CYCLOSPORINE A-INDUCED NEPHROTOXIC SPRAGUE-DAWLEY RATS ARE MORE SUSCEPTABLE TO ALTERED VASCULAR FUNCTION AND HAEMODYNAMICS

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ABSTRACT

Cyclosporine A (CsA) is widely employed in the management of solid organ transplantation but often brought occurrence of nephrotoxicity. The present study investigates its potential deleterious effects on the cardiovascular and renal excretory function as well as on the renal haemodynamics. Male Sprague Dawley rats (n=16) were randomized into two groups viz. control (CT) and CsA treated group (CS). CS rats received CsA for 21 days at 25mg/kg/day p.o. Renal excretory functions were determined at day 0 and day 21 respectively. The vascular and renal heamodynamics were measured at the end of treatment period. Pulse pressure and pulse wave velocity was used as the markers of vascular stiffness. Data are presented as Mean±SEM and were analyzed by *student's t-test* with significance at (p<0.05). CS rats showed significant (P<0.05) reduction in body weight (CT: 271±8 g; CS: 215±9 g) fluid intake (CT: 27±1 ml; CS: 24±1 ml), creatinine clearance (CT: 5.1±0.2 ml/min/kg; CS: 3.4±0.1 ml/min/kg), glomerular filtration rate (CT: 1.4±0.2 ml/min/kg; CS: 0.5±0.1 ml/min/kg), fractional excretion of sodium (CT: 0.7±0.1 %; CS: 0.1±0.0%) and fractional excretion of potassium (CT: 29.0±4.8 %; CS: 10.9±1.1 %) compared to CT rats. Systolic blood pressure (CT: 117±2 mmHg; CS: 145±1 mmHg) together with urinary protein excretion (CT: 25.8±1.9 mg/ml/kg; CS: 49.3±1.3 mg/ml/kg) were elevated (P<0.05) in CS rats. The baseline renal cortical blood perfusion (CT: 181±8 BPU; CS: 158±3 BPU) was blunted in CS group compared to CT group. Besides that, the pulse pressure (CT: 32±2 mmHg; CS: 38±1 mmHg) and pulse wave velocity (CT: 3.7±0.1 m/s; CS: 5.0±0.2 m/s) were observed slightly higher in CS group than CT group. The current study suggests that CsA induced renal failure rats experienced attenuation in renal cortical blood flow and high blood pressure. This was the consequence of the higher sympathetic and renin-angiotensin system activity. High index of pulse pressure and pulse wave velocity indicated that CsA treated rats were more susceptible to cardiovascular disorder than non CsA treated rats.

Key words: Cyclosporine A, Haemodynamics, Pulse wave velocity, Renal failure, Renal cortical blood perfusion.

INTRODUCTION

Cyclosporine A (CsA), the primary calcineurin inhibitor which is now widely employed as an immunosuppressive agent for clinical application after solid organ transplantation procedure to improve the long term survival and prevention of graft rejection[1]. However, its toxic side effects are common. Hence, its clinical use is somehow limited by its nephrotoxicity effect and the development of hypertension[2]. The most often reported complications include cardiovascular dysfunction such as arterial stiffness and tachycardia are observed. These latter indications of toxicity have been attributed to the stimulation of sympathetic nervous system and renin-angiotensin system which are in part, driven by the local angiotensin II type 1 (AT[1]) receptor activation by angiotensin II[3].

The adverse nephrotoxicity is often characterized by the histological and functional studies respectively. An elevation of plasma creatinine (PCr), reduction in creatinine clearance (ClCr), and a decrease in the sodium and potassium excretion could be an obvious indication of renal functional insufficiency. The renal morphological damages such as interstitial fibrosis, tubular atrophy and arteriolopathy might also be an indication for the development of renal failure[4]. Therefore, the manifestation of the decreased glomerular filtration rate (GFR) and retention of salt and water, as well as proteinuria were related to the nephrotoxic effect of CsA administration which later leads to the constellation of renal haemodynamics[5,6].

Hypertension has been noted to occur as early as a few weeks after administration of CsA in heart transplantation patients[7]. Recent studies have shown that increased aortic stiffness in many pathological conditions, with aortic stiffness is used as a strong indicator and independent predictor of coronary risk in subjects with normal or high blood pressure and chronic renal failure[8]. Therefore, Pulse Wave Velocity (PWV) and pulse pressure (PP) is a suitable index of arterial stiffness and a strong determinant of the cardiovascular disorder. Furthermore, its prognostic value has been repeatedly emphasized. To better understand the effect of the chronic administration of CsA on renal and cardiovascular function, the aims of this work were to evaluate whether CsA affects the renal haemodynamics and vascular dysfunction in rats model.

MATERIAL AND METHODS

Animals

Sixteen Male Sprague-Dawley (SD) rats [180g-230g] were procured from the Central Animal Facility of Universiti Sains Malaysia, Penang, Malaysia. All experiments were performed in accordance to the approval of the Animal Ethics Committee of University Sains Malaysia (PPSG/07(A)/044/2010/57/187). Firstly, all rats were allowed to acclimatize to the environment in the animal transit room for a period of 7 days in a standard condition (25ºC, 60-70% humidity) with a 12h:12h day light dark cycle. All animals had free access to water and commercial rodent chow (Gold Coin Sdn. Bhd., Penang, Malaysia). The rats were then randomly assigned into two groups. Control (CT; n=8) and CsA treated renal failure (CS; n=8) and continued to receive standard chow and tap water ad libitum. Administration of CsA (Neoral®, Novartis, Basel, Switzerland) was done by oral gavage at a dose of (25 mg/kg/day) as previously mentioned for a period of 21 days[9].

Measurements

Body weight, fluid intake, urine excretion and systolic blood pressure of each rat were measured at the beginning and at the end of treatment period. Blood and urine samples were also collected from rats placed in metabolic cages (Nalgene®, Thermo Scientific, Philadelphia, USA) for 24 hours. The collected samples were immediately frozen at -70 ºC for further analyses of sodium, potassium, total protein and creatinine content. Creatinine clearance (CrCl), fractional sodium excretion (FE$_{Na^+}$), fractional potassium excretion (FE$_{K^+}$) and glomerular filtration rate (GFR) were calculated

using standard equations and studied[10]. The kidney index (KI) was also measured using both left and right kidneys after the renal haemodynamic experiment[14]; the right kidney was collected and stored in 10% formalin for histological examination using heamatoxylin and eosin staining[11].

Blood Pressure measurements

Systolic blood pressure (SBP) and heart rate (HR) were measured in conscious rat using a tail cuff plethysmography technique (NIBP Controller, ADInstruments®, Sydney, Australia) on day 0 and day 21 following a 3-day training period. The measurements were performed in a noise-free and dark room without applying external heat as previously reported[12]. An average of five readings were taken from each rat with the difference of each reading not more than 5 mmHg are preferably selected for the study.

Urine and plasma collection for renal functional estimations

Urine and blood samples were collected and centrifuged at 3500 rpm for 10 minutes immediately after the metabolic studies, to eliminate any precipitants in urine and blood samples. Then, plasma and urine supernatant were collected and kept frozen at -70ºC until analyzed. Creatinine and protein were measured using a spectrophotometric (Power Wave X340, Bio.Tek Instrument Inc., USA) method while sodium and potassium were measured using a flame photometer (Hitachi, Japan)[13].

Animal surgical preparation for haemodynamic study

The animals were fasted overnight with at least 12 hours and more but had free access to drinking water. Then, 60 mg/kg i.p. sodium pentobarbitone (Nembutal, Ceva, Santé Animale, Libourne, France) was used to anesthetize the animals. The loss of conscious state was determined by the loss of reflexes of eyelid and when the tail or leg was pinched. Following this, the animal was placed with its dorsal side on the surgical board. Tracheostomy was performed via a small incision in the neck region and cannulated (PE 240, Portex, Kent, UK) to provide clear air passage throughout the experiment and the left jugular vein was cannulated (PE 50, Portex, Kent, UK) as a preparatory line for maintenance dose of anesthesia or fluids whenever required. Another cannula was inserted into the left carotid artery and connected to a pressure transducer (Model P23 ID Gould, Statham Instrument, UK) which was linked to a computerized data acquisition system (PowerLab®, ADInstruments, Sydney, Australia) for continuous monitoring of blood pressure. A midline abdominal incision was done to expose the left kidney and its renal artery using an electrical cutlery knife (Medel HDCS, Rimmer Brothers) to minimize abdominal bleeding. Immediately, a piece of damp cotton wool was placed on the exposed kidney to prevent the organ from dehydration. Later, the cannulation of the left common iliac artery were done with its cannula attached to a

second pressure transducer (Model P23 ID Gould, Statham Instrument, UK) which was also connected to a computerized data acquisition system (PowerLab®, ADInstruments, Sydney, Australia) for the measurement of renal arterial pressure[14].

Measurement of baseline renal cortical blood perfusion (RCBP), PWV and Pulse Pressure (PP)

Upon completion of the operative procedure, one hour stabilization period was given to allow equilibration. A laser flow meter probe (EP 100 series, Carolina Medical Instrument, USA) was then placed on the kidney for renal blood flow measurement. This probe was connected to a Square-Wave Electromagnetic Flow Meter (Model FM501 King, NC, Carolina Medical Electronic Inc.) that was also linked to the PowerLab® computerized data acquisition system. The PWV and PP data were also recorded using this system[8,16]. The distance between the proximal aorta catheter and dorsal aorta catheter were measured and to be used as one of the parameter for PWV measurements. PWV was calculated by dividing the propagation distance (d) by the propagation time (t) and measured in meters per second[7].

Statistical Analysis

The results in renal haemodynamic measurements were determined off-line using (Lab Chart 6, ADInstruments, Sydney, Australia) software. Data were presented as mean±S.E.M. Statistical analysis of the metabolic and heamodynamic studies and other data were performed under student's *t-test* followed by the Benferroni *post hoc test* using the statistical package (Superanova, Abacus In., CA, USA). The differences between the means were considered significant at 5% level.

RESULTS

Metabolic and renal functional parameters (Table 1) summarizes the metabolic and renal functional data for each group of animals. Animals in CT group showed normal body weight gains for the whole experiment period. Whereas, body weight in CS group reduced by approximately 20% (P<0.05) on day 21 compared to day 0. A significant (P<0.05) decreased in fluid intake was also observed in CS group by 12% (P<0.05) after 21 days. There was a 33% (P<0.05) reduction in CrCl observed in this experiment by which the GFR were found to be 62% (P<0.05) less compared to CT animals. A similar observation was also found on FE_{Na^+} and FE_{K^+} in CS animals with the reduction 83% (P<0.05) and 64% (P<0.05) respectively. There was a 35% reduction (P<0.05) of urinary Na+: K+ found at the end of the treatment period. The ability of the animal's kidney to retain the protein substances were also drastically reduced as this was indicated by the urinary protein excretion (UPE) with 48% (P<0.05) of protein substances were detected in urine sample. Indeed, the KI was 31% (P<0.05) higher compared to CT group.

Table 1: Metabolic and renal functional parameters of 21 days of CsA administration

| Parameter	Group	n	Day 0	Day 21
Body weight(g)	C	8	229±7	271±8
	CS	8	244±4	215±9*
Fluid Intake(ml/kg/day)	C	8	36±1	27±1
	CS	8	35±3	24±1*
CrCl (ml/min/kg)	C	8	5.2±0.1	5.1±0.2
	CS	8	5.1±0.1	3.4±0.1*
FE_{Na} (%)	C	8	0.7±0.1	0.7±0.1
	CS	8	0.6±0.1	0.1±0.0*
FE_{K} (%)	C	8	30.0±5.5	29.0±4.8
	CS	8	29.8±4.8	10.9±1.1*
GFR (ml/min/kg)	C	8	1.4±0.1	1.4±0.2
	CS	8	1.3±0.1	0.5±0.1*
UPE (mg/ml/kg)	C	8	27.9±2.2	25.8±1.9
	CS	8	25.2±2.4	49.3±1.3*
UrinaryNa+: K+ratio	C	8	0.24±0.00	0.23±0.01
	CS	8	0.22±0.01	0.15±0.02*
Kidney index (%)	C	8	-	0.33±0.01
	CS	8	-	0.43±0.01*

Values are presented as mean±S.E.M, * indicates P<0.05 compared to Day 0. Data were analyzed by repeated measures ANOVA followed by Bonferroni *post hoc* test.

Histopathology study

(Fig.1A) and (Fig.1B) showed the results of the histology study using haematoxylin and eosin staining light microscopy technique in CT group and CS group respectively. The kidney from those animals treated with CsA exhibited mild ischaemic tubular damage which was labeled as **[5]**. Besides that, subcapsular area also showed inflammation with abscess neutrophils and surrounded by foamy macrophages, this was labeled as **[4]**. The glomerular apparatus of CsA treated animals showed deformation and was classified as striped interstitial fibrosis with thickening of the tubular basement membranes as well as in Bowmen's capsules where it was labeled as **[3]. (**Fig.1A) showed normal architecture of a healthy kidney with glomerulus and proximal tubules labeled as **[1]** and **[2]** respectively.

Haemodynamic study

(Fig.2A) depicted the systolic blood pressure of CT and CS animals respectively. There was a normal SBP on CT group animals throughout the whole experiment period. However, animals from CS group showed significant elevation of SBP from 117 mmHg to 145 mmHg with extra 28 mmHg (P<0.05) of BP were raised. (Fig.2B) showed the pulse pressure (PP) difference found in CT and CS group. CS animals were found to have approximately 19% (P<0.05) of PP increased compared to CT animals. (Fig.2C) demonstrated the difference of PWV between the two studied groups. CS animals had 35% (P<0.05) PWV higher than CT animals. (Fig.2D) represent heart rate per minutes, CS animals found to have 20% higher (P<0.05) beat per minutes compared to CT animals. As for the study of the renal cortical blood perfusion (RCBP), CS animals had 15% less (P<0.05) blood flow into their kidney as compared to CT animals (Fig.2E). (Fig.3A&3B) showed the time difference between CT and CS group taken during the surgery heamodynamics study. The time difference at the minimal values of proximal and distal blood pressure (t) demonstrated 50% shorter (P<0.05) in CS group compared to CT group.
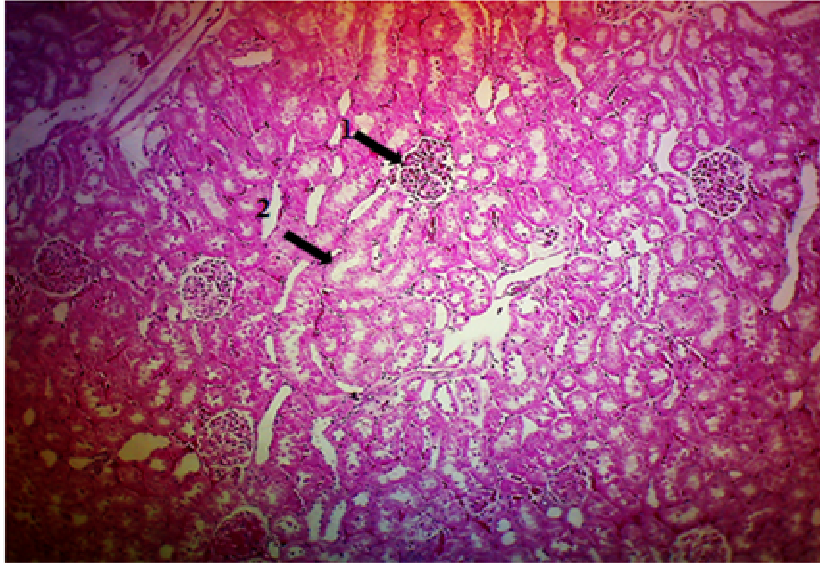
Fig. 1A: Photomicrographs of haematoxylin and eosin staining light microscopy in SD rat renal tissue from C group. (Magnification: 100x). [1] Indicate normal appearance of glomerulus; [2] indicate normal appearance of proximal convoluted tubules.
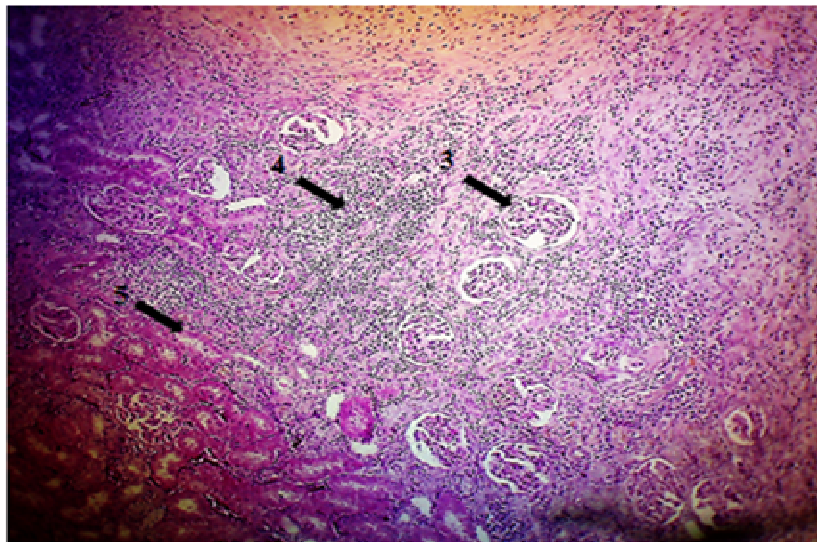
Fig. 1B: Photomicrographs of haematoxylin and eosin staining light microscopy in SD rat renal tissue from CS group. (Magnification: 400x). There is severe disruption in the glomerulus [3] and tubular structure [5] mainly in subcapsular area with [4] abscess (neutrophils) inflammation surrounded by inflammatory cells and foamy macrophages.
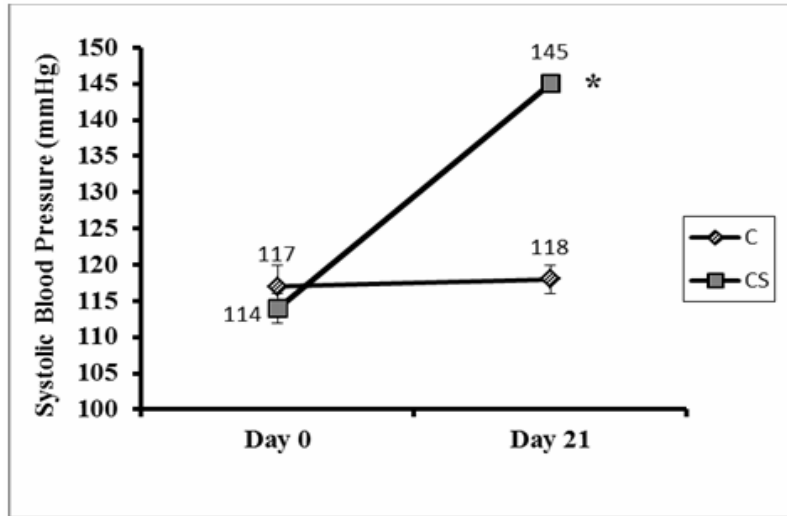
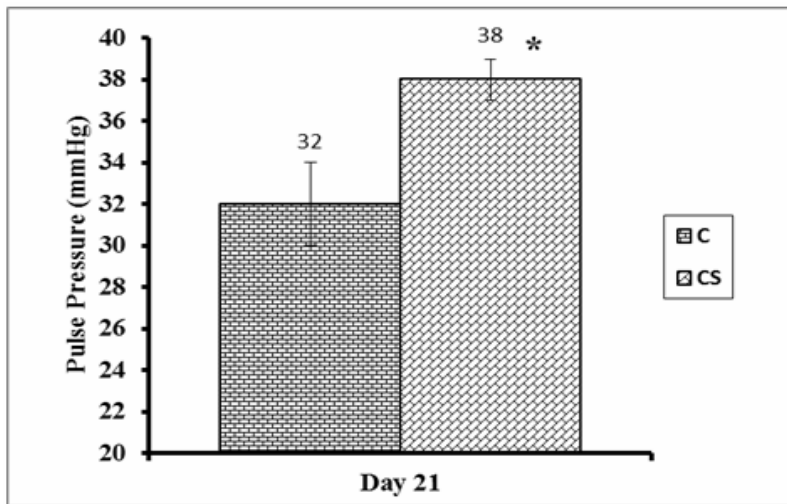**Fig. 2A: Effect of 21 days of CsA administration on Systolic Blood Pressure.**

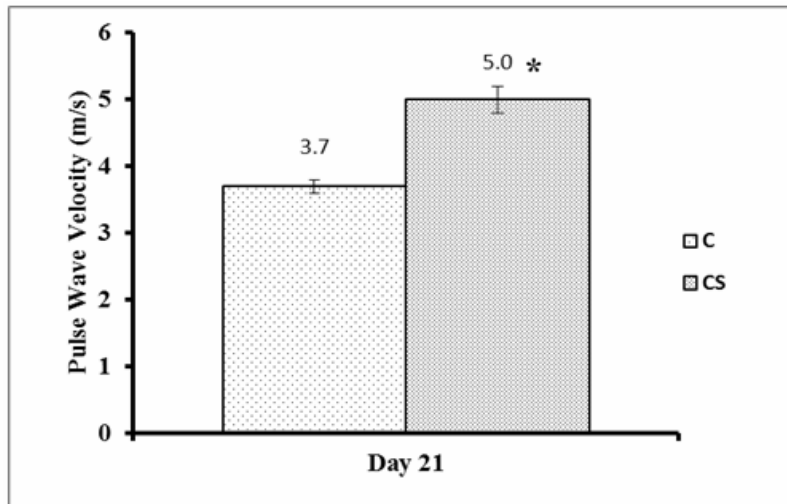**Fig. 2B: Effect of 21 days of CsA administration on Pulse Pressure.**

Fig. 2C: Effect of 21 days of CsA administration on Pulse Wave Velocity.
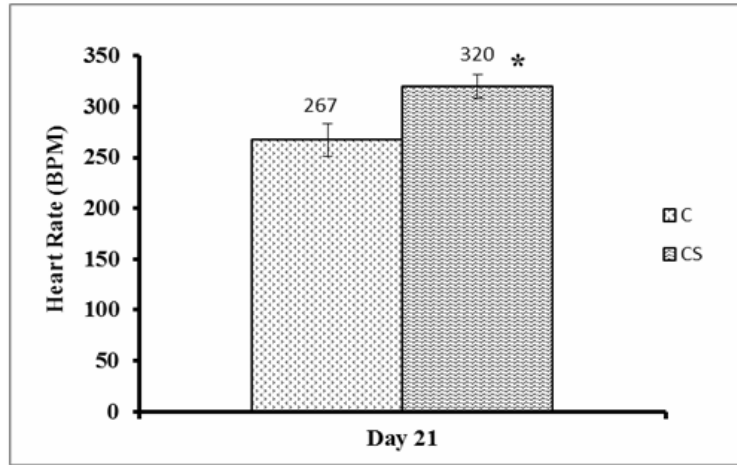
Fig. 2D: Effect of 21 days of CsA administration on Heart Rate.
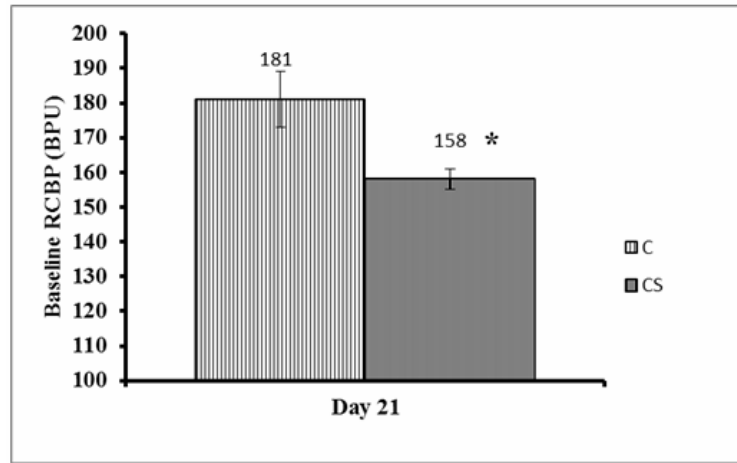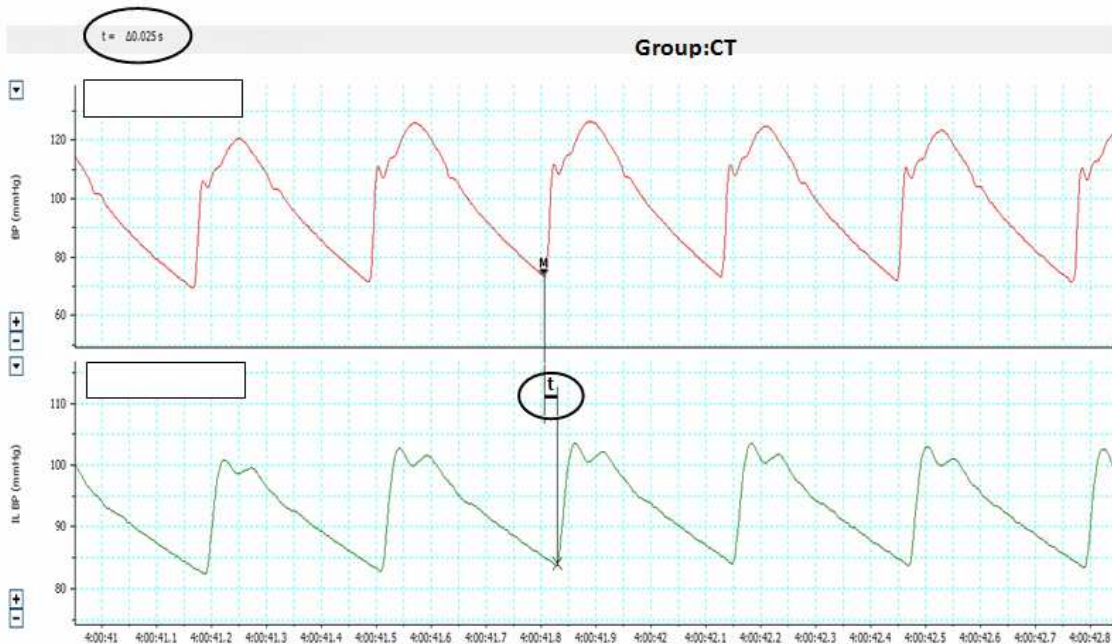

Fig. 2E: Effect of 21 days of CsA administration on Baseline Renal Cortical Blood Perfusion.
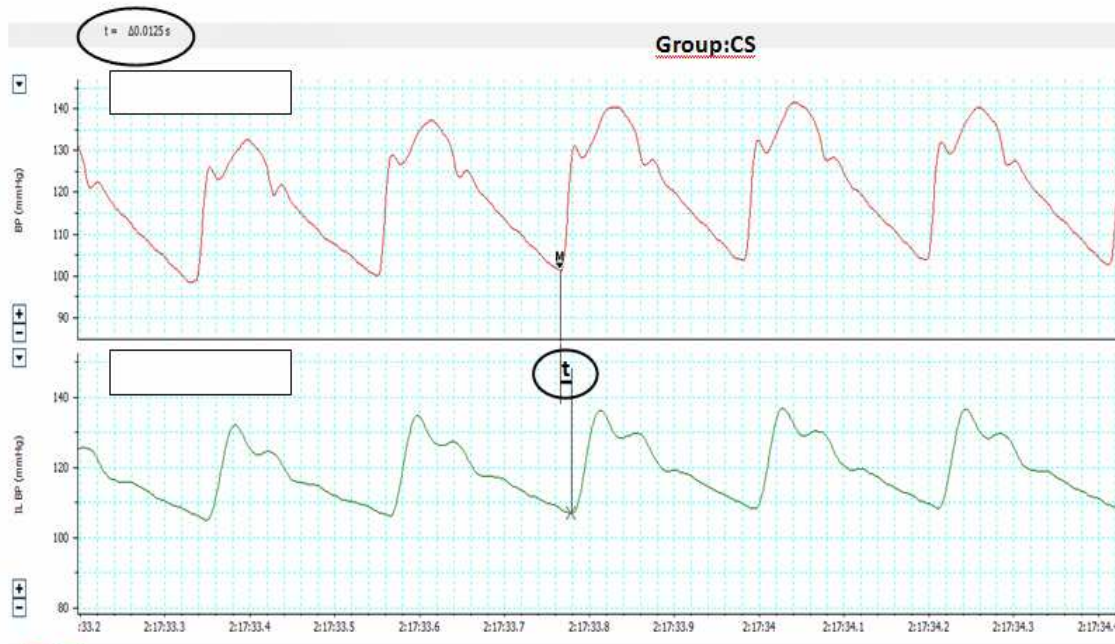
Fig. 3A & 3B: Schematic illustration of the measurement of PWV.

PWV is the ratio of between the distance (D) between the tips of the 2 catheters and the time difference at the minimal values of proximal and distal blood pressure (T). CT group showed (t = 0.025s) whereas CS group showed (t = 0.0125s).

DISCUSSION

The kidney is affected by many drugs and chemicals, some of which contribute to renal insufficiency and thus considerably to health care cost[15]. The present study yields additional information that suggested 21 days administration of CsA could induce renal dysfunction and morphological alteration in the kidney. Treatment with CsA was also associated with systemic and renal vasoconstriction, leading to arterial hypertension[17]. In fact, the manifestation of this situation was due to the renal circulation which richly endowed with sympathetic nerves that allowed autonomic nervous system to regulate the renal haemodynamic and tubular function as well as body fluid homeostasis and blood pressure[18,19]. Therefore, sympathetic nerves found in the renal system have always played a major role in regulating the kidney functions appropriately in different pathological conditions.

In current studies, animals that treated with 25mg/kg/day of CsA for 21 days experienced the reduction of body weight. The same observation was also reported by Parra et al., 1998. The reasons behind this could be due to the decreased in food intakes, other factors such as oxidative stress and kidney dysfunction might also be a direct catabolic effect of CsA administration[20]. The reductions in the volume of water intake by CS animals were also manifested throughout the experimental period, along with a remarked decreased in creatinine clearance and a fall in glomerular filtration rate. This finding was consistent to that reported by others[21,23]. In contrary, there was more than 5.6 fold decrease in fractional sodium excretion and 2.6 fold of fractional potassium excretion, which may indicate a remarkable increased in reabsorptive capacity although others have reported sodium depletion to be associated with CsA treatment[23]. Gerkens, (1989), summarized that the decrease in food intake could be the culprit that contributed to the loss of body weight. The elevation of SBP could be due to the activation of sympathetic nerve function, prostaglandin-thromboxane production and renin-angiotensin system (RAS) when urinary excretion of sodium is impaired by CsA. The increased of sodium reabsorption in CsA treated animals could also be the factors that responsible development of hypertension. The impairment of urinary sodium to potassium ratio caused the activation of renin angiotensin system by which the elevation of angiotensin II level will eventually lead to

more aldosterone hormone being produced. This phenomenon increased the activity of sodium reabsorption. Another argument that can be put for this phenomenon was the vasoconstriction of renal arterioles that eventually leading to the reduction in perfusion pressures which results in sodium retention and therefore, a rise in blood pressure[24].

To study the mechanism of CsA induced nephropathy, animals which were administered with CsA displayed histologic alteration similar to human lesions of chronic CsA nephropathy. Indeed, this view is supported by the histological observation, where the proximal convoluted tubules and glomeruli showed clear destruction of their structural integrity. The KI were reported increase by 1.3 folds and 1.9 folds increased of UPE in CS animals compared to CT animals. Both of these observations actually explained the reason of the reduction in the clearance of creatinine in CsA treated animals[25,26,27]. The functional apparatus in the nephron: such as mesangium that usually regulates the single nephron glomerular filtration rate were also found to be destructed. When the destruction occurred in glomeruli, contraction of the mesangium will close and prevent the perfusion of anatomically associated glomerular capillary loops; this eventually results in the decreased of the total surface area available for glomerular filtration and hence, reduced the glomerular ultrafiltration coefficient[28]. Furthermore, the existence of abnormally filtered proteins may interact adversely with the mesangium or with the cell lining the tubular space which will further enhance glomerular and interstitial damage[29]. This is brought by the infiltration of macrophages and deposition of extracellular matrix protein in mesangial cells[30]. Accumulation of these macromolecules produced mesangial cell proliferation and glomerulosclerosis[31].

CsA reduced RCBP by afferent arteriole vasoconstriction[32]. The present experiment experiences the same observation as reported by the above author. The potential mediators of CsA induced vasoconstriction again involved the renal sympathetic nerves, others factors such as angiotensin II, reduced nitric oxide production and alteration of prostaglandin-thromboxane cascade may also account for the pathogenesis of renal dysfunction[33]. Long term CsA treatment rapidly reduces GFR and RCBP by inducing intrarenal vascular resistance, which ultimately consequences in low grade ischemic injury[34].

In the current study, CsA administration for 21 days was sufficient to increase the blood pressure by approximately 13% as the elevation of blood pressure may be due to the participation of adrenergic system. CsA changes the vascular response to norepinephrine, and also affect α_1-adrenoceptors by changing their transduction mechanism which directly stimulates a target on upstream G protein, possibly at the receptor level[35]. This activation results in a potentiation of inositol phosphate formation and intracellular calcium influx thus, inducing vascular contraction. Another explanation could be the activation of afferent impulses from the diseased kidney to central integrative structures in the brain to cause increased sympathetic nerve discharge and eventually contributing hypertension[36].

In SD rats, the PWV changes were associated with an increase and decrease of central SBP for instances, when there was an increased in PP[37]. Till now, there are not many study investigated the effect of chronic CsA administration HR, SBP, PP and PWV on SD rats. Therefore, this study has further investigated the relationship of all the above factors towards the development of arterial stiffness. CsA treated animals were found to have 6 mmHg PP higher than untreated animals. Besides that, the 1.3 m/s difference in pulse wave transmission was also observed in this experiment. HR was also reported to be 53 beat per minutes higher than the non CsA treated animals. Safar and Laurent, (2003), explained that the biological factors such as SBP and PP amplification actually modulating the PWV in rat models where persistent and sustained increase of BP may trigger the arterial wall hypertrophy. The development of hypertension mainly involved the nitric oxide and vasoconstriction agents, extracellular matrix and also sodium. Therefore, CsA altered renal vascular and systemic heamodynamics was confirmed via this experiment by which the vascular stiffness develops from a complex interaction between a stable and dynamics changes that might involving structural and cellular elements of the vessel wall[38]. Indeed, the haemodynamic forces are actually influencing these vascular systems. Other factors, like hormones such as renin angiotensin aldosterone and salt regulation might also take part in this situation. In hypertensive rats, high sodium concentration found in the blood vessels was accounted to be responsible for the structural alterations in renal arteries. Under high salt conditions, the increased of wall thickness involves a substantial increase of collagen content together with abnormal HR exerts a significant influence on PWV. Arterial elasticity was impaired with CsA induced hypertension[39].

CONCLUSION

Accordingly, we conclude that chronic administration of CsA altered the renal function and renal haemodynamics via renal nephrotoxicity. Besides that, the amplication of PWV, SBP and PP were observed in CsA treated animals based on the statistical evaluations.

ACKNOWLEDGEMENT

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