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Research Paper

Immunohistochemistry of kidney and mesenteric tissues of heterozygous *Pkd2* knock out mice as an investigatory tool for detecting endothelial dysfunction in polycystic kidney disease

Adekoya Adebowale Olayinka (PhD)
Nephrology Unit, Department of Medicine, Lagos State University Teaching Hospitaland Lagos State University College of Medicine, Ikeja, Lagos State

ABSTRACT: ***Introduction:*** *Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the commonest inherited cause of chronic kidney disease (CKD) and hypertension is an important feature in affected individuals, even those with normal kidney function. Also, endothelial dysfunction (ED) has been reported to precede hypertension in other disease conditions. However, there is paucity of data on ED hence, the need for more research. The aim of this study is to investigate activities of biomarkers of ED in PKD using immunohistochemistry (IHC) of kidney and mesenteric tissues. These biomarkers are superoxide dismutase 2 (SOD2), hemeoxygenase 1 (HO1), matrix metalloproteinase 2 (MMP2) and 8 hydroxydeoxyguanosine (8OHdG).* ***Methods:****The experimental animal were ischaemic-reperfusion models of $Pkd2^{+/-}$ founder mice with a null allele (ws183) for pkd2 which results from homologous recombination at exon-1 of the Pkd2 locus. Wild type mice tissues were used as controls. Activities of SOD2, HO1, MMP2 and 8OHdG were studied using IHC. Also, semi quantitative assessments of the expressions of these biomarkers was performed.* ***Results:*** *Heterozygous Pkd2 mice showed reduced expression of SOD2 and a greater level of HO1 staining when compared with wild type mice kidneys ($p<0.0001$ and $p<0.05$ respectively). Similarly, 8OHdG immunostaining in mesenteric vessels of wild type and heterozygous Pkd2 mice showed significant difference in the intensity ($p<0.05$). Lastly, MMP2 expression was predominantly extracellular but there was a statistically significant difference in staining intensity between the wild and Pkd2 KO mice ($p<0.05$).****Conclusion:*** *Superoxide dismutase 2, hemeoxygenase 1, matrix metalloproteinase 2 and 8 hydroxydeoxyguanosine are significant biomarkers of endothelial dysfunction and immunohistochemistry is an acceptable tool for their detection.*

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I. INTRODUCTION:

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the commonest inherited cause of chronic kidney disease and hypertension is an important feature in affected individuals, even those with normal kidney function. However, endothelial dysfunction (ED) has been reported to precede hypertension in other disease conditions. The global prevalence of ADPKD is 1:400 - 1:1000 (1). Although the disease affects all races in every part of the world, there is a wide variation in the prevalence figure from one region to another. There are about 12.5 million people with ADPKD worldwide (2). Immunohistochemistry is a recognised laboratory method for the detection, localization, and quantification of proteins in tissues. Optimal detection of antigen is determined by antibody specificity, tissue fixation and processing. The importance of localization and quantification of biomarkers of oxidative stress ispertinent in the search for ways to halt disease progression and onset of cardiovascular events especially in a disease condition like ADPKD where cardiovascular complications have been reported as a major cause of morbidity and mortality (3). This is important because oxidative stress and endothelial dysfunction precede these cardiovascular events (4).There is paucity of data on ED. Hence, the need for more investigatory tool. The aim of this study is to investigate activities of biomarkers of ED in PKD using immunohistochemistry of kidney and mesenteric tissues. These biomarkers are superoxide dismutase 2 (SOD2), hemeoxygenase 1 (HO1), matrix metalloproteinase 2 (MMP2) and 8 hydroxydeoxyguanosine (8OHdG)

II. METHODS:

Experimental animals. The experimental animals used for this study were $Pkd2^{+/-}$ founder mice obtained from Yale university, courtesy of Prof Somlo. They carried a null allele (ws183) for pkd2 which results from homologous recombination at exon-1 of the *Pkd2* locus (5). Details of the animal handling have been previously described (6).The left renal artery and veins of these animals were clamped using non-traumatic clampsfor 25minutes following which they were perfused for 48hours. These kidney sections were fixed with formalin and embedded in paraffin following which immunostainings were performed.

Immunohistochemistry of kidney tissue and mesenteric vessel of heterozygous Pkd2 KO mice: Immunohistochemistry was performed to investigate oxidative stress (SOD2, HO1 and 8OHdG antibodies) and endothelial dysfunction (MMP2 antibodies).

Immunohistochemistry protocol for paraffin embedded sections: Paraffin embedded tissue was prepared for staining by first dewaxing twice in xylene for 3 minutes each. This wasfollowed by hydrating twice through 100% alcohol series for 3 min per session. The process was repeated through 95% ethanol for 3 minutes (x 1)followed by 70% ethanol for 3 minutes (x1). Then through dH2O for 3 minutes (x1). The tissue was then immersed in a solution of 3% hydrogen peroxide in methanol for 20 minutes and rinsed in distilled water (dH2O). This quenched endogenous peroxidase activity. Antigen retrieval was performed through heat induced method. The section was microwaved for 10 minutes on high power in 0.01M Tri Sodium Citrate at a pH of 6.0 and then cooled under running water. This was followed by rinsing in PBS. Then serum from Impress universal kit (Vector Systems) was used to block the section for 30 minutes followed byincubation with primary antibody for 1 hour at room temperature. (Primary at a dilution of 1/1000, made up in sterile PBS). The section was then washed with PBS thrice and incubated for 30 minutes with the secondary biotinylated antibody from Impress kit at room temperature. Washing in PBS was repeated for 5 minutes. Chromogen (DAB) was prepared as follows: 1ml of diluent + 30µl (or 2 drops) of DAB solution. The section was incubated in this chromogen preparation, observing the development under a microscope. The reaction was stopped in dH2O and counterstained for 15-30seconds with haematoxylin blue and 10 seconds in Scott's tap water. Finally, the stained section was mounted with consul mount, protected with a cover slip and allowed to dry in room air.

Vector kits: Impress Universal Kit.DAB cat no. SK4100

Protocol for mouse on mouse immunohistochemistry: Working solutions were prepared as contained in manufacturer guide from Vector Laboratories, United Kingdom (catalogue number BMK-2202). These solutions were M.O.M- mouse IgG blocking reagent, M.O.M diluent and M.O.M biotinylated anti mouse IgG reagent.

Tissue sections were deparaffinised and hydrated through xylene, graded alcohol series and rinsed for 5 minutes as described above. Antigen unmasking was done using citrate based solution (H-3300). Sections were incubated with 'BLOXALL' blocking solution (SP-6000) for 10 minute to block endogenous enzyme activities. This was followed by 2 minutes' wash in PBS solution (twice). Avidin/Biotin blocking agent (SP-2001) was added to the sections. This was followed by 1 hour incubation in working solution of M.O.M mouse IgG blocking reagent and two-minute wash in PBS. Then, another 5-minute incubation in working solution of M.O.M diluent was performed. Sections were then incubated in diluted (1:1000) primary antibody (mouse monoclonal antibody) for 30 minutes after tapping off excess M.O.M diluent. This was followed by 2 minutes' wash in PBS (twice). Working solutions of M.O.M biotinylated anti mouse IgG reagent were then added to the sections and incubated for 10minutes followed by another PBS wash. Lastly, avidin based detection system was performed.

There was strict adherence to timing as described above. Also, kit was stored at 4 degree Celsius as instructed by product manufacturer.

Immunohistochemistry protocol for 8OHdG, SOD2, HO1 and MMP2.
8OHdG immunohistochemistry

Details of the methods is as described above and summarised in the table 2.1

Table 2.1: 8 OHdG immunohistochemistry

Method	IHC-paraffin embedded
Primary antibody	Goat polyclonal anti 8 OHdG
Negative control IgG	Goat IgG
Blocking serum	Horse serum
Secondary antibody	Horse anti goat antibody
Tissue development	DAB chromogen staining
Microscopy	Bright field, inverted microscope

SOD2 immunohistochemistry: Details of the methods is as described above and summarised in table 2.2

Table 2.2. SOD2 immunohistochemistry

Method	IHC-paraffin embedded
Primary antibody	Rabbit monoclonal antibody
Negative control IgG	Rabbit IgG
Blocking serum	Horse serum
Secondary antibody	Horse anti rabbit/mouse antibody
Tissue development	DAB chromogen staining
Microscopy	Bright field, inverted microscope

Heme oxygenase Immunohistochemistry: The details of method used for this immunostaining is as described above and summarised in table 2.3 below.

Table 2.3 Heme oxygenase immunohistochemistry

Method	IHC-paraffin embedded
Primary antibody	Rabbit polyclonal antibody
Negative control IgG	Rabbit IgG
Blocking serum	Horse serum
Secondary antibody	Horse anti rabbit/mouse antibody
Tissue development	DAB chromogen staining
Microscopy	Bright field, inverted microscope

Matrix metalloproteinase 2 immunohistochemistry: This is a mouse on mouse immunohistochemistry and details of the methods is described above and summarised in table 2.4 below.

Table 2.4: Matrix metalloproteinase 2 immunohistochemistry

Method	IHC-paraffin embedded, mouse on mouse
Primary antibody	Mouse monoclonal antibody (1:1000)
Negative control IgG	Mouse IgG (1:1000)
Blocking serum	MOM mouse IgG blocking reagent
Secondary antibody	MOM biotinylated anti-mouse antibody
Tissue development	Avidin/Biotin staining
Microscopy	Bright field, inverted microscope

Microscopy

Slides were viewed using the Leica DMI 4000B inverse microscope. The intensity was set to 465ms, exposure was 6.9, saturation was 44 and gain was 71. Lens power of 20 was used for microscopy. The intensity score was determined by examining 10 consecutive overlapping fields in the cortical and medullary areas of the kidney. Scoring was 1 when there was no staining and 2 when the staining was mild. Moderate staining was scored as 3 while deeply intense staining was scored as 4. Scores were entered on excel spreadsheet and the average stain intensity score calculated. These average scores were then entered in graphpad prism 6. The result was expressed as mean± SD for each slide.

III. RESULTS:

Semi-quantitative assessment of expression of superoxide dismutase (SOD2) in wildtype and Pkd2 -/+ heterozygous knockout mice.

Figure1 shows SOD2 immunostaining in non-stressed as well as ischemic reperfusion injury (stressed) kidneys of heterozygous Pkd2 KO and wild type mice. When quantified and there was a statistically significant difference in staining intensity score between wild type and heterozygous Pkd2 mice kidneys ($p<0.0001$).

Pkd2 mice showed reduced expression of SOD2.

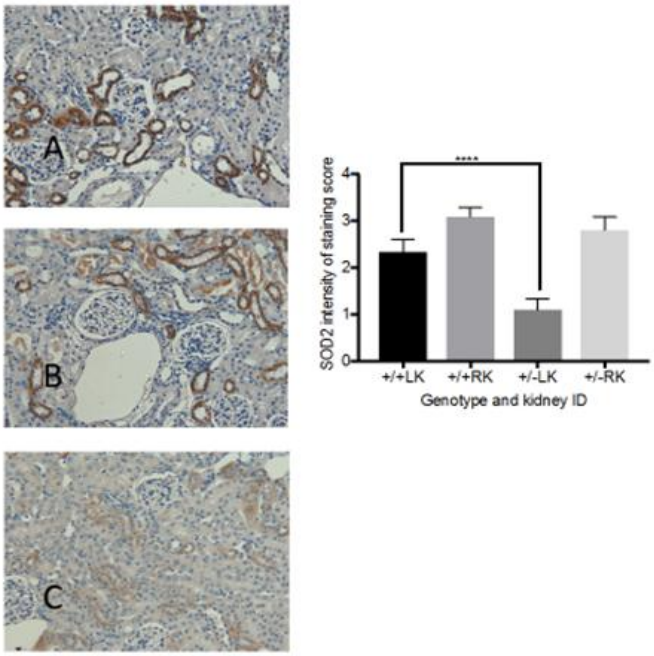

Figure1. Super oxide dismutase 2 immunostaining of non-stressed (A) and ischaemic reperfusion injury (IRI) kidneys of wild type (B) and heterozygous Pkd2 (C) mice. (****=p<0.0001).

Heme oxygenase 1 immunostaining results.

Figure 2 shows heme oxygenase 1 (HO1) immunostaining in non-stressed as well as ischemic reperfusion injury kidneys of wild type and heterozygous Pkd2 mice. When quantified, I found a greater level of HO1 staining in pkd2 heterozygous knockouts compared with wild type and this was statistically significant ($p<0.05$).

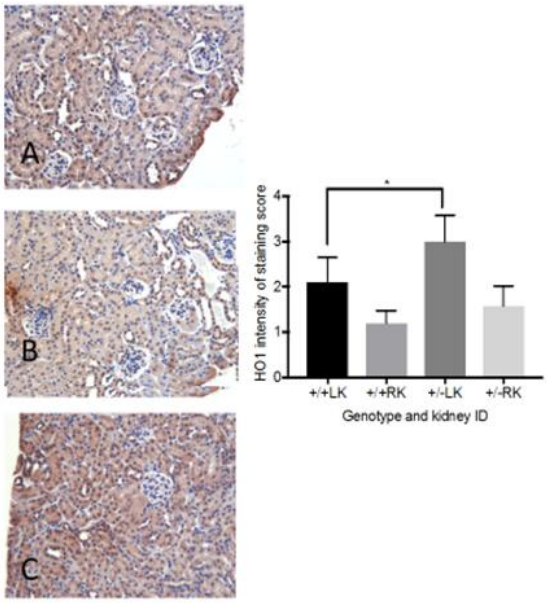

Figure 2 Heme oxygenase 1 immunostaining of non-stressed (A) and ischaemic reperfusion injury (IRI) kidneys of wild type (B) and heterozygous Pkd2 (C) mice. (*=p<0.05).

Hydroxyl deoxy guanosine (8 OHdG) immunostaining in mice mesenteric vessel and kidney tissue.

Figure3 shows 8OHdG immunostaining in non-stressed as well as ischemic reperfusion injury kidney (IRI) kidneys of wild type and heterozygous Pkd2 mice. There was significant difference in the intensity of staining between wild type and heterozygous Pkd2 mice ($p<0.05$)
Figure 4 shows 8OHdG immunostaining in mesenteric vessels of wild type and heterozygous Pkd2 mice. There was significant difference in the intensity of staining between wild type and heterozygous Pkd2 mice ($p<0.05$).

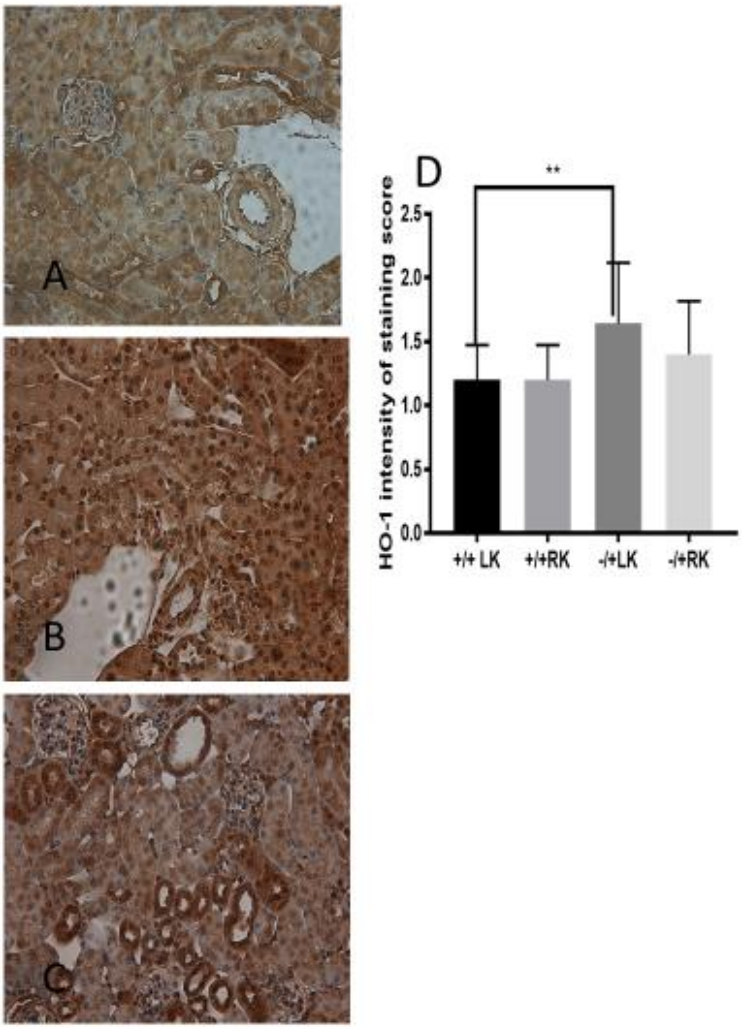

Figure 3. 8 Hydroxyl deoxyguanosine (8OHdG) immunostaining of non-stressed (A) and ischaemic reperfusion injury (IRI) kidneys of wild type (B) and heterozygous Pkd2 (C) mice. There was significantly greater

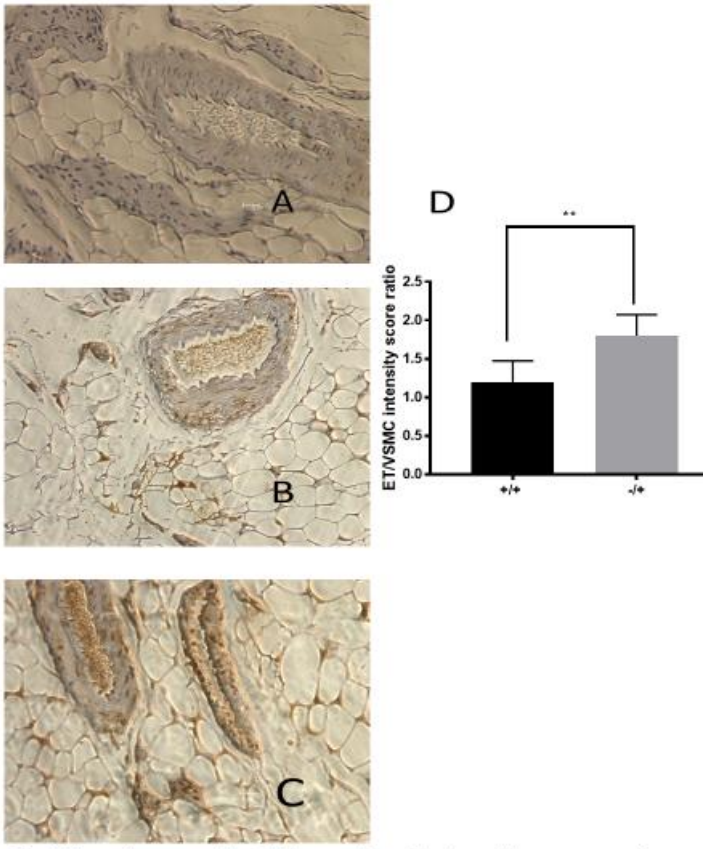

Fig 4 Negative control IgG (A) as well as 8 hydroxyl deoxyguanosine immunostaining of WT (B) and Heterozygous Pkd2 © mice mesenteric vessels. There was greater expression in HET Pkd2 (D)

Matrix metalloproteinase 2 immunostaining of kidney tissue.

Figure 5 shows mouse on mouse matrix metalloproteinase 2 immunostaining of wild type and heterozygous Pkd2 mice as well as IgG negative immunostaining. MMP2 expression was predominantly extracellular and was more significant (*=$p<0.05$) in HET Pkd2 KO mice

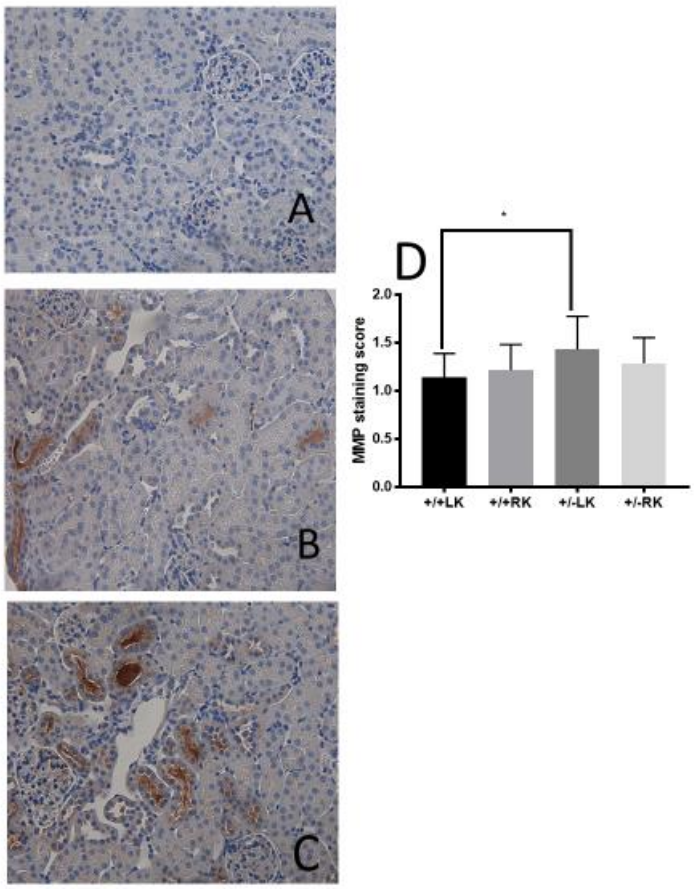

Fig 5 Negative control IgG (A) as well as matrix metalloproteinase 2(MMP2) immunostaining of WT (B) and HET Pkd2(C) mice kidney tissue. There was a significantly greater expression(*p<0.05) in HET Pkd2 KO mice.

IV. DISCUSSION

In this study, immunohistochemistry of three biomarkers of oxidative stress (8OHdG, SOD2 and HO-1) and one novel biomarker of endothelial dysfunction (MMP2) was performed. There was significant oxidative stress in heterozygous Pkd2 knock out mice as evidenced by detectionand quantification of SOD2 as well as HO-1. Similarly, there was increasedactivity of 8OHdG and MMP2 proteins in heterozygous Pkd2 KO mice.

Superoxide anions (SOA) are the commonest ROS under physiologic condition and they are spontaneously produced around the electron rich mitochondrial membrane, the respiratory chain(7) and endoplasmic reticulum(8). Superoxide dismutase is an enzyme that catalyse the dismutation of SOA into hydrogen peroxide or molecular oxygen. In this study, the activity of one of its isotypes, superoxide dismutase 2 was investigated in kidney tissue of wild type as well as heterozygous Pkd2 KO mice using immunohistochemistry. Agharazi and colleagues had reported decreased SOD2 expression in CKD rats (9). Similarly, Krueger et al investigated SOD2 protein expression in CKD patients and they reported that lower expression of this protein was associated with better survival(10).However, very little is known about the activity of this enzyme in ADPKD and this is despite its reported therapeutic potential as a cardio protective agent. There lower expression of SOD2 in heterozygous Pkd2 mice and this was statistically significant when compared with wild type. This suggested a greater level of oxidative stress in Pkd2. In a related study, Menon et al reported lower serum SOD in hypertensive ADPKD patients with no graded difference across the CKD stages(11). Also, in a study of mice with cardiac specific SOD2 overexpression, Kang and colleagues observed supernormal cardiac function which was produced due to the ability of this enzyme to effectively enhance mitochondrial function and subsequently promote metabolic vasodilation (12).

Furthermore, significantly greater expression of HO-1 in was detected in heterozygous Pkd2 when compared with wild type mice kidney. Heme oxygenase is an important enzyme for all organisms that depend on aerobic oxidation(13, 14). HO1, the inducible isoform of HO has been reported to be cytoprotective through its anti-inflammatory, antiproliferative and antiapoptotic effects(15). Reports about activities of HO1 in ADPKD is sparse. HO1 induction has beneficial effects and It has been reported as a potential therapeutic target in acute

kidney disease (16). This beneficial effects of HO1 were partly attributed to some of its metabolites such as Iron, carbon monoxide and bile pigments. There is paucity of information on tissue detection of this protein in non ADPKD as well as ADPKD chronic kidney disease, The finding in this study support the need for more investigations into role of HO-1 as a therapeutic option in ADPKD.

The observed significant activity of SOD2 as a biomarker of oxidative stress is of interest when considered alongside that of HO1. Both biomarkers had previously been reported to be important biomarkers of oxidative stress in other disease conditions with very few reports in ADPKD patients.This becomes more important now that there is search for effective treatment options (11, 17).

Furthermore, 8OHdG was detected and localised in mesenteric vessels as well as kidney tissue of heterozygous Pkd2 mice. Single electron reduction in molecular oxygen leads to production of reactive oxygen species (ROS) which have beneficial effect in aerobic organisms as it is involved in cell survival, signalling pathway and protection. Conversely, accumulation of ROS produces deleterious effects (18). Repair process that follows ROS-mediated DNA damage results in the formation of 8OHdG and it is the most recognised marker of oxidative stress when considering DNA damage products(19). However, there is paucity of data on its activity in ADPKD. 8OHdG was more significantly expressed in kidney tissue of Pkd2 KO mice, using immunohistochemistry. This is in agreement with reported activity of 8OHdG in other disease processes.(20, 21). While investigating the beneficial effect of KT on evolution of oxidative stress in patients with end stage kidney disease, Cerrillos-Guitierrez and colleagues reported significantly higher level of 8OHdG in patients with end stage kidney disease compared with healthy individuals. The level returned to normal within six months following kidney transplantation (22). Similarly, increased level of 8OHdG has been reported to be an independent predictor of all-cause mortality in hemodialysis patients(22). However, there are sparse reports about the activities 8OHdG as a biomarker of oxidative stress in ADPKD patients hence the finding of increased activity of 8OHdG in Pkd2 suggest a further investigation into its activities in ADPKD

In addition, there was a significantly increased expression of MMP2 in PKd2 mice and this was predominantly extracellular. Nagaredy el al investigated the role of MMP2 in endothelial function in animal model of acquired systolic hypertension and insulin resistance. They reported that increased activities of this protein produced impaired endothelial function and promoted hypertension(23). They suggested that MMP2 played a role in endothelial dysfunction probably through its ability to uncouple eNOS. This was novel as previous reports on MMP2 were mostly about its traditional role in enhancing digestion of extracellular structure.

In conclusion, there was significant oxidative stress in heterozygous Pkd2 mice as evidenced by increase activities of SOD2, HO1, 8OHdG and MMP2.

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