κ B and

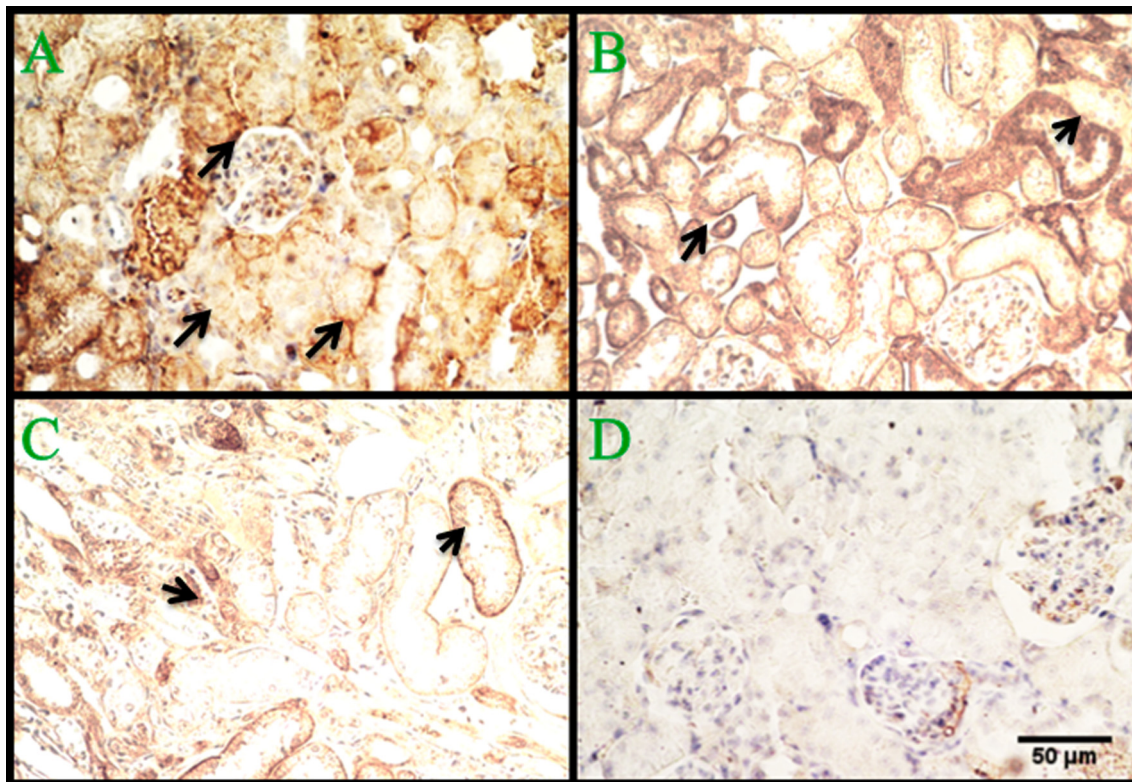


Figure 6 Immuno-histological labeled sections of E-cadherin within *P. obesus* kidney. E-cadherin (depicted by arrows) normal expression can be observed at the level of renal parenchyma structures, in normal kidney of *P. obesus* fed with ND (A) and in kidney of animals after three months of starting a high energy diet (B), whereas its expression is reduced at 6 (C) and 9 (D) months. Scale bar 50 μ m.

E-cadherin proteins as markers to predict possible diabetic nephropathy evolution toward cancer.

5. Conclusions

We have demonstrated that HED modifies *P. obesus* homeostasis leading to a diabetic process evidenced by lesions on different kidney structures which are associated with changes in the expression levels of NFκB and E-cadherin. Since the modification on the expression levels of these two proteins is associated with tumor processes and metastasis when studied independently, we propose that taken together they can be used as prognosis markers of the evolution from diabetic nephropathy toward cancer.

Acknowledgments

The authors would like to express their sincere appreciation to the Histology facility of the Institut Curie and to PICT-IBiSA Institut Curie Orsay for facilitate sample preparation and provide access to equipment. We also want to thank Dr. Florent Poyer for his advice on immune-labelling.

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