

The effect of Mastin® on expression of Nrf2 in the rat heart with subtotaly nephrectomy chronic Kidney disease model

J Nathania and V Soetikno*

Department of Therapeutic Pharmacology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

*E-mail: vivian_09st@yahoo.com

Abstract. Chronic kidney disease (CKD) is increasingly prevalent in Indonesia and worldwide. One of the major causes of morbidity and mortality in CKD is the complication of cardiovascular disease. Mastin® is a supplement that is locally produced in Indonesia and is made from extract of mangosteen pericarp, which is reported to have antioxidative, anti-inflammatory, and antitumor properties. The present study aimed to investigate whether Mastin® could improve antioxidant responses in the rat heart during CKD by measuring the expression of nuclear factor erythroid-2-related factor (Nrf2), a master regulator of antioxidant response elements. RNA was extracted from the heart tissue of three groups of rats: a normal group, a nephrectomy group, and a nephrectomy with Mastin® group. Two-step real-time RT-PCR was then conducted to calculate the relative expression of the Nrf2 gene. Nrf2 expression was markedly decreased in the nephrectomy group vs the normal group, but slightly increased in the nephrectomy with Mastin® group vs the nephrectomy group. CKD resulted in impaired activation of the Nrf2 pathway in the rat heart. Although the administration of Mastin® slightly increased Nrf2 expression, it was not enough to confer cardioprotective effects through the Nrf2 pathway.

1. Introduction

The increasing prevalence of chronic kidney disease (CKD) in developing countries, such as Indonesia, presents a major health problem due to the many complications with which it is associated. The risk of developing cardiovascular disease (CVD) is particularly increased in CKD that is associated with a decreased glomerular filtration rate (GFR) [1]. CVD complications have become a serious cause of morbidity and mortality in patients with CKD, resulting in a startling 10 to 30 times higher mortality rate in dialysis patients than in the general population [1]. Thus, the markedly increased burden of CVD in people with CKD has sparked numerous research efforts to identify treatments that minimize the clinical manifestations of CVD.

The progression of CVD has been attributed to both traditional (diabetes, obesity, hypertension) and non-traditional (oxidative stress, decreased GFR, proteinuria, RAS activity) risk factors, among which oxidative stress appears to play a central role [2,3]. Oxidative stress is the link between CKD and CVD and is responsible for the increasing occurrence of this reno-cardiac syndrome. There is considerable evidence to show that the nuclear factor erythroid-2-related factor 2 (Nrf2)-Keap1 signaling pathway plays a protective role during renal injuries [4]. Nrf2 is a transcription factor that regulates antioxidant-response-element (ARE)-dependent gene expression encoding antioxidant proteins, detoxifying enzymes, stress response proteins, and other products, which can reduce oxidative and inflammatory



damage [5]. Therefore, a pharmacological intervention to stimulate Nrf2 signaling is potentially beneficial in protection against CKD and its manifestations.

Mastin[®] is a supplement that is locally produced in Indonesia, and consists of mangosteen pericarp extract. *Garcinia mangostana*, also known as mangosteen, has generated increasing interest due to the variety of bioactive compounds contained within the pericarp, such as xanthenes, phenolic acids, tannins, and other compounds reported to have strong antioxidative, anti-inflammatory, anti-bacterial, and antitumor properties [5]. However, the effect of mangosteen-derived compounds on CKD remains unknown. Therefore, the present study aimed to elucidate the potential antioxidative effects of Mastin[®] by measuring the expression of Nrf2 in 5/6 nephrectomized rats.

2. Materials and Methods

This experimental research was conducted in the Department of Pharmacology and Therapeutics, Faculty of Medicine, Universitas Indonesia. The samples were obtained from a previous study in which CKD was induced in rats via the 5/6-nephrectomy procedure, before treatment with Mastin[®] for 8 weeks. The heart tissues of the rats were then extracted, and RNA was isolated using Tripure Isolation Reagent. Next, cDNA synthesis was carried out using a transcriptor first strand cDNA Synthesis Kit (Roche) before two-step real-time RT-PCR was performed. A Nanodrop 2000 spectrophotometer was used to assess the purity and concentration of cDNA in the samples. Real-time RT-PCR was then carried out to detect expression of the Nrf2 gene and the reference gene, beta actin. The relative expression of Nrf2 was calculated using the Livak method by comparing the quantitation cycle (Cq) values of the treated group to those of the control group. These data were then statistically analyzed using Statistical Package for the Social Sciences software, version 17.

3. Results and Discussion

3.1 Results

The forward primer used had a sequence of P_f (5' → 3'): **AGC-ATG-ATG-GAC-TTG-GAA-TTG**, while the reverse primer had a sequence of P_r (5' → 3'): **CCT-CCA-AAG-GAT-GTC-AAT-CAA**. The primers that were used for the real-time RT-PCR were analyzed with NCBI primer-BLAST and Oligo Analyzer 3.1 (Integrated DNA Technologies) to ensure their quality and suitability. The results of the spectrophotometry using the Nanodrop 2000 are shown in Table 1. The purity of cDNA obtained from RNA extraction can be assessed by the ratio of absorbance at 260 nm and 280 nm. A ratio of approximately 1.8 indicates a pure sample. Therefore, it can be concluded that all the samples were sufficiently pure. The nucleic acid concentration is used to calculate the amount of cDNA that will be used for the real-time RT-PCR.

Table 1. Nucleic acid concentration (ng/μl), absorbance at 260 nm, and ratio of absorbance at 260 nm and 280 nm of collected samples

Sample	Nucleic Acid Concentration			A260			260/280		
	1	2	Mean	1	2	Mean	1	2	Mean
B11	126.9	127.3	127.1	3.846	3.856	3.851	1.82	1.81	1.815
B12	124.7	125.0	124.9	3.778	3.788	3.783	1.82	1.80	1.81
B16	143.5	143.5	143.5	4.347	4.349	4.348	1.82	1.81	1.815
B17	145.7	146.4	146.1	4.414	4.437	4.425	1.83	1.82	1.825
B19	128.5	128.7	128.6	3.893	3.901	3.897	1.83	1.81	1.82
B20	120.3	121.8	121.1	3.647	3.692	3.669	1.82	1.82	1.82
R7	119.2	119.6	119.4	3.611	3.625	3.618	1.81	1.81	1.81
R8	156.2	157.5	156.9	4.734	4.772	4.753	1.82	1.83	1.825
R10	122.0	122.3	122.2	3.698	3.705	3.701	1.82	1.82	1.82
R16	134.0	134.1	134.1	4.059	4.063	4.061	1.83	1.82	1.825
R17	130.6	131.2	130.9	3.958	3.976	3.967	1.83	1.82	1.825
R21	135.6	136.6	136.1	4.111	4.141	4.126	1.83	1.83	1.83
R22	154.2	154.8	154.5	4.673	4.690	4.681	1.82	1.83	1.825

Two-step real-time RT-PCR was conducted to obtain the relative expression of Nrf2 mRNA in the three sample groups, and Cq values were obtained from the PCR, as shown in Figure 1. The results were then normalized using beta actin as a reference gene and calculated using the Livak method to obtain the relative expression ratio. The results were then analyzed using Welch analysis of variance and the Games-Howell *post hoc* test.

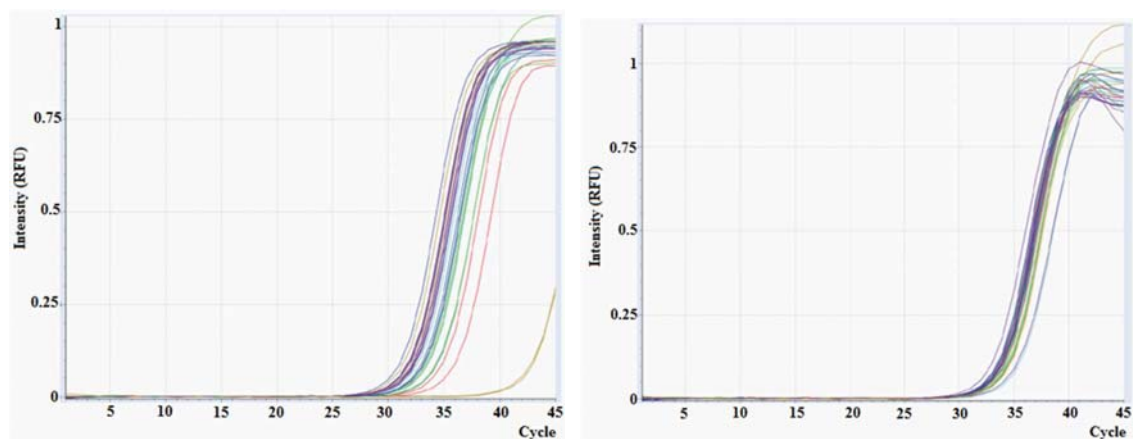


Figure 1. Fluorescent curves depicting the amplification point of beta actin (left) and of nuclear factor erythroid-2-related factor (right)

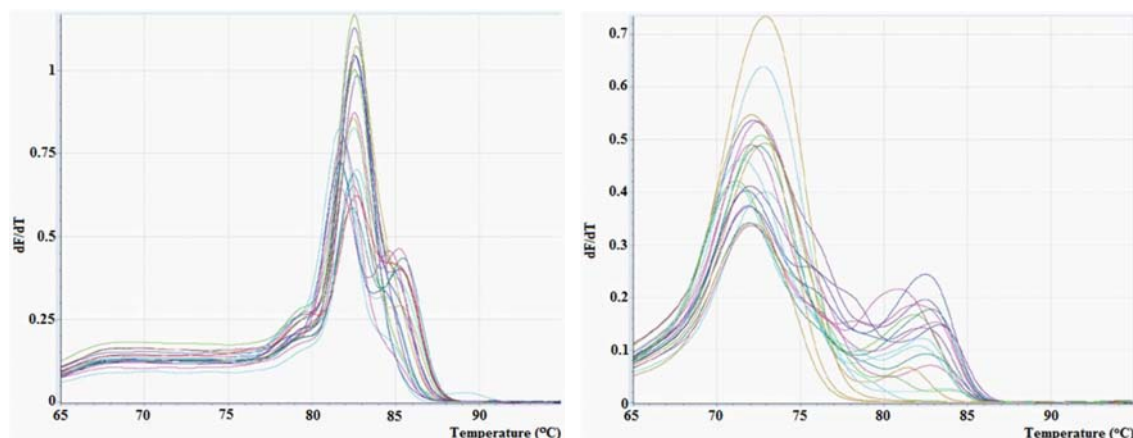


Figure 2. The melting curve of beta actin (left) and Nrf2 (right)

The one-peak presentation obtained from the melting curve analysis indicates the suitability of the primer and the absence of contamination in the sample.

As shown in Figure 3, the relative expression of Nrf2 was markedly decreased in the nephrectomy group compared to the normal group. A significant difference was also observed between the normal and the nephrectomy+mastin[®] group. Although there was an increase in mean expression of Nrf2 in the nephrectomy group compared to the nephrectomy + Mastin[®] group, it was not significant.

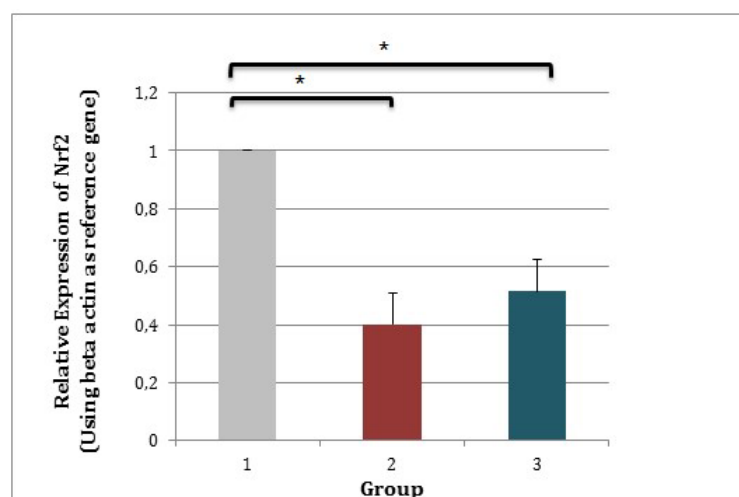


Figure 3. The relative expression of nuclear factor erythroid-2-related factor in the normal, nephrectomy, and nephrectomy+mastin[®] groups. The data are presented as means \pm SE, and * denotes $p < 0.05$ after analysis using Welch analysis of variance and the Games-Howell *post hoc* test. 1= normal 2= nephrectomy, 3= nephrectomy + Mastin[®]

3.2 Discussion

The pathogenesis and progression of CKD is intrinsically linked to oxidative stress and inflammation [6], which accompanies the disease. Physiologically, an oxidative imbalance promotes up regulation of endogenous antioxidants, together with cytoprotective proteins, to ameliorate tissue damage and dysfunction. Nrf2 is the master regulator of this process, as it regulates basal activity and the induction of several genes that code for antioxidants and phase II enzymes [7]. The proteins encoded by Nrf2 include, but are not limited to, SOD, HO-1, and glutathione peroxidase [7]. However, it has been shown that the activity and expression of Nrf2 is paradoxically decreased during chronic renal injuries. Aminzadeh et al. showed that Nrf2 activation was impaired in rats undergoing CKD due to tubulointerstitial nephropathy [8]. Similarly, a 5/6 nephrectomy CKD model showed a significant reduction in the nuclear abundance of Nrf2, accompanied by an elevation of cytoplasmic Keap [9]. Therefore, the Nrf2 expression level was used in the present study to determine whether Mastin[®] can improve antioxidant responses to CKD.

As shown in Figure 9, Nrf2 expression was significantly decreased in the 5/6 nephrectomy group compared to the normal group. This is in accordance with results obtained in previous studies, as mentioned above. The mechanism causing this impairment in Nrf2 activity remains unknown, although it is hypothesized that the accompanying systemic inflammation during kidney injuries is partly responsible, as a similar decrease in Nrf2 activity has been identified in various chronic inflammatory diseases, including asthma [10], and chronic granulomatous disease [11]. In the event of chronic inflammation, the release of Nrf2 from Keap1 and the interaction of Nrf2 and ARE is disrupted by the p65 and p53 subunit of NF- κ B [12]. NF- κ B regulates the expression of pro-inflammatory mediators, including cytokines, as well as adhesion molecules [5].

Mastin[®] is a herbal supplement made from extract of mangosteen pericarp. The bioactive compounds isolated from the shell of this fruit are known to have powerful antioxidative properties [13] that can protect various tissues, such as the heart, kidney, retina, and brain [14]. These compounds may directly and indirectly confer antioxidative effects; they may directly act as ROS scavengers by reducing singlet oxygen and superoxide anions [15] or indirectly activate endogenous antioxidants [16]. In a recent study, Fang et al. showed that alpha-mangostin, derived from *Garcinia mangostana*, protected retinal cells against oxidative stress by enhancing Nrf2 transcription and nuclear translocation of Nrf2

[16]. However, other studies have shown that mangosteen-derived compounds may also activate antioxidant responses independent of the Nrf2 pathway [17].

The results obtained in the present study show that the increase in Nrf2 expression in rats treated with Mastin® compared to the nephrectomy only group was not statistically significant. We may therefore state that the bioactive compounds in Mastin® do not activate the Nrf2 pathway. This result was not in accordance with our hypothesis. It is notable that in the previous studies that showed a significant increase in Nrf2 expression due to mangostin, the compounds were given as a pretreatment and not as a post treatment, as was the case in the present study. Pretreatment conditioning with mangosteen-derived compounds may have resulted in a more significant increase in Nrf2. Since the present study did not investigate other possible pathways of antioxidant activity, the antioxidative mechanism of Mastin® remains unknown.

4. Conclusion

5/6 nephrectomy-induced CKD significantly decreases Nrf2 expression. The bioactive components of Mastin® slightly increase Nrf2 expression, but cannot return it to normal expression levels.

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