LAPORAN AKHIR GERAN JANGKA PENDEK

"ASSOCIATION OF MALONDIALDEHYDE (MDA) LEVEL AND RENAL IMPAIRMENT IN SPONTANEOUSLY HYPERTENSIVE RATS"
(RUJ: FAIL : FPP 2001/018)

PROF. MADYA NOR AKMAL WAHAB

4 Mac 2004

Kepada :

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Tuan

Laporan Akhir Projek Penyelidikan Jangka Pendek bagi projek bertajuk :

"Association of 8-Isoprostanes level and Renal Impairment in Spontaneously Hypertensive Rats " (Ruj. Fail: FPP 2001/018)

Merujuk kepada perkara di atas, bersama ini dikepilkan laporan terakhir projek penyelidikan jangka pendek untuk makluman dan tindakan Tuan.

Saya ingin merakamkan terima kasih kerana menyalurkan geran kewangan yang membolehkan kajian ini dijalankan dan juga membolehkan pelajar MSc (Cheng Yew Chean) untuk menyiapkan tesisnya.

Sekian, terima kasih

Prof Madya Nor Akmal bin Wahab
Jabatan Patologi Kimia
Pusat Pengajian Sains Perubatan

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BAHAGIAN PENYELIDIKAN & PEMBANGUNAN CANSELORI
UNIVERSITI SAINS MALAYSIA

Laporan Akhir Projek Penyelidikan Jangka Pendek

1) **Nama Penyelidik :** Nor Akmal bin Wahab

Nama Penyelidik-Penyelidik Lain *(Jika berkaitan)* : Harbindar Jeet Singh
Mutum Samarendra Singh
Cheng Yew Chean (Pelajar MSc)

2) **Pusat Pengajian/Pusat/Unit :** Pusat Pengajian Sains Perubatan

3) **Tajuk Projek :** " Association of 8-Isoprostanes level and Renal Impairement in Spontaneously Hypertensive Rat" (Ruj. Fail: FPP 2001/018)

4) (a) **Penemuan Projek/Abstrak**
(Perlu disediakan makluman di antara 100 –200 perkataan di dalam Bahasa Malaysia dan Bahasa Inggeris. Ini kemudiannya akan dimuatkan ke dalam Laporan Tahunan Bahagian Penyelidikan & Pembangunan sebagai satu cara untuk menyampaikan dapatan projek tuan/puan kepada pihak universiti).

Prolonged uncontroled hypertension is known to cause renal impairment . The precise mechanism by which raised systemic blood pressure leads to renal impairment is unclear. Free radicals have been implicated in cell damage and hypertensive has been shown to have higher level of plasma lipid peroxidation end product malondialdehyde (MDA) than normal individuals . This study aims to ascertain the levels of MDA in spontaneously hypertensive rats (SHR) and determine the age at which changes in the levels of MDA and renal impairment begin to occur in SHR.

Sixty-six male SHR, aged 8 weeks, were divided into eleven groups. A similar number of male Wistar Kyoto rats (WKY) were also similarly divided to act as parallel controls. Body weight , systemic blood pressure and plasma MDA were measured every four weeks starting from the age of 8th weeks until the age of 48th week. Plasma MDA was measured using High Performance Liquid Chromatography (HPLC). Every four weeks, six rats per group were anaesthetized for measurement of Glomerular Filtration Rate (GFR), using standard clearance procedures, to assess the renal function.

Mean body weight of SHR was consistently lower than that of WKY rats from the age of 12 weeks onwards ($p < 0.05$). Blood pressure, on the other hand, was significantly higher in the SHR when compared to that in age-matched WKY rats from the age of 8 weeks onwards ($p < 0.05$). Interestingly, plasma MDA levels was higher in the SHR than in the controls only from the age of 12 weeks, reaching statistical significance by the age of 24, 44 and 48 weeks. GFR was lower in SHR from the age of 24 weeks onwards ($p < 0.05$). It appears that there is an age-related decline in GFR in SHR, implying renal impairment , particularly from the age of 24 weeks onwards when compared to WKY rats but plasma MDA levels was higher in the SHR than in the controls from the age of 12 weeks. It is unclear if the decrease in GFR is a result of the raised levels of MDA, but it appears that renal impairment occurs subsequent to changes in plasma MDA levels. Clearly, more studies are needed to ascertain the relationship between the changes in GFR and MDA in hypertension.

(Abstrak dalam Bahasa Malaysia)

Adalah umum diketahui hipertension tak terkawal yang berpanjangan boleh menyebabkan gangguan/kerosakan fungsi renal. Mekanisme yang tepat bagaimana tekanan darah yang tinggi boleh menyebabkan kerosakan ginjal masih belum jelas. Radikal bebas telah disabitkan dengan proses kerosakan sel dan pesakit hipertension didapati mempunyai aras plasma malondialdehid (MDA) yang lebih tinggi berbanding individu normal. Kajian ini bertujuan untuk menentukan aras MDA dalam tikus hipertensif spontaneous (SHR) dan umur tikus di mana perubahan kepada aras MDA dan kerosakan fungsi ginjal berlaku.

Enam puloh enam tikus jantan SHR, berumur 8 minggu, dibahagikan kepada sebelas kumpulan. Jumlah yang sama tikus jantan Wistar Kyoto (WKY) juga dibahagikan kepada sebelas kumpulan sebagai mewakili kumpulan kontrol. Berat badan, tekanan darah dan plasma MDA diukur setiap empat minggu dari tikus mula berumur 8 minggu sehingga berumur 48 minggu. Aras plasma MDA diukur dengan menggunakan kaedah 'High Performance Liquid Chromatography' (HPLC). Setiap empat minggu, enam tikus dalam setiap kumpulan dibius untuk penentuan Kadar Penurasan Glomerulus (GFR), menggunakan kaedah penurasan piawai, untuk menilai fungsi ginjal.

Berat badan min SHR didapati secara konsisten lebih rendah daripada tikus WKY dari mula berumur 12 minggu dan seterusnya ($p < 0.05$). Tekanan darah tikus SHR adalah lebih tinggi dari tikus WKY yang sama umur bermula dari umur tikus 8 minggu dan seterusnya ($p < 0.05$) . Aras plasma MDA adalah lebih tinggi dalam tikus SHR jika dibandingkan dengan kumpulan tikus kontrol bermula dari umur 12 minggu, dan mencapai tahap signifikan statistik pada umur tikus 24, 44 dan 48 minggu. GFR adalah lebih rendah dalam tikus SHR bermula dari umur 24 minggu dan seterusnya ($p < 0.05$). Didapati bahawa nilai GFR pada tikus SHR menurun mengikut umur , menandakan gangguan/kerosakan fungsi ginjal, bermula dari umur 24 minggu jika dibandingkan dengan tikus WKY, tetapi

aras plasma MDA tikus SHR adalah lebih tinggi jika dibandingkan dengan kontrol bermula dari umur tikus 12 minggu. Adalah tidak pasti penurunan nilai GFR pada tikus SHR adalah akibat dari peningkatan aras MDA , tetapi nampaknya gangguan/kerosakan ginjal berlaku selepas berlaku peningkatan aras MDA. Adalah jelas, banyak lagi kajian perlu dijalankan untuk mengetahui kaitan/ perhubongan di antara perubahan GFR dan MDA dalam hipertension.

4) (b) Senaraikan Kata Kunci yang digunakan di dalam abstrak :

Bahasa Malaysia	**Bahasa Inggeris**
Tikus Hipertensif Spontan	Spontanoeusly Hypertensive Rat
Radikal bebas	Free radicals
Plasma Malondialdehid	Plasma Malondialdehyde
Kadar Penurusan Glomerulus	Glomerular Filtration Rate
Gangguan/Kerosakan renal	Renal impairment

5) Output Dan Faedah Projek

(a) Penerbitan (termasuk laporan/kertas seminar)
(Sila nyatakan jenis, tajuk, pengarang, tahun terbitan dan di mana telah diterbit/dibentangkan)

Persembahan poster di '8th National Conference on Medical Sciences –2003' USM Kubang Kerian , Kelantan , bertajuk :
Age related changes in renal function in spontaneously hypertensive rats.
Cheng YC, Nor Akmal Wahab, Harbindar Jeet Singh, Mutum Samarendra

(b) Faedah-Faedah Lain Seperti Perkembangan Produk, Prospek Komersialisasi Dan Pendaftaran Paten . *(Jika ada)*

- tiada -

(b) Latihan Gunatenaga Manusia

i) Pelajar Siswazah : Cheng Yew Chean (Pelajar MSc di PPSP-USM)

ii) Pelajar Prasiswazah : - tiada –

iii) Lain-Lain : - tiada –

6. **Peralatan Yang Telah Dibeli :**

- tiada -

UNTUK KEGUNAAN JAWATANKUASA PENYELIDIKAN UNIVERSITI

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TANDATANGAN PENGERUSI
JAWATANKUASA PENYELIDIKAN
PUSAT PENGAJIAN SAINS PERUBATAN

AGE-RELATED CHANGES IN PLASMA MALONDIALDEHYDE (MDA), URINARY 8-EPI-PROSTAGLANDIN $F_{2\alpha}$ AND RENAL FUNCTION IN SPONTANEOUSLY HYPERTENSIVE RATS (SHR)

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Abstract:

Compared to normotensives, hypertensives and patients with renal failure have higher levels of plasma malondialdehyde (MDA) and urinary 8-epi-prostaglandin $F_{2\alpha}$. The exact significance of the raised levels of MDA and urinary 8-epi-prostaglandin $F_{2\alpha}$ activity in hypertension is unclear. It is however unclear if they are involved in the pathogenesis of hypertension or are a consequence of hypertension. This study attempts to ascertain the age at which changes in blood pressure, levels of plasma MDA, urinary 8-epi-prostaglandin $F_{2\alpha}$ and renal impairment begin to occur in Spontaneously Hypertensive rats (SHR).

Sixty-six, 8-week old Spontaneously Hypertensive Rats (SHR) were divided equally into eleven groups with age-matched Wistar-Kyoto rats (WKY) as controls. Every 4 weeks, blood pressure was recorded by the tail-cuff method on six SHR and six WKY rats. After the measurement of blood pressure, the rats were then individually housed in metabolic cages for collection of 24-hr urine samples for 8-epi-prostaglandin $F_{2\alpha}$ and urine electrolyte determinations and from 36 to 48 weeks onwards for the measurement of food and water intake. After the urine collection the rats were anaesthetised for measurement of glomerular filtration rate (GFR) using a standard ^{3}H–inulin clearance protocol. Immediately upon completion of the measurement of glomerular filtration rate, the animals were exsanguinated for the determination of plasma malondialdehyde levels.

Mean body weight of SHR was consistently and significantly lower than that of WKY rats from the age of 12 weeks onwards ($p<0.01$). Systolic blood pressure was significantly higher in the SHR when compared to those in age–matched WKY rats from the age of 8 weeks onwards ($p<0.01$). Plasma MDA and urinary 8-epi-prostaglandin $F_{2\alpha}$ were all significantly higher in SHR when compared to the controls from the age of 16 and 12 weeks onwards respectively ($p<0.05$ for MDA; $p<0.01$ for 8-epi-prostaglandin $F_{2\alpha}$). GFR was significantly lower ($p<0.05$) in SHR from the age of 28 weeks onwards when compared with age–matched normotensive WKY rats. No significant differences were noted in plasma creatinine concentrations between the two groups throughout the study period.

In conclusion, it seems, evidence for increased free radical activity appears four to six weeks after the development of hypertension in SHR rather than the cause of it. In addition, as renal impairment only becomes evident after changes in free radical activity, increased free radical activity might have a role in some of the complications associated with hypertension.

Keywords: Free radicals, hypertension, renal impairment, MDA, urinary 8-epi-prostaglandin $F_{2\alpha}$, SHR.

Introduction

Free radicals are unstable, reactive normal products of cellular metabolism [1]. Due to their high reactivity, free radicals can cause severe damage to biological molecules, especially to lipids, DNA and protein [2]. All cells either generate or make use of a variety of lipid or water-soluble antioxidant compounds (Vitamins E, C, A, gluthathione etc), and synthesise a series of antioxidant enzymes (catalase, superoxide dismutase, gluthathione peroxidase), which are responsible for deactivating these reactive intermediates of oxygen. Under normal conditions, there exists a fine balance between free radical production and antioxidant defence system such that little damage results to the cells.

An imbalance between free radical production and antioxidant activity, often referred to as oxidative stress, has been implicated in cardiovascular [3,4,5,6,7] and renal diseases[8,9,10]. There is abundant evidence implicating oxidative stress in various animal models of hypertension. Vascular $O_2^{\cdot-}$ is increased in the DOCA-salt [11,12], angiotensin II-infusion [13,14], and 1-kidney 1-clip [15] models of hypertension. Increased activity of NAD(P)H oxidase in angiotensin II-induced hypertension has been shown to activate NAD(P)H oxidase by up-regulating $p22^{phox}$ mRNA levels, and infusion of recombinant heparin-binding superoxide dismutase (SOD) decreased both blood pressure and $p22^{phox}$ mRNA expression [16]. Administration of heparin-binding SOD within the vessel wall has been shown to normalise partially the blood pressure of the SHR [12,17]. Interestingly, norepinephrine-induced hypertension does not increase $O_2^{\cdot-}$ production or oxidase expression suggesting that $O_2^{\cdot-}$ could be variably involved in the syndrome of hypertension [14]. Despite of the considerable evidence implicating oxidative stress in

hypertension, the point of time when there occurs a detectable change in free radical production or oxidative stress remains unclear. Whether it occurs before the development of hypertension or along with the development of hypertension or sometime after the establishment of hypertension has still to be clearly established. Suzuki et al [18] showed an increased generation of $O_2^{\cdot-}$ in arterioles and venules in SHR. Wu et al [19] noted that NADH stimulated $O_2^{\cdot-}$ production in cultured smooth cells (SMCs) from 6-week old SHR was not different from SMCs from age-matched Wistar-Kyoto rats. However, $O_2^{\cdot-}$ production was significantly higher in SMCs from 9 and 12 week old SHR when compared to that in SMCs from age-matched Wistar Kyoto rats. Incidentally, hypertension is usually well established in SHR by the time they reach 9 -12 weeks of age. These observations imply that hypertension either precedes or occurs concomitantly with the oxidative stress.

Prolonged uncontrolled hypertension often results in renal impairment in humans. The precise mechanism by which raised systemic blood pressure leads to renal impairment is still unclear. High levels of lipid peroxidation activity has been reported in patients with chronic renal failure [20,21,22]. Besides, reactive oxygen species (ROS) like hydrogen peroxide [23] and superoxide radicals [24] have been shown to cause tubular and glomerular damage. Although, hypertensives have been shown to have higher levels of oxidative stress, as indicated by raised levels of lipid peroxidation end products like malondialdehyde (MDA) [25,26] and 8-epi-prostaglandin $F_{2\alpha}$ [27,28,29] and lower levels of antioxidant activity [30,31], it remains unclear how long after the increased oxidative stress that significant renal damage becomes apparent. Animal studies looking at the time point

changes in oxidative stress and the development of hypertension in SHR and its renal complications are lacking. The purpose of this study therefore was to investigate the time point at which evidence for increased oxidative stress becomes apparent in hypertension, and whether renal function precedes or follows this change in oxidative stress in Spontaneously Hypertensive Rats (SHR), using plasma MDA and urinary 8-epi-prostaglandin F2α as markers of oxidative stress.

Material and Methods

Sixty-six, 8-week old Spontaneously Hypertensive Rats (SHR) were divided equally into eleven groups. A similar number of age-matched Wistar-Kyoto rats (WKY) were similarly divided to act as corresponding controls. All animals were exposed to a similar light-dark cycle and had access to food and water *ad libitum*. Every 4 weeks, blood pressure was recorded by the tail-cuff method on six SHR and six WKY rats. After the measurement of blood pressure, the rats were then individually housed in metabolic cages for a period of 5 days. The animals were allowed to adapt to the metabolic chambers for the first two days. For the next three days afterwards, 24-hr urine samples were collected for 8-epi-prostaglandin $F_{2\alpha}$ and urine electrolyte determinations. After the last urine collection, rats were allowed to recover for 24 hours before they were anaesthetised for measurement of glomerular filtration rate (GFR) using a standard ^{3}H–inulin clearance protocol for anaesthetised animals. Immediately upon completion of the measurement of glomerular filtration rate, the animals were exsanguinated for the determination of plasma malondialdehyde levels.

Blood pressure measurement

Arterial blood pressure was measured by tail plethysmography (Life Science, IITC, USA). To help reduce anxiety in the animals during the measurement of blood pressures, all animals, from the age of 5 weeks, were handled daily and repeatedly exposed to a perspex cylindrical animal holder used to restrain the animals during the measurement of blood pressure. During the actual measurement, conscious rats were first placed under an angle–poised lamp for 10 minutes to ensure good tail blood circulation before being placed inside an animal holder in a chamber, where the temperature was maintained at 32 $\pm$ 0.5 ^{0}C, for the measurement of blood pressure. Rats were allowed to rest inside the chamber for a period of ten minutes before blood pressure measurements were obtained. On each occasion three consecutive measurements were recorded using a physiograph (NARCO BIO SYSTEM, USA), and a mean of three measurements was recorded.

Determination of glomerular filtration rate

Glomerular filtration rate was estimated using ^{3}H-Inulin clearance as previously described [32]. Briefly, after an overnight fast, but with access to water *ad libitum* the rats were anaesthetized with an intraperitoneal injection of thiopentone sodium (ROTEX, USA), at a dose of 50 mg/kg body weight. After anesthetization, the rats were transferred to a preheated thermostat controlled animal operating table. A mercury thermometer was introduced into the rectum to monitor the body temperature throughout the procedure. The left jugular vein, for saline infusion, and right carotid artery were cannulated for saline infusion and the recording of blood pressure and blood collection respectively. A tracheotomy was performed to keep the airway clear. A bolus of 0.03 ml of normal saline

containing 20μ Ci ml^{-1} of ^{3}H Inulin (AP Biotech, England) was injected into the jugular vein. This was followed by an infusion of normal saline at the rate of 100μl min^{-1}, containing 1μ Ci ml^{-1} of ^{3}H-Inulin. Urinary bladder was cannulated suprapubically for collection of urine samples. After an equilibration phase of 2 hours, samples of arterial blood and urine were obtained every 30 minutes for one hour. Arterial blood (100μl) was collected in heparinized hematocrit tubes and following centrifugation, plasma was retained for measurement of ^{3}H-Inulin. Urine and plasma ^{3}H-Inulin activities were estimated using a β scintillation counter (Packard, TRI–CARB 3100TR, USA).

Measurement of plasma MDA

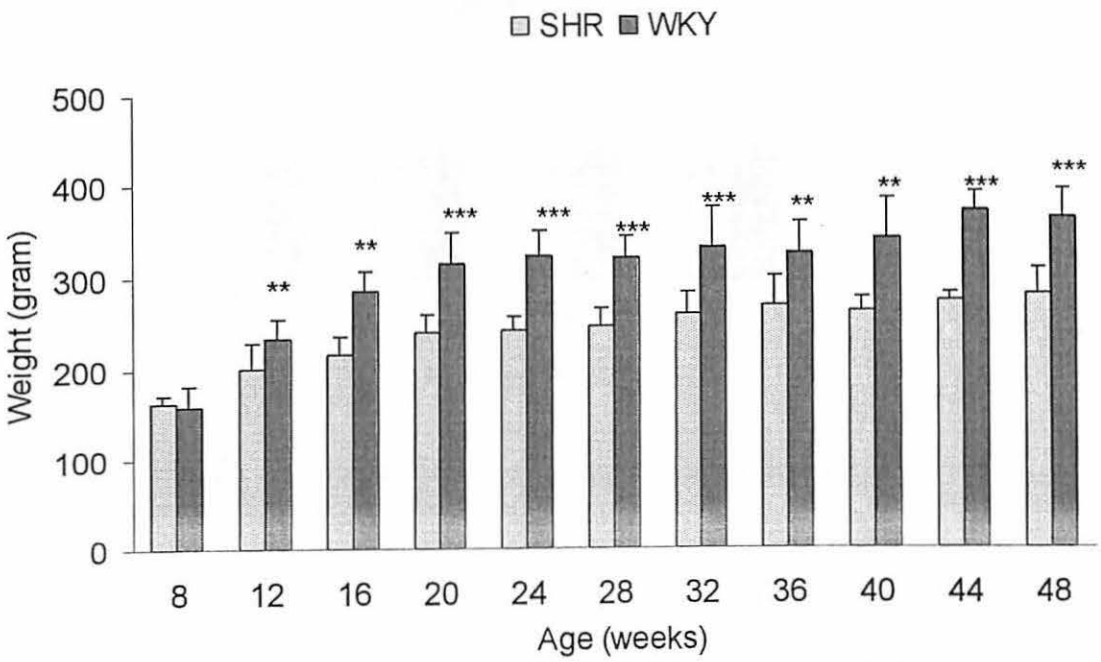
Plasma MDA was quantified using High performance liquid chromatography (HPLC) as described previously, [33,34,35] using a Micro–Tech HPLC system (Micro – Tech Instrument, model 200M, USA) consisting of a Micro –Tech HPLC pump (M –PMOD) with a 50μL injection loop, and a UV detector (Linear Instrument, modal 200, USA) was used. The column was a LiChroCART ® 250–4, packed with 5 mm LiChrospher 100 RP-18 (Merck). It was equipped with a guard column: LiChroCART 4–4 packed with 5 mm LiChrospher 100 RP-18. The elution was carried out at a flow rate of 0.5 ml min^{-1}. The mobile phase consisted of 10 mmol L^{-1} potassium dihydrogen phosphate (pH 6.0) and methanol (BDH, UK) in a ratio of 60: 40 by volume. The column effluent was quantified at a wave-length of 532 nm. After thawing, 100μl of plasma was added into a 5 ml Pyrex centrifugation tube containing 700μl of 1% orthophosphoric acid (Merck, German) and then vortex-mixed for 10 seconds. 200μl of 42 mmol/L 2-TBA solution (Merck, German) was then added, and vortex-mixed for another 10 seconds. The sample was then heated in a water bath at 100 °C for 60 minutes. Hereafter, the sample was kept on ice until 10

minutes before HPLC analysis. At the time of analysis the sample was vortex-mixed for 10 seconds, and 200µl of it was transferred into a 2 ml tube containing 200µl of 1:12 (by volume) 2 mol L^{-1} sodium hydroxide (Merck, German): methanol solution. The sample was vortex-mixed for 10 seconds and centrifuged for 3 minutes at 13,000 rpm and 200µl of the supernatant was transferred to a 300µl glass vial and 50µl of this solution was injected into the column and absorbance measured at 532 nm. MDA standard solution was prepared from 1,1,3,3-Tetraethoxypropane (TEP) (Sigma, USA) by serial dilution with the concentrations of 5µmol/L, 2.5µmol/L, 1.25µmol/L and 0.625µmol/L. MDA standard concentration was plotted against the area under the curve. The concentration of each sample was identified on the standard curve and read from the corresponding values on the y-axis (MDA standard concentration).

Urine 8-epi-prostaglandin $F_{2\alpha}$ was measured using commercially available 8-Isoprostane EIA Kit (Cayman, USA).

Statistical analysis was performed using repeated measures-ANOVA and Student's *t* test. All results are presented as mean ± standard error of mean and a 'p' of <0.05 was considered significant.

Results



**p<0.01, ***p<0.001

Figure 1: Body weight in age-matched SHR and WKY rats.

No significant difference was evident in body weight between the two groups at the age of 8 weeks (Figure 1). Body weight increased significantly in both groups over the period of study. The rate of increase was somewhat greater up to about twenty weeks of age, particularly in the WKY rats, after which the rate of increase became slower. From the age of 12 weeks, however, WKY rats were significantly heavier than age–matched SHR. The body weight of SHR was about 78% of their WKY counterparts at the age of 48 weeks.

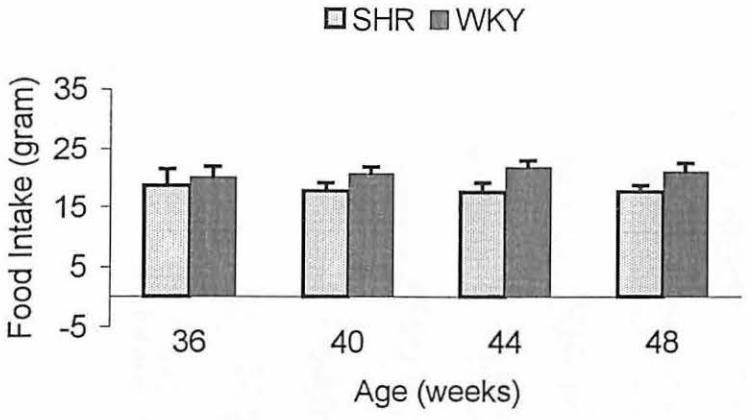


Figure 2: Daily food intake between 36 and 48 weeks in SHR and WKY rats

Food intake in both groups averaged between 15 – 25 grams per day over that period of study (Figure 2). There was no significant difference in food intake between the two groups.

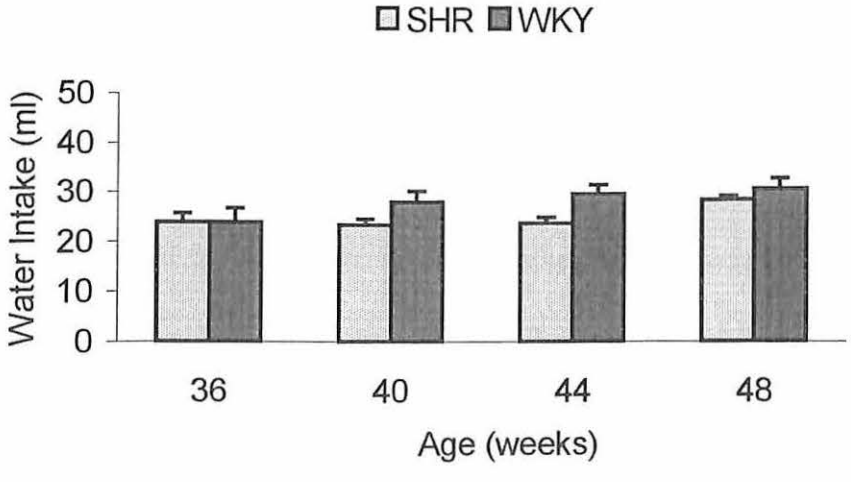
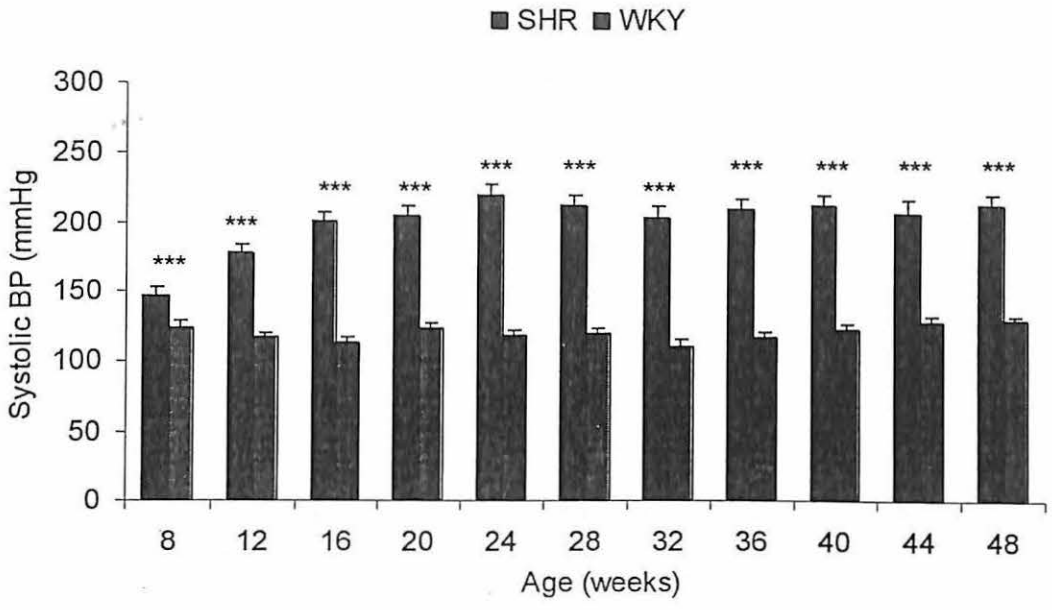


Figure 3: Water intake between week 36 and 48 in SHR and WKY rats

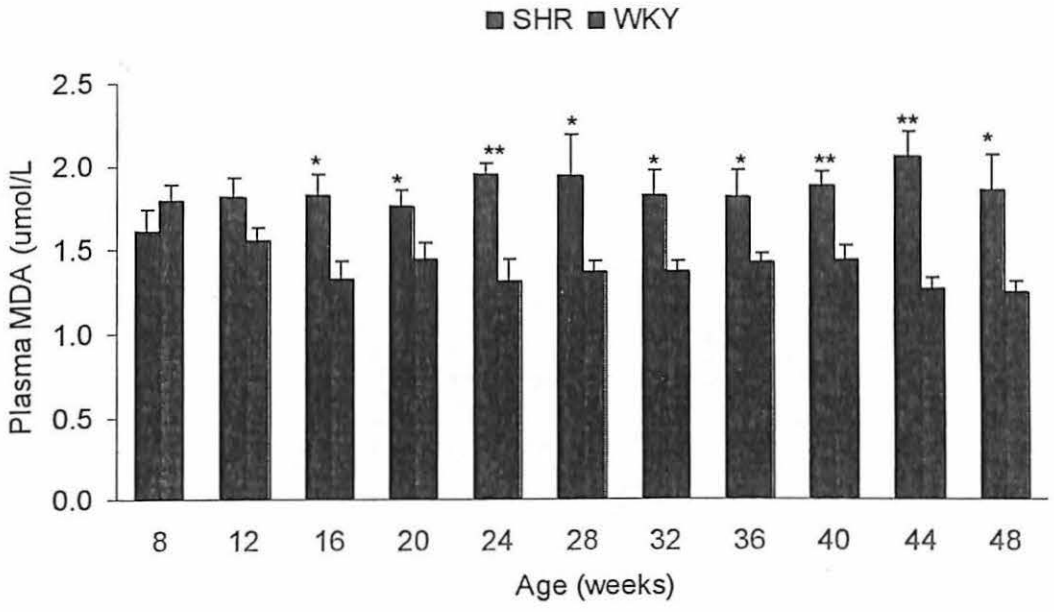
Water intake in both groups averaged between 25 – 30 ml per day over that period (Figure 3). There was no significant difference in water intake between the two groups.



**p<0.01; ***p<0.001

Figure 4: Systolic blood pressure in age-matched in SHR and WKY rats.

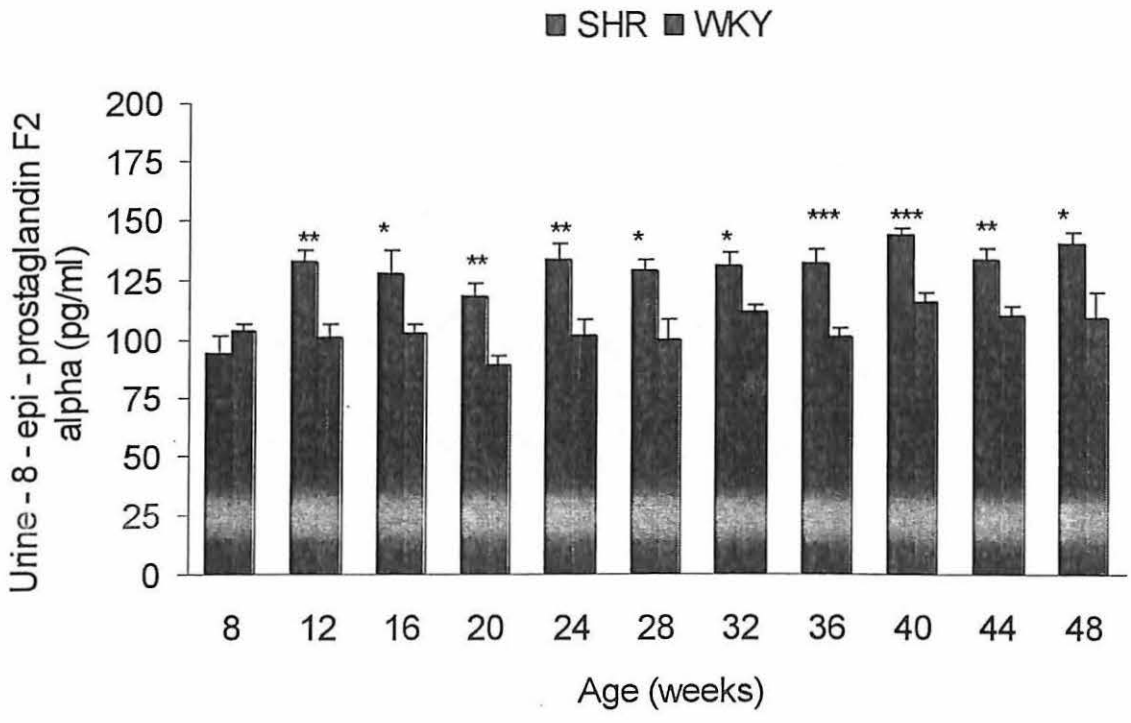
Mean systolic blood pressure in SHR was significantly higher from the age of 8 weeks onwards when compared to age–matched WKY rats (Figure 4). Although systolic blood pressures remained almost unchanged between the age of 8 and 48 weeks in WKY rats, in the SHR, however, systolic blood pressure appears to increase significantly with age until about the age of 20 weeks, remaining somewhat unchanged thereafter. Mean systolic pressures increased from a mean of 146 ± 7 mmHg at week 8 to a mean of 212 ± 8 mmHg at week 28 in the SHR. It remained within that level until the termination of the study at the age of 48 weeks.



*p<0.05; **p<0.01

Figure 5: Plasma MDA levels in SHR and age-matched WKY rats.

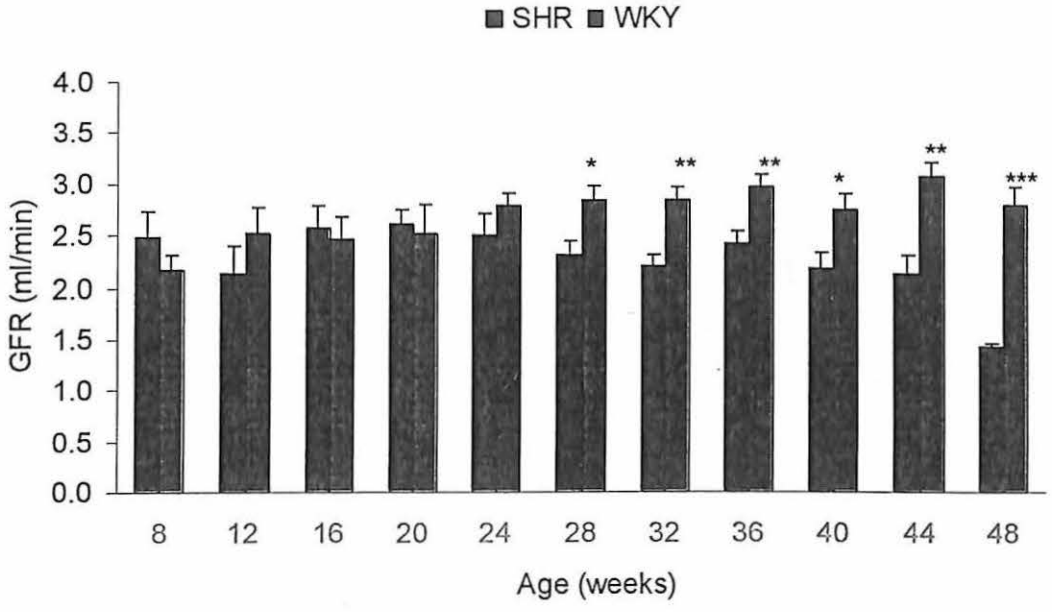
No significant difference was evident in plasma MDA levels between the two groups at the age of 8 weeks (Figure 5). However, plasma MDA levels were higher in SHR from the age of 12 weeks reaching statistical significance from the age of 16 weeks onwards ($p < 0.05$).



*p<0.05; **p<0.01, ***p<0.001

Figure 6 Mean urinary 8-epi-prostaglandin F2 alpha in SHR and age-matched WKY rats

No significant difference was evident in urinary 8–isoprostane concentrations between the two groups at the age of eight weeks (Figure 6). However, urinary 8 – isoprostane concentration increased significantly in SHR from the age of 12 weeks ($p<0.01$) and remained within that levels until end of study at the age of 48 weeks.



*p<0.05; **p<0.01, ***p<0.001

Figure 7: Mean glomerular filtration rate (GFR) in SHR and age-matched WKY rats

No significant difference was evident in glomerular filtration rate between the two groups up until the age of 24 weeks. However, glomerular filtration rate was significantly higher from the 28^{th} – 48^{th} week in WKY rats (Figure 7). The difference appears to be due to a slight age related increase in the WKY rats ($r = 0.422$; $p<0.01$) and a slight age–related decrease in GFR in SHR ($r = -0.424$; $p<0.01$).

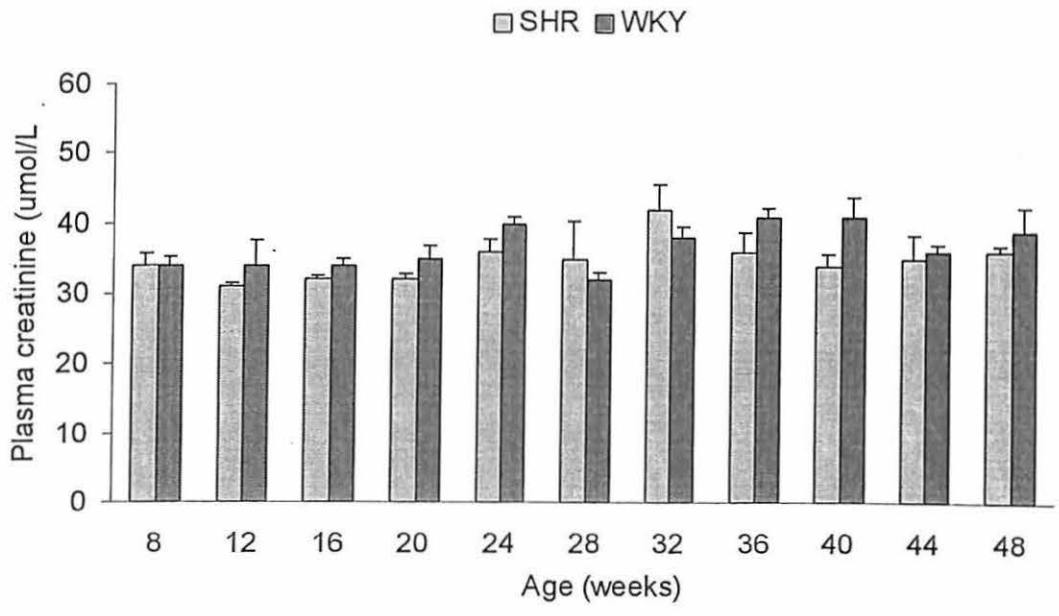


Figure 8: Mean plasma creatinine concentration in SHR and age-matched WKY rats

No statistically significant difference was evident in plasma creatinine levels between SHR and WKY rats (Figure 8). It also appears to have remained unchanged with age in both the groups.

Discussion

There were three important observations of note in this study. Body weight of SHR was significantly lower than that in age-matched WKY rats from the age of 12 weeks onwards. In addition, there were significant differences in urinary excretion of 8-epi-prostaglandin F_2 alpha and plasma MDA between the two groups, which became evident from the age of 12 and 16 weeks onwards respectively. More importantly, these differences only became evident after the establishment of high blood pressure in the SHR. The third important observation was the difference in GFR between the two groups, which became evident from the age of 28 weeks.

Age related increase in body weight is a well-established and reported phenomenon even in adult rats [36,37,38,39]. What has not often been reported is the tendency to a lower increase in body weight in SHR. Although body weight was not significantly different between the two groups at the age of 8 weeks, but from then onwards body weight of SHR was consistently lower than WKY rats (Figure 1; $p < 0.01$). By the age of 48 weeks, body weight of SHR was about 78% of that in age–matched WKY rats. The precise reason for the differences in body weight is uncertain. Although food intake was only recorded between weeks 36 and 48, no significant difference was evident in food intake between the two groups (Figure 2). Two possibilities that could explain for the lower body weight in SHR include either impaired gastro-intestinal absorption of nutrients or the effect of increased stress or both. In this regard, significant ultrastructural and functional changes in the jejunal epithelium in SHR [40] have been reported and numerous

studies *in-vitro* have shown impaired calcium [41,42] and glucose [43] absorption in SHR. Weight loss in rats exposed to repeated acute restraint stress has been reported [44,45]. The SHR in this study always appeared tensed and squeaked excessively during handling. Although the precise mechanism by which stress causes weight loss again remains unknown, there is evidence to suggest that the weight loss may be dependent upon acute central release of corticotropin-releasing factor [45], and leptin from the adipose tissue. Leptin secretion, which increases during stress in man [46], is known to suppress appetite and food intake in rodents and man, in addition to increasing sympathetic activity and possibly metabolism. Serum leptin was not measured in this study. Although mean food intake was slightly lower in SHR (Figure 2), it however was not statistically significant. It therefore appears that the reason for the lower bodyweight is probably due to a combination of slight lower food intake, impaired gastrointestinal absorption and stress related increase in metabolic rate.

At the age of 8 weeks, SHR were moderately hypertensive as evident by mean systolic pressure, which was 19% higher than that in WKY rats ($p<0.01$) at this age (Figures 4). Systolic pressure remained unchanged from the age of 8 to 48 weeks of study in the WKY rats. In the SHR however, systolic blood pressure increased significantly until about the age of 24 weeks after which it remained unchanged until the end of the study at the age of 48 weeks. The exact cause for the raised blood pressure in SHR remains unknown but similar results have also been reported before [36,38,47,48]. Increase peripheral resistance is a possible factor that results in the raised blood pressure, but what causes this remains unknown. The toxic effects of free radicals are now well recognised and

altered free radical activity in hypertension has been reported in the rat [5,6,7] and man [31,49,50]. It is however, unclear if the altered free radical production and activity is primary or secondary to the hypertension. Plasma MDA concentration was not significantly different between SHR and WKY rats at the age of 8 weeks, although by that time the hypertension was already evident in the SHR (Figure 5). In WKY rats, plasma MDA levels were significantly lower by about 26% at 16 weeks when compared to 8-week old WKY rats. The levels remained unchanged thereafter in the rest of the groups. The reason for the lower levels from the age of 16 weeks in WKY rats is uncertain. In the SHR, however, plasma MDA levels were higher in all age categories above 12 weeks when compared to WKY rats, the difference reaching statistical significance from the age of 16 weeks onwards ($p < 0.05$). It remained significantly higher thereafter for rest of the period of study. The reason for the higher plasma MDA levels in SHR is unclear. It is possible that there is either an increased production of free radicals and consequently their activity or a decrease in the natural antioxidant activity. Decreased antioxidant activity has been reported in patients with essential hypertension [26] and in hepatocytes from SHR [51]. A decrease in antioxidants could induce an increased cellular generation of radical species and lipid peroxidation. Of more interest however, is the time point at which changes in free radical activity became apparent in this study. The difference in plasma MDA between the two groups only became evident from the age of 16 weeks onwards. This is about 8 weeks after the establishment of hypertension. This finding therefore suggests that higher levels of lipid peroxidation and elevated free radical activity only become evident after the establishment of moderate to severe hypertension and not before. Whether the increased free radical activity is a result of the raised blood

pressure per se or is an associated abnormality remains to be confirmed but our finding does, however, imply or postulate that the raised blood pressure is not a result of increased free radical activity or caused by oxidative stress. This postulation appears in contrast to numerous studies showing the benefits of antioxidant supplementation in the treatment of hypertension [7,39,47,52,53]. Although supplementation of antioxidants like vitamin C, E, tempol, lazaroid and PUFA reduced systemic arterial pressures in both rat and humans, it was interesting to note that in none of the studies the hypertension was completely ameliorated. The inference from this is that increased oxidative stress may not be the primary cause of hypertension, but elevated free radicals may play an important role in the maintenance of high blood pressure.

No significant difference was evident in urinary 8-epi-prostaglandin $F_{2\alpha}$ concentration between SHR and WKY rats at the age of 8 weeks (Figure 6). However, urinary concentration of 8-epi-prostaglandin $F_{2\alpha}$ in 12-week old SHR was significantly higher, not only from their 8-week old counterparts but also when compared to age – matched WKY rats. Urinary concentration of 8-epi-prostaglandin $F_{2\alpha}$ in the remaining SHR age–categories remained the same to that in their 12-week old counterparts, it was nevertheless consistently and significantly higher in the SHR when compared to age – matched WKY rats. The concentration of 8-epi-prostaglandin $F_{2\alpha}$ was not different between the various ages–categories in the WKY rats. Raised urinary 8-epi-prostaglandin $F_{2\alpha}$ has also been reported in SHR [54,55], in patients with pulmonary hypertension [56], in patients with reno – vascular disease and essential hypertension [29], and in pigs with renovascular hypertension induced following unilateral renal occlusion [6]. In addition,

hypertension secondary to chronic angiotensin II infusion in pigs has also been shown to increase plasma 8-epi-prostaglandin $F_{2\alpha}$ [27]. As was the case with changes in MDA levels in this study, elevated urinary 8-epi-prostaglandin $F_{2\alpha}$ was only evident after establishment of hypertension in SHR. These observations suggest that the levels of 8-epi-prostaglandin $F_{2\alpha}$, which is also an indicator of oxidative stress, may be a result of raised systemic arterial pressure and not a cause of it. This is also supported by a recent observation where levels of urinary 8-epi-prostaglandin $F_{2\alpha}$ were increased only in moderate to severe hypertension, but not in mild hypertension [56]. Although detectable changes in oxidative stress only became evident from the age of 12 weeks in this study, it is possible that increased production of free radicals may have occurred together or soon after the beginning of hypertension. Cultured smooth muscle cells from 9-week, but not from 6-week, old SHR were shown to have increased NADH stimulated superoxide production [19]. The reason for the delay in the development of detectable oxidative stress could be due to concomitant increase in endogenous anti-oxidant activity. It is only when there is an imbalance between the rate of production of free radicals and anti-oxidant capacity, that oxidative stress appears. When taken together these observations and those from our study suggest that increased oxidative stress is not directly involved in the aetiology or pathogenesis of hypertension, at least not in the early stages, but sustained hypertension increases free radical activity. Once again it appears that free radicals may not be involved in the aetiology or pathogenesis of hypertension but may be involved in the maintenance of raised blood pressure. In this regards, vasoconstrictor effects of 8-epi-prostaglandin $F_2 \alpha$ have been reported [57].

The consequences of raised blood pressure include cerebral vascular stroke and damage to organs like the heart and kidney. Precisely how the latter happens is unclear. They could be caused either by the raised blood pressure itself or by some co–existing abnormality in hypertension. When compared by age–categories, no significant difference in GFR was evident between SHR and WKY rats up to the age of about 24 weeks (Figure 7). From the age of 28 weeks onward however, GFR was significantly lower in SHR when compared to WKY rats. Glomerular filtration rate in the SHR declined with progressive age categories from the age of 20 weeks, whereby at the age of 48 weeks it was significantly lower than that in 8-week old SHR. There was a negative correlation between GFR and age in SHR. In contrast, in the WKY rats GFR appeared to be rising or increasing with increasing age – categories. The reason for the differing GFR pattern in the WKY and SHR appears to be due to the hypertension as renal impairment only develops after several weeks of sustained hypertension. Our finding of a lower GFR in SHR is in contrast to observation of Tolbert *et al* [48], who found no significant difference in GFR between 36 week old SHR and WKY rats. The reason for the difference between this study and that of Tolbert et al [48] is not immediately apparent but it may be related to the clearance protocol used. In their study, Tolbert *et al* had used very low infusion rates (0.5 ml/hr versus 6 ml/hr in our study) and shorter equilibration phase. The extracellular volume expansion was small in their study. Sufficient extracellular volume expansion is necessary to uncover latent renal impairment, as low volume challenges may not be sufficient to reveal the presence of such renal impairment at this stage of the hypertension. The finding of impaired renal function confirms the presence of this complication in SHR, albeit evident only when challenged with a high

saline load. Not surprisingly, plasma creatinine concentration was not significantly different between the two groups at this stage (Figure 8). The reason for this is that the renal impairment was not sufficient at this stage to compromise renal function under normal circumstances. It does however indicate of decreasing renal reserve which is progressive and would lead impaired renal function under normal circumstances. The precise mechanism by which hypertension impairs renal function is unclear. Of the numerous possibilities currently suspected, the involvement of free radicals or their metabolites is increasingly recognised. In this regard, administration of 8-epi-prostaglandin $F_{2\alpha}$ has been shown to cause significant renal vasoconstriction, causing reductions in GFR of between 40 – 45% in rats [57]. Besides, male normotensive Sprague – Dawley rats aged 22 month have been shown to have a 60% lower GFR and a 3–fold higher renal 8-epi-prostaglandin $F_{2\alpha}$ concentration when compared to 3 to 4 month old rats [58]. In the same study, supplementation of vitamin E was shown to prevent the drop in GFR and at the same time was also accompanied by a 60% reduction in renal 8-epi-prostaglandin $F_{2\alpha}$ concentration. It appears the 8-epi-prostaglandin $F_{2\alpha}$ increases not only with hypertension but also with age. It is both a marker of free radical activity, and by itself, it also has vasoconstrictor activity. However, it is also not possible to say if the lowered GFR in the SHR was due to the vasoconstrictor effects of 8-epi-prostaglandin $F_{2\alpha}$ and its toxic effects on the renal glomeruli or both.

In conclusion, this study further confirms that oxidative stress per se may not be directly involved in the aetiology of hypertension in SHR, as increased oxidative stress is only detectable a few weeks after the establishment of blood pressure in this species. It

however, does not exclude a possible increase in free radical production before the evident oxidative stress. It further confirms that impaired renal function occurs in SHR, but it only becomes apparent at the age of about 28 weeks following a large saline challenge. Although it does not provide any direct evidence to show that renal impairment in hypertension is due to increased oxidative stress, the time interval between the establishment of hypertension and evidence for increased oxidative stress and the emergence of renal impairment seem to suggest a role for free radicals in renal impairment of hypertension. Clearly more work is still required to ascertain the role of free radicals in hypertension and hypertensive renal disease, an understanding of which would certainly help in improving the current management of human hypertension and in the reduction of its morbidity and mortality.

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