Hibiscus sabdariffa L. Extract Improved Plasma Catalase Activity but not Malondialdehyde Level in Hind Limb Immobilized Rats

Zainie Aboo Bakkar*, Nooraqilah Rubaai, Nur Afiqah Mahazi, Nurhanisah Hosni, Nik Nur Nadia Nik Mat, Nur Sahira Al-Fattah Yahaya, Hanan Kumar Gopalan

Universiti Kuala Lumpur, Institute of Medical Science Technology (UniKL MESTECH), 43000 Kajang, Selangor, Malaysia

Article History:
Received on: 01 Nov 2019
Revised on: 01 Dec 2019
Accepted on: 07 Dec 2019

Keywords:
Catalase, disused muscle atrophy, DNA damage, limb immobilization, malondialdehyde

INTRODUCTION

Disused muscle atrophy (DMA) that may be triggered by immobilization, lack of physical activity and weightlessness, is suggested to be caused by an increased protein degradation with decreased protein synthesis (Powers et al., 2012). Studies reported that prolonged muscle disuse increased the exposure of proteins and lipids to oxidative damage. This occurs due to reduced endogenous antioxidant enzymes capacity including catalase, superoxide dismutase and glutathione peroxidase, and elevated reactive oxygen species (ROS) accumulation in inactive muscle fibers (Jackman and Kandarian,
A study by (Min et al., 2011) demonstrated that muscle immobilization in mice for 14 days led to significant muscle atrophy resulting from muscle oxidative damage and increased mitochondrial ROS production. This suggested that metabolic processes in mitochondria are the origin of ROS production during DMA. Furthermore, increased ROS may trigger the proteolytic pathway associated with atrogin-1, ubiquitin ligases and muscle-specific ring finger protein 1 (MuRF-1) that are important in the development of DMA (Reich et al., 2010).

Increasing endogenous antioxidant defence could protect against oxidative damage induced muscle atrophy in DMA. Hibiscus sabdariffa L. or roselle contains high flavonoids particularly anthocyanins (cyanidin and delphinidin) (Frank et al., 2005) in its red calyces thus possess profound antioxidant and genotoxic actions (Poddar, 2014). This tropical herbal plants that belongs to the Malvaceae family has been shown to possess hepatoprotective (Adeyemi et al., 2014), antihypertensive and antidiabetic (Hopkins et al., 2013) properties. Recently, a study by (Murata et al., 2016) reported that oral administration of delphinidin (anthocyanin that highly present in HS) may prevent DMA in animal tail suspension model. Thus, we sought to investigate the effect of HS aqueous extract on oxidative damage and antioxidant enzyme in hind limb immobilization animal model of DMA.

MATERIALS AND METHODS

Preparation of HS Aqueous Extract

Dried HS calyces were collected from Bangi, Selangor, Malaysia and preparation of aqueous extract from the calyces was adapted from (Mohamed et al., 2013). First, distilled water was added into dried calyces that has been ripped to smaller pieces in 1:2 ratio. Then, the mixture was ground using a blender for 10 minutes and subsequently filtered. The filtrate was stored at 4°C in a bottle covered with aluminium foil until use.

Animal Experimentation and Sample Collection

The animal ethics approval was obtained from Universiti Kuala Lumpur, Institute of Medical Science Technology (UnIKL MESTECH)’s Animal Ethics Committee (FYP/AEC/MESTECH-UNIKL/2017/011/JULY-2017-NOV-2017). 24 male Sprague-Dawley rats (200-250g) aged approximately eight weeks acclimatized for seven days in laboratory cages under standard conditions (12 hours light and 12 hours dark cycle) and provided with standard rat chow and water ad libitum. The rats were divided into 4 groups: Control, Immobilized (I), Hibiscus sabdariffa L. (HS) and Immobilized + HS (I+HS). The rats in the control group received no intervention while HS received 100 mg/kg/bw orally through force feeding for 28 days. The rats in I and I+HS groups were subjected to unilateral hind limb immobilization for 5 days and followed by HS treatment with same dosage until day-28 for I+HS group. The immobilization procedure was conducted according to (Madaro et al., 2008). Following immobilization procedure, the rats were separated in an individual cage. At the end of intervention for each group, the rats were anaesthetized with sodium pentobarbital and the blood was collected by cardiac puncture. The blood samples were processed for DNA damage analysis and centrifuged for plasma separation and stored at -20°C. The plasma sample was used for the oxidative marker and antioxidant activity analysis.

Alkaline Comet Assay

Alkaline comet assay was adapted from the method describe by (Chuang and Hu, 2004). First, 3ml of whole blood was pipetted onto the top of 3 ml of Histopaque (Sigma-Aldrich, Missouri, USA). in a centrifugation tube (ratio 1:1). After centrifugation (30 minutes at 1300 rpm), mononuclear cells layer that appeared between blood plasma and Histopaque was transferred into a tube containing 5ml of phosphate buffer saline (PBS). The cells were then washed twice with PBS and centrifuged at 1300 rpm for 15 minutes. The cell pellet was resuspended in 1ml of PBS before proceeded with the comet assay.

0.6% normal-melting-point (NMP) agarose in PBS was prepared and coated onto a frosted glass microscope slide. These slides were then allowed to solidify at room temperature. Then, 1ml of mononuclear cell suspensions in PBS was transferred to 1.5ml Eppendorf tubes and centrifuged for five minutes at 4000 rpm. The resulting cell pellet was mixed with 160 μl of 0.6% low-melting-point (LMP) agarose in PBS at 37°C. Then, 100 μl of this mixture was transferred onto the slide pre-coated with NMP and left for ten minutes at 4°C to allow solidification. The slides were then submerged in lysing solution (2.5M NaCl, 100mM EDTA, 10mMTris, 1% Triton X-100, 4°C) for one hour at 4°C. The slides were subsequently placed in an electrophoresis tank filled with electrophoresis alkaline solution (300mM NaOH and 1mM EDTA) and allowed to run at 200mA, 20V for 20 minutes at room temperature allowing the DNA to unwind. The slides were then neutralized (0.4M Tris–HCl buffer, pH 7.5) and stained with ethidium bromide. The image was analysed for comet scores according to the method by (Collins
et al., 2008). Type 0: No damage; Type 1: Mild damage; Type 2: Moderate damage; Type 3: Severe damage; Type 4: Very severe damage. shown in Figure 1.

**Oxidative Marker and Antioxidant Activity**

Level of lipid peroxidation was measured based on the production of MDA following the method by (Yoshioka et al., 1979). First, 0.25ml of plasma was added into test tubes and mixed with 1.25ml of 20% trichloroacetic acid (TCA). Then, 0.5ml of 0.67% thiobarbituric acid (TBA) was added and heated for 30 minutes in boiling water bath. After that, 2ml of n-butanol were added to each tube. The mixture was centrifuged at 3000 rpm for 10 minutes. Finally, the supernatant layer was taken out and its absorbance was measured at 535nm. Plasma protein concentration was measured using NanoPhotometer (P330, Implen, Schatzbogen, Germany). The MDA concentration was expressed as nM/mg of protein.

Catalase enzyme activity was determined using the method adapted from (Sinha, 1972). 0.1ml of plasma and 0.4ml of hydrogen peroxide were added to 0.9ml of PBS. Then, the mixture was left for one minute and 2ml of dichromate acetic acid was added to the mixture. The tube was heated in boiling water bath for ten minutes to decompose blue precipitate. Then, the green solution of chromic acetate was formed. After cooled at room temperature, the solution was measured at absorbance of 530nm using spectrophotometer. From the catalase standard curve, the activity of catalase enzyme was expressed as inversely proportional to the concentration of hydrogen peroxide $[H_2O_2]$ in $\mu g/ml$.

**Data Analysis**

All values were expressed as mean ± SEM and anal-
yzed using one-way ANOVA followed by Bonferroni post-hoc test for multiple mean comparisons. Statistical significant was set at P-value ≤ 0.05.

RESULTS AND DISCUSSION

The mean body weight at the beginning of the study were similar in all groups (Control: 248.6±10.5 g; I: 361.0±2.6 g; HS: 248.2±8.3 g; I+HS: 366.2±2.6 g, P≥0.05). A recent study by (Murata et al., 2016) showed that delphinidin (anthocyanin from HS), given orally to rats significantly inhibited the weight loss of muscle that induced by DMA in tail suspension rat model.

DNA Damage

Figure 2 shows the comet scores for DNA damage for all groups. There were significant differences in mild (type 1) and moderate (type 2) DNA damage between groups. The results in Table 1 indicated that there were 86.5±1.4%, 37.0±5.5%, 25.0±3.5% and 56.7±7.9% of mild DNA damage in Control, HS, I and I+HS group respectively (P=0.003). For moderate DNA damage, there were only 5.7±1.4% in Control as compare to other groups (HS: 36.9±1.2%; I: 33.4±6.1%; I+HS: 23.0±3.9%; P=0.033). There were no significant differences in type 0 (no damage), type 3 (severe damage) and type 4 (very severe damage) comet scores between groups (P>0.05). Data are presented in mean ± SEM. HS: Hibiscus sabdariffa L.; I: Immobilised; *: P<0.05 for One-way ANOVA; a: Bonferroni post-hoc test P<0.05 vs Control; b: Bonferroni post-hoc test P<0.05 vs I.

Data from the present study demonstrated that HS has protective effects against unilateral hind limb immobilisation-induced DNA damage in rat model. The HS extract prevented the increase of severe and very severe comet scores after limb immobilisation. The previous study by (Fujita et al., 2011) confirmed that 8-hydroxy-2 ′- deoxyguanosine (8-OHdG), an index of oxidative damage in DNA, accumulated in the gastrocnemius muscle during unloading. Interestingly, our findings are supported by previous study form (Poddar, 2014) which reported that seven days administration of HS extract (50-150 mg /kg body weight), similar dosage range used in the present study, in albino rats prior to induction of sodium arsenite significantly reduced the DNA damage. This effect was reported to be dose-dependent. HS aqueous extract with different concentrations (50, 100, 150 and 400 mg/kg) given daily to rats was also reported to show potential protective effects against damage induced by sodium arsenite and cyclophosphamide as assessed by erythrocytes micronucleus test (Adetutu et al., 2004; Gheller et al., 2017).

Additionally, (Hamid et al., 2014) evaluated genoprotective potency of HS on oxidative damage using H2O2 as the ROS-induced model and the level of DNA damage was assessed using Comet Assay. After preincubation of murine bone marrow cells with HS for 24 hours, a significant protection effect against oxidative damage by H2O2 was observed with 500ng/ml HS conferring better protection than the higher dose of 1000ng/ml. This study demonstrated that smaller dosage of extract is effective for ex vivo intervention as compared to in vivo. Collectively, previous and present studies demonstrated that HS may offer potential role of antioxidants to reduce oxidative stress changes and chances of DNA damage, may be associated with phenolic compound possess by HS (Murata et al., 2016), especially cyanidin and delphinidin (Frank et al., 2005).

Oxidative Marker and Antioxidant Activity

Figures 3 and 4 exhibited the plasma lipid peroxidation (MDA content) and catalase enzyme activity respectively in all groups. There were no significant differences in plasma MDA between groups (Control: 12.1±2.4 nmol/mg; I: 31.1±10.1 nmol/mg; HS: 31.3±10.5 nmol/mg; I+HS: 18.2±8.1 nmol/mg, P>0.05). Interestingly, there was a significant increase in plasma catalase activity after HS treatment (plasma [H2O2]) in Control: 72.5±0.3 μg/m; I: 65.1±1.3 μg/ml; HS: 68.3±3.2 μg/ml; I+HS: 56.5±4.9 μg/ml, P=0.006). Data are presented in mean ± SEM. HS: Hibiscus sabdariffa L.; I: Immobilised. Data are presented in mean ± SEM. HS: Hibiscus sabdariffa L.; I: Immobilised; a: Bonferroni post-hoc test P<0.05 vs Control.

Table 1: Comet scores for DNA damage.

<table>
<thead>
<tr>
<th></th>
<th>Type 0 (%)</th>
<th>Type 1 (%)</th>
<th>Type 2 (%)</th>
<th>Type 3 (%)</th>
<th>Type 4 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.0±0.6</td>
<td>86.5±1.4</td>
<td>5.7±1.4</td>
<td>0.9±0.6</td>
<td>-</td>
</tr>
<tr>
<td>HS</td>
<td>0.8±0.5</td>
<td>37.0±3.5</td>
<td>36.9±1.2</td>
<td>25.3±2.8</td>
<td>-</td>
</tr>
<tr>
<td>I</td>
<td>9.7±4.1</td>
<td>25.0±5.5</td>
<td>33.4±6.1</td>
<td>30.1±10.6</td>
<td>2.5±1.3</td>
</tr>
<tr>
<td>I+HS</td>
<td>12.4±3.9</td>
<td>56.7±7.9</td>
<td>23.0±3.9</td>
<td>7.3±5.5</td>
<td>0.7±0.7</td>
</tr>
</tbody>
</table>

Data are presented in mean ± SEM. HS: Hibiscus sabdariffa L.; I: Immobilised.
Findings from the present study demonstrated that HS aqueous extract administration after 5 days of hind limb immobilisation showed protective effects against oxidative damage by increasing endogenous catalase enzyme activity and reduction of plasma lipid peroxidation level, although this reduction is not significant. A previous study had shown that levels of glutathione peroxidase and catalase enzymes were significantly reduced in rat’s muscle after hind limb unloading (Lawler, 2003) which also correspond to human study (Reich et al., 2010). Our current findings that HS extract administration increased endogenous enzyme activity and reduced oxidative damage were in agreement with previous studies (Adeyemi et al., 2014; Ali et al., 2016; Murata et al., 2016). Analysis of microarray samples in DMA rat model also showed that delphinidin could suppress the upregulation of gene expression involved in the oxidative stress pathway (Murata et al., 2016). Studies suggested that the HS extract contain antioxidant activity as potential scavengers of ROS and free radicals (Adeyemi et al., 2014; Poddar, 2014) thus acting as cell defence mechanism from oxidative damage especially at the membrane via inhibition of MDA formation (Mohamed et al., 2013; Zainalabidin et al., 2016). The antioxidative potential of HS in combating the effects of DMA may be due to its ability to downregulate ubiquitin ligase casitas B-lineage lymphoma-b (Cbl-b) which is associated with the loss of muscle volume (Murata et al., 2016).

CONCLUSIONS

The mechanism of DMA was suggested to involve oxidative stress lead by mitochondrial dysfunction that promote protein degradation and damage to cellular components. Flavonoids, particularly anthocyanin, was demonstrated to alleviate hind limb immobilised-induced oxidative damage by reducing DNA damage, plasma MDA level and increasing plasma catalase enzyme activity. The present study provided evidence of HS efficacy as antioxidant to prevent progression of DMA especially in bedridden people, although more studies should be carried out to elucidate its underlying mechanism of action.

ACKNOWLEDGEMENT

This work was supported by Universiti Kuala Lumpur Final Year Project research grant.

Conflict of interest

The authors declare that they have no conflict of interest for this study.

Funding support

The authors declare that they have no funding support for this study.

REFERENCES


