

Expression of Circulating CD146 Associated with Endovascular Dysfunction in Adenine-induced Chronic Renal Damage in Rats Using an EvaGreen Real-time RT-PCR Assay

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ABSTRACT

The integrity of the endothelial layer of the blood vessel, intima, is critical since damage can lead to atherosclerosis. Assessment of its integrity is therefore very important although this can not be done through routine diagnostic analysis. An alternative non-invasive method should be established to assess its integrity. The objective of this study was to develop a quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) using an EvaGreen dye for the assessment of vascular endothelial damage-associated with chronic renal damage in rats via quantification of the expression level of CD146, which is one of the blood biomarkers of endovascular dysfunction and/or damage. Comparison of the sensitivity of the EvaGreen qRT-PCR and the conventional RT-PCR was also performed in this study. For this purpose, a total of 16 male Sprague Dawley rats were divided equally into two groups, namely the control and adenine. Results showed that rats fed with diet containing 0.75% adenine for six consecutive weeks developed chronic renal damage through elevations in the blood urea nitrogen (BUN) and blood creatinine (Cr) levels, increased urine protein to creatinine ratio (UPC) and decreased creatinine clearance (CrCl). The confirmatory diagnosis of chronic renal damage was made through gross and histopathological examinations of the kidneys. The results were consistent with lesions of chronic renal damage. The expression of circulating CD146 was mildly increased in rats with chronic renal damage analysed using the EvaGreen qRT-PCR or the conventional RT-PCR, suggesting a mild degree of endovascular damage. The intensity of the EvaGreen qRT-PCR products, however, was brighter than the conventional RT-PCR, indicating that the EvaGreen qRT-PCR is more sensitive compared to conventional RT-PCR, which is further recommended for analysis of CD146 expression in rats.

Keywords: Adenine, CD146, vascular dysfunction and damage, chronic renal damage, real-time RT-PCR, conventional RT-PCR

INTRODUCTION

The National Kidney Foundation Task Force on Cardiovascular Disease in Chronic Renal Disease reported patients with chronic kidney

disease are at a higher risk of developing cardiovascular disease (CVD) (Levey *et al.*, 1998). The evidence of the relationship between renal dysfunction and adverse cardiovascular (CV) events was first recognised in the dialysis

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patients who showed high incidence of CV death. Several studies have reported approximately 50% of individuals with end stage renal disease (ESRD) die from CVD-related events, such as heart attacks (Foley *et al.*, 1998; Herzog *et al.*, 1998; Tonelli *et al.*, 2006). The development of CVD is suggested as the result of endothelial dysfunction and excessive oxidative stress. Endovascular dysfunction is an early feature of vascular disease in different diseases, such as chronic renal failure, diabetes, hypertension, hypercholesterolaemia, and coronary heart disease.

CD146, a membrane glycoprotein, is expressed in all human endothelium or vessel calibres. Expressed by the endothelial cells, it is one of the genes that is localised at the endothelial junction (Bardin *et al.*, 1996; 2001). In blood, the vast majority of blood cells that express CD146 are lymphocytes (Duda *et al.*, 2006). Hence, low level of the CD146 gene expression could be detected in healthy blood. Meanwhile, the increase in the number of circulating cells, including circulating endothelial cells (CECs) and lymphocytes expressing the membrane glycoprotein CD146, was observed in a wide variety of unhealthy conditions such as inflammatory, immune, infectious, neoplastic, and cardiovascular diseases which are likely to be evidence of profound vascular insult (Blann *et al.*, 2005). Koc *et al.* (2005) reported that elevation of CEC numbers conveys a risk of future vascular events. Studies conducted by Bardin *et al.* (2003), Malyszko *et al.* (2004), Faure *et al.* (2006) and Sally *et al.* (2009) demonstrated that circulating CD146 and CD144 were significantly elevated in human patients with uraemic chronic renal failure. Those patients usually show various pathologies associated with endothelial dysfunction, such as acute coronary syndromes (Mallat *et al.*, 2000), preeclampsia (Bretelle *et al.*, 2003) or antiphospholipid syndrome (Dignat-George *et al.*, 2004). The detection of circulating CD146 in those studies was performed via several means or methods such as ELISA, flow cytometry and real-time RT-PCR assays.

The objectives of this study were to develop a quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) using an EvaGreen dye for the assessment of vascular endothelial damage-induced by chronic renal damage in rats via quantification of the expression level of CD146, and to compare the sensitivity of the EvaGreen qRT-PCR and the conventional RT-PCR.

MATERIALS AND METHODS

Experimental Design

Both the control and Adenine groups equally comprised eight 6-week-old Sprague Dawley male rats. The rats in the control group were fed with normal rat pellets and water *ad libitum* throughout the 10-week experimental period. Meanwhile, the rats in the Adenine group were fed with ground rat pellets that were mixed with 0.75% Adenine (Sigma-Aldrich) and water *ad libitum* for 6 weeks (the treatment period), and this was continued with normal rat pellets and water *ad libitum* for 4 more weeks thereafter throughout the observation period. The rats were daily observed for clinical signs of renal failure.

Blood and Urine Analyses

Blood creatinine (Cr) and blood urea nitrogen (BUN) were analysed fortnightly during the treatment period. The blood samples were directly collected from the tail vein at weeks 0, 2, 4, 6, 8, and 10, and immediately analysed using an Automated Dry Chemistry Analyser (scil Reflotet® Plus, Roche Diagnostics) as per manufacturer's instruction. Both the blood and 12-hour urine samples were collected at the end of the experimental period. Urine volume was measured using a standard method. Serum Cr, urine Cr, and urine protein concentrations were analysed using an Automatic Biochemistry Analyser (Biorex, TRX7010, Manheim, Japan).

RNA Extraction from Fresh Whole Blood

The blood samples were taken from each rat via cardiac puncture at the end of the experiment.

RNA was extracted from the whole blood using a QIAamp RNA Blood Mini Kit from Qiagen (USA). The optical density (OD) of the extracted RNA was determined by measuring the absorbance at A260/A280 nm using a spectrophotometer (Eppendorf, German) and kept in -80°C until reverse transcription for cDNA synthesis.

Conventional RT-PCR Assay for Amplification of CD146 and GADPH Genes

The extracted RNA was converted into cDNA using a GeneAmp RNA PCR Core Kit (Applied Biosystems, USA), as per manufacturer's instruction. The cDNA products were stored at -20°C.

Master mix for the conventional PCR was prepared using sterile deionised water with the total reaction mixture of 20 uL containing 2 mM MgCl₂ solution, 1x PCR Buffer II, 200 uM for each dNTPs (Applied Biosystems, USA), 1.5 uM CD146/GAPDH forward and reverse primers (1st BASE, Malaysia), 0.05U AmpliTaq DNA polymerase (Applied Biosystem, USA), and 2 uL of sample containing 3 - 4 ng of cDNA. The amplification of the cDNA via conventional PCR was performed using a conventional PCR machine (Little Genius, BIOER). The amplification conditions were as follows: 1 cycle of 95°C for 2 minutes (predenaturation), followed by 30 cycles of 95°C for 30 seconds (denaturation), 51.2°C (for CD146) and 50.0°C (for GAPDH) for 30 seconds (annealing), 72°C for 30 seconds (extension), and 1 cycle at 72°C for 7 minutes for the final extension.

Meanwhile, the primers for the GADPH gene (designed using the Oligo Primer Analysis Software v.7) were: GAPDH forward: 5'-TGTGAAACGGATTTGGCCGTA-3', and GAPDH reverse: 5'-CATTGATGTTAGCGGGATC-3'. The size of the amplification product is 240 bp (GenBank accession number: NM017008). All the CD146 amplification products were normalised with the GAPDH gene.

Real-time RT-PCR Assay for the Amplification of CD146 Gene

The same cDNA samples that were converted from the total RNA were used. Meanwhile, the master mix for the qPCR was prepared with the total reaction mixture of 20 uL containing 10 uL ImmoMix™ (Bioline, USA), 1 uL EvaGreen™ Dye (Biotium, USA), 1.5 um CD146 forward and reverse primers (1st BASE, Malaysia), as well 2uL of sample containing 3 - 4 ng of cDNA mixed with sterile deionised water in a single qPCR tube for each reaction. The amplification of CD146 gene was done using a real-time PCR machine (Biorad CFX96), with the following amplification conditions: 1 cycle of 94°C for 15 minutes (predenaturation), followed by 35 cycles of 94°C for 15 seconds (denaturation), 51.2°C for 30 seconds (annealing), 72°C for 45 seconds (extension).

The primers for CD146 gene used in this study were those previously described by Taira *et al.* (2004) and Hazilawati *et al.* (2010). The size of the PCR products is 100 bp.

Gel Electrophoresis

The PCR products were electrophoresed at 80V for 60 minutes in 1.5% agarose gel, stained with 0.5 ug/mL ethidium bromide solutions for 15-20 minutes, and visualised under UV transillumination (Alpha Imager, GEL DOC). The intensity of the bands of CD146 and GADPH in the control and Adenine groups was also compared.

Gross Lesion and Histopathological Examination

All the rats were sacrificed at the end of the 10-week experimental period. Both the left and right kidneys were collected and fixed in 10% formalin. The kidneys were processed with a standard protocol for Haematoxylin and Eosin (H&E) staining for histopathological examination.

Statistical Analysis

The results were analysed using SPSS version 17.0 for ANOVA and differences at $p<0.05$ were considered as significant.

RESULTS AND DISCUSSION

Blood Urea Nitrogen (BUN) and Blood Creatinine (Cr)

Figs. 1 and 2 show the blood urea nitrogen (BUN), and blood creatinine (Cr) concentrations of the control and Adenine groups throughout the 10-week experimental period. It was found that the baseline value for BUN and blood Cr concentrations of the control and Adenine groups were 9.33 ± 0.57 mmol/L and 7.97 ± 0.63 mmol/L, respectively, and 44.20 ± 0.00 μmol/L for both groups. The rats in the Adenine group had gradual increase in the blood Cr and BUN concentrations throughout the treatment period. The highest values were observed at week 6; BUN was significantly increased to 38.05 ± 7.11 mmol/L and blood Cr was significantly increased to 201.30 ± 43.85 μmol/L. Those parameters were decreased about half of

the peak values during the observational period, although it was still significantly higher than the control group and/or normal ranges. A similar study conducted by Okada *et al.* (1999) showed Wistar rats were more susceptible to chronic renal failure induced by adenine. Blood Cr and BUN levels in their study were higher than those obtained in this study, whereby they reported 67% of the adenine-treated rats died of chronic renal failure during the study. In this study, the mortality rate was 0%.

Urine Protein to Creatinine Ratio (UPC) and Creatinine Clearance (CrCl)

Figs. 3 and 4 show UPC and CrCl of the control and adenine-treated rats. Even though BUN and blood Cr concentrations dropped during the observation period, albeit still significantly higher than the normal values, the UPC and CrCl results were consistent with the renal insufficiency. The UPC of the control rats was 3.38 ± 1.61 , while the UPC of the adenine-treated rats was 16.07 ± 19.37 , which was significantly five times higher than the control.

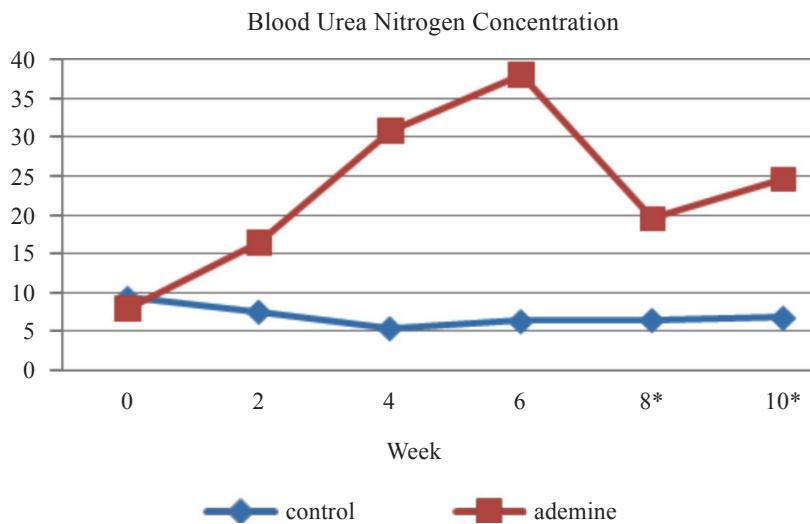


Fig. 1: The BUN concentrations of the Adenine group were gradually increased from week 0 to 6 (treatment period), and decreased at weeks 8 and 10 (observation period*).

The BUN results (weeks 2 to 10) for the rats in the Adenine group were significantly different at $p<0.05$ from those rats in the control group

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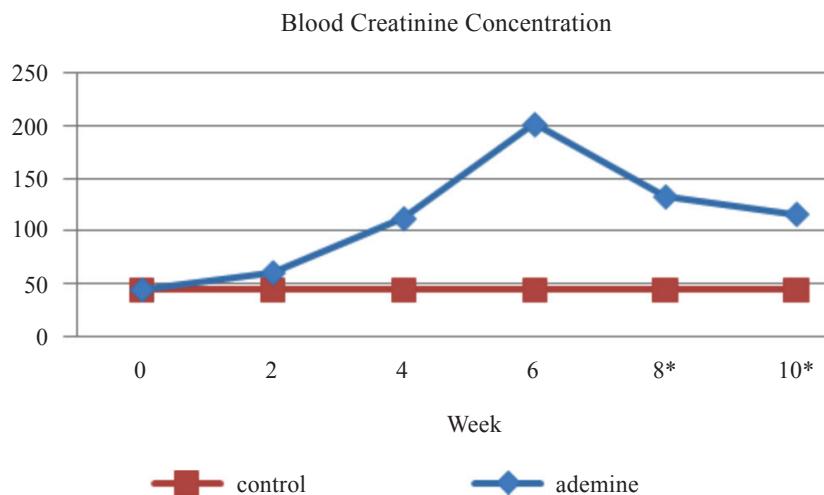


Fig. 2: The blood creatinine concentrations of rats in the Adenine group show a similar trend in their BUN concentrations. The results (weeks 4 to 10) were significantly different at $p<0.05$ from those rats in the control group

The CrCl for the control and adenine groups were significantly different, with 59.72 ± 38.30 and 12.48 ± 10.91 mL/hour, respectively. It was clearly showed that the rats in the Adenine group were unable to remove creatinine in the body as a result of the damage to the renal.

Detection of Circulating CD146 via EvaGreen qRT-PCR and Conventional RT-PCR

In this study, the transcription of CD146 in the blood was determined by both the qRT-PCR and conventional RT-PCR assays. Other than the comparison of the threshold cycle (C_t) from the amplification graph of the control and adenine groups, the intensity of CD146 bands from the

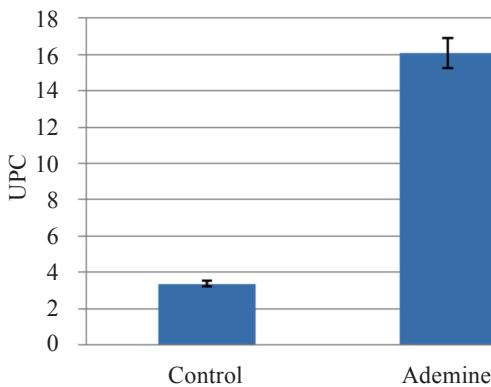


Fig. 3: The UPC of the rats in the Ademeine group was significantly ($p<0.05$) increased five times higher than the control group

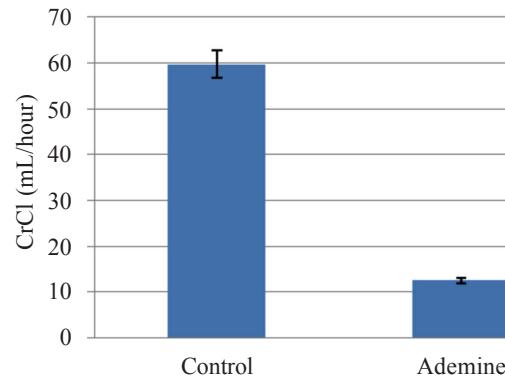


Fig. 4: The clearance of creatinine (CrCl) from the body through the urine of rats in the Ademeine group was significantly ($p<0.05$) decreased compared to those rats in the control group. The results were comparable to the blood creatinine concentrations of the rats shown in Fig. 2

EvaGreen qRT-PCR and the conventional RT-PCR assays were also compared.

Figs. 5 and 6 show the amplification products of GAPDH and CD146 genes, respectively, from the control and adenine-treated rats amplified using the conventional RT-PCR. All the rats in both groups had expressed the GAPDH gene (*Fig. 5*). Meanwhile, the intensity of the bands was almost similar, indicating that the quality and quantity of the extracted RNAs were similar between the groups. The amplification of CD146 transcripts via the conventional method, however, was more difficult as both groups showed very faint bands, albeit the intensity of the bands was slightly brighter in the adenine group (*Fig. 6*).

Further investigation on the level of transcription of CD146 in the control and Adenine groups, via the EvaGreen qRT-PCR assay, revealed that the mean threshold cycle (C_t) of the adenine-treated rats was not significantly different from the control, 21.23 ± 0.38 (Adenine group) and 20.96 ± 0.79 (control group) (*Fig. 7*). The band intensity of the EvaGreen qRT-PCR products of the adenine-treated rats was slightly brighter and thicker than the control rats (*Fig. 8*),

although the concentrations of the PCR products were almost comparable to the control (*Fig. 8*). This indicates that rats with chronic renal damage had a very mild increase in the expression of CD146 in the blood circulation, reflecting the dysfunction or damage of the endovascular wall. The findings of this study therefore suggested that the daily feeding of 0.75% adenine diet to rats for 6 weeks, followed by normal diet for 4 more weeks after that might have not induced significant damage to the endothelial layer of the blood vessels. Some previous studies reported that significant higher expression of CD146 has been observed in chronic renal failure patients analysed using ELISA (Baldin *et al.*, 2003; Malyszko *et al.*, 2004), flow cytometry (Faure *et al.*, 2006) and also real-time RT-PCR assay (Sally *et al.*, 2009).

A comparison of the intensity of bands between the conventional RT-PCR and the EvaGreen qRT-PCR assays clearly showed that the EvaGreen qRT-PCR assay is far more sensitive as compared to the conventional RT-PCR assay (*Figs. 6 and 8*). The results are in agreement with the findings reported by Di Trani *et al.* (2006) and Gurukumar *et al.* (2009) for the

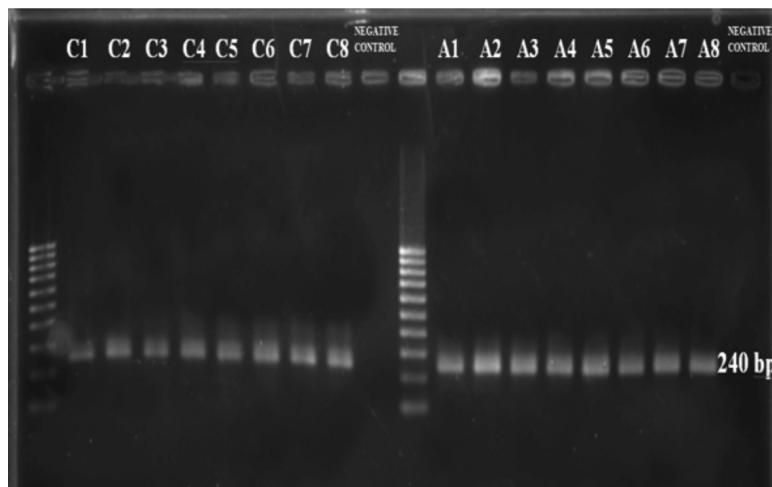


Fig. 5: Bands of the GADPH amplification products (240 bp) of the control (C1 to C8) and chronic renal damage rats (A1 to A8) amplified using the conventional RT-PCR assay. The amplifications of the control rats are labelled as C1 to C8, whereas the amplifications of the Adenine rats are labelled as A1 to A8. The DNA markers (Bioline, UK) are in lanes 1 and 11. Negative controls showed no amplification

detection of avian influenza and dengue viruses, respectively.

Gross and Histopathological Findings of the Kidneys

Fig. 9 shows gross and histopathological lesions of the kidneys of rats with chronic renal damages. All the rats that were treated with adenine had enlarged kidneys (*Fig. 9A[a]*) with generalised yellowish discolouration (stone like colour) of the renal parenchyma, which were consistent with severe renal damage. Microscopically, the kidneys had abnormal glomeruli (*Fig. 9B[c]*), crystal of adenine in the interstitial tissues (*Fig. 9B[d]*), infiltration of inflammatory cells in the interstitial tissues (*Fig. 9B[e]*), infiltration of inflammatory cells in the renal tubules (*Fig. 9C[f]*), necrotic cells (*Fig. 9D[g]*), tubular dilatation (*Fig. 9D[h]*), interstitial fibrosis (*Fig. 9E[i]*) and protein casts in the renal tubules (*Fig. 9F[j]*). The results were comparable to those of the previous reports (Yokozawa *et al.*, 1986; Tamagaki *et al.*, 2005).

Several studies, via ultrasound, vascular markers' measurements, vessel wall movement detector and occlusion plethysmography, have

suggested that chronic renal failure is associated with impaired endothelial function (Guldener *et al.*, 1998; Bolton *et al.*, 2000). However, the precise mechanisms of chronic renal failure that induces endovascular dysfunction are still unclear. Several possibilities have been proposed, and these include oxidative stress-related accumulation of uraemic toxins and chronic inflammation (Stenvinkel *et al.*, 1999). Other possible causes of endovascular dysfunction are accumulation of oxidised low-density lipoprotein (oxLDL) (Stenvinkel *et al.*, 1999) and elevation of asymmetric dimethylarginine (ADMA), a competitive inhibitor of endothelial nitric oxide (NO) production (Zoccoli *et al.*, 2001).

CONCLUSION

In conclusion, the study has demonstrated that circulating CD146 is mildly expressed in rats with chronic renal damage, and the EvaGreen qRT-PCR assay is more sensitive compared to conventional RT-PCR assay, and it is thus recommended for the analysis of CD146 expression in rats with endovascular dysfunction and/or damage.

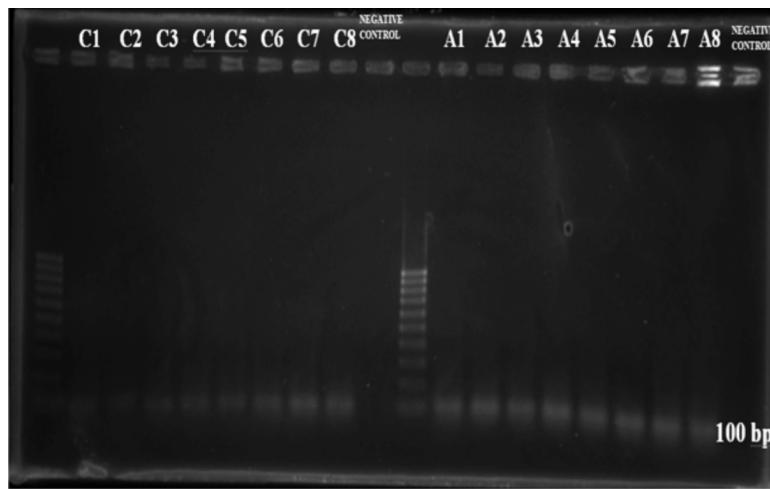


Fig. 6: Bands of CD146 amplification products (100 bp) of control (C1 to C8) and chronic renal damage rats (A1 to A8) amplified using the conventional RT-PCR assay. Amplicons of control rats are labeled as C1 to C8 and amplicons of Adenine rats are labeled as A1 to A8. DNA markers (Bioline, UK) are on lanes 1 and 11. Negative controls show no amplification

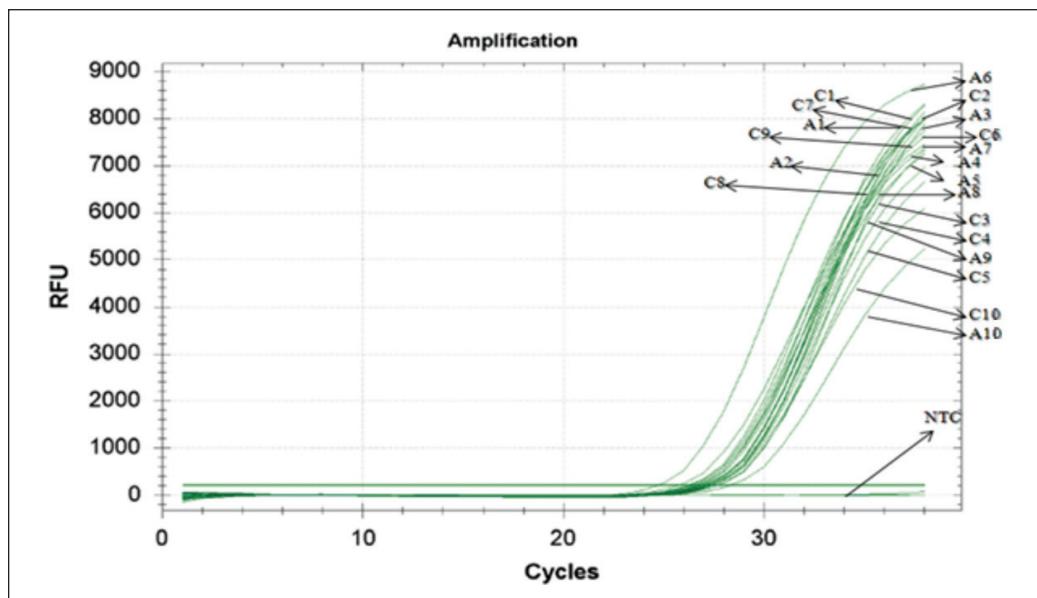


Fig. 7: Amplification graph of CD146 of the control (C1 to C8) and adenine-treated rats (A1 to A8). The melting curve peak for each sample clearly showed that all the amplified genes are products specific of CD146 with the melting temperature of $80.5^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$

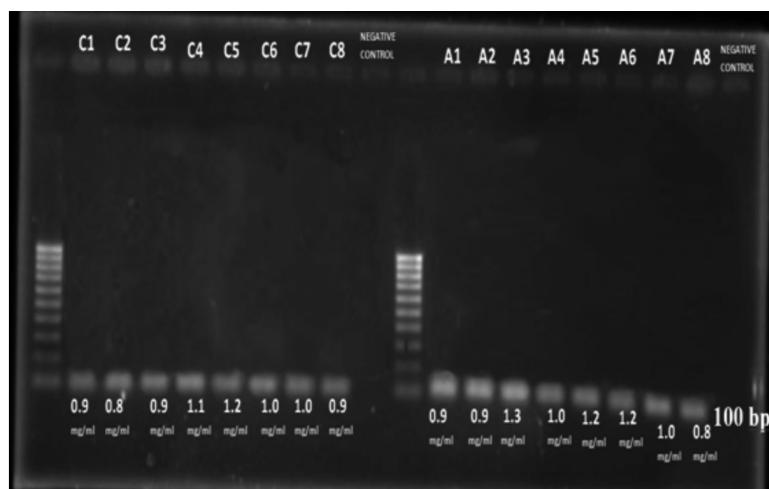


Fig. 8: Bands of CD146 amplification products (100 bp) amplified using the EvaGreen qRT-PCR assay. The amplifications of the control rats are labelled as C1 to C8, whereas the amplifications of the Adenine rats are labelled as A1 to A8. The DNA markers (Bioline, UK) are given in lanes 1 and 11. Negative controls show no amplification

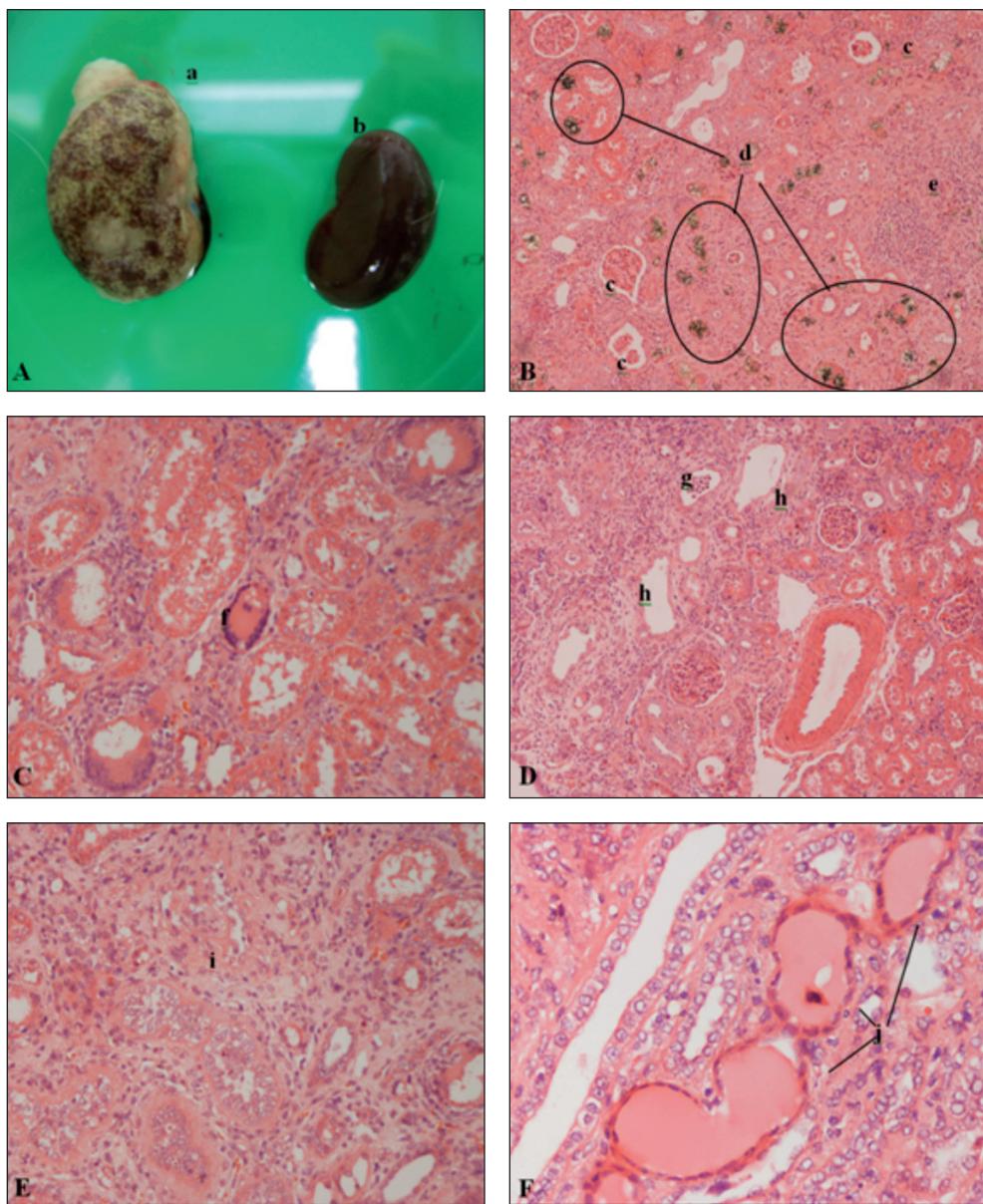


Fig. 9(A) a: Enlarged kidney with generalised yellowish discolouration, b: normal kidney; (B) c: abnormal glomeruli, d: adenine crystals, e: infiltration of inflammatory cells in the interstitial tissues; (C) f: infiltration of inflammatory cells in the renal tubule; (D) g: necrotic cells, h: tubular dilatation; (E) i: interstitial fibrosis; (F) j: protein casts

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