






RESEARCH ARTICLE

REVISED The effect of red-fleshed pitaya (*Hylocereus polyrhizus*) on heat shock protein 70 and cortisol expression in strenuous exercise induced rats [version 2; peer review: 2 approved]

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Abstract

Background: Oxidative stress from exercise can contribute to damaging cells, increasing heat shock protein 70 (HSP70) and suppressing the immune system in the body. This research aimed to determine the antioxidant potential of red-fleshed pitaya extract on HSP70 and cortisol expression in rats which were subjected to strenuous exercise.

Methods: The subjects of this research were 32 Sprague Dawley male rats, aged 3 months, with an average weight of 200 g. Red-fleshed pitaya extract was obtained from methanol extraction process; a maceration technique was performed and the extract was concentrated using an air-drying method. Rats were randomly divided into four groups. Group 1 were subjected to strenuous exercise and treated with distilled water only; while Groups 2, 3 and 4 were subjected to strenuous exercise and treated with 100 mg/kg body weight, 200 mg/kg body weight and 300 mg/kg body weight of red-fleshed pitaya extract, respectively. Strenuous exercises in rats was performed by intense swimming of 20 min/day, 3 days a week for 3 weeks. HSP70 expression and cortisol were measured with Enzyme-Linked Immune Sorbent Assay (ELISA) method.

Results: There was a significant reduction of HSP70 ($p=0.000$) and cortisol expression ($p=0.000$) between the groups. Also, there was a significant difference in the average decreasing of HSP70 expression between group 4 and either groups 1 or 2 ($p=0.000$). However, a significant difference between groups 4 and 3 was not observed ($p=0.813$). Lastly, a significant difference was found in the average decrease of cortisol expression between groups 4 and 1 ($p=0.000$), 2 ($p=0.000$), and 3 ($p=0.000$) respectively.

Open Peer Review

Reviewer Status  

Invited Reviewers

1

2

version 2

(revision)

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report



version 1

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report



report

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Any reports and responses or comments on the article can be found at the end of the article.

Conclusion: Red-fleshed pitaya is potential to be utilized as antioxidant to decrease the HSP70 and cortisol expression.

Keywords

Red-fleshed pitaya, HSP70, cortisol, strenuous exercise

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REVISED Amendments from Version 1

The difference between this new version and version 1 is that in this new version, we can explain the antioxidant activity contained in red-fleshed pitaya that can affect heat shock protein 70 and the expression of cortisol. Red fleshed pitaya contains powerful antioxidants because it consists of various antioxidants, namely flavonoids, and polyphenol. Thus Red fleshed pitaya which has the potential as an antioxidant can reduce ROS and respond to HSP70 expression. We can explain that giving 3 doses of 100 mg/kg body weight, 200 mg/kg body weight, and 300 mg/kg body weight to find out what is the effective dose to neutralize oxidative stress due to strenuous physical exercise. So it is necessary to experiment with 3 doses, in accordance with the purpose of the study to find out the potential of Red-fleshed pitaya as an antioxidant and once to know the effective dose to reduce oxidative stress which is influence on HSP70 and cortisol expression in rats that were subjected strenuous exercise.

Any further responses from the reviewers can be found at the end of the article

Introduction

Physical activity is an activity which has various influences and significant effects on the body. The effect of regular physical activity is a positive influence on biological functions, and will improve health and the antioxidant defense system in order to protect body from the negative effects of oxidative damage¹. Strenuous exercise tends to trigger free radical compound production. Moreover, this impairs the balance of free radicals and antioxidants as a result of oxidative stress²⁻⁴. Research has discovered that oxidative stress from strenuous exercise reduces performance as it damages cells⁵, causing pain and muscles fatigues⁶, lowering antioxidant levels^{7,8}, increasing the expression of heat shock protein 70 (HSP70)^{9,10} and suppressing the immune system¹¹. Increased RONS (Reactive Oxygen and Nitrogen Species) production during exercise has potential negative effects, transient increases in RONS seem to be a trigger for many exercise-induced adaptations in skeletal muscle. Exercise can stimulate increased concentration of HSP70 in muscles and plasma¹².

During an intense workout, self-defense and self-adaptation depend on the body condition which can be observed from HSP70 protein expression. Increases of HSP70 in muscles indicates a response to protect muscles cells from oxidative stress. HSP70 expression is an adaptation mechanism and a sign of damaging cells caused by oxidative stress¹³. Previous research reported that workouts increase HSP70 expression¹⁰. Strenuous exercise is a physical stressor in the body and as a result, adrenocorticotropic hormone (ACTH) is secreted by hypothalamus hypo-physis anterior and triggers the adrenal cortex to produce cortisol¹⁴. The escalation of cortisol is influenced by the intensity and duration of training that leads to a suppression of the immune system, resulting in a decline of antibody. Cortisol can be a sign that the body encounters a decline in the immune system due to heavy training¹⁵. Antioxidants can detoxify the lipid peroxide produced during exercise, which can eliminate radicals and reduce the inflammatory response to exercise. Therefore, it can prevent a muscle damage from exercise¹⁶.

The body needs exogenous antioxidants to neutralize and prevent chain reactions from free radicals formed from heavy physical trainings¹⁷. Sources of exogenous antioxidants are Vitamin E, C and also beta-carotene. External antioxidants from food or supplements can help the body to fight an excess of free radicals. In a previous study, proanthocyanidin from grape seed was given to rats for 2 weeks. As a result, it lowered malondialdehyde level and increased the superoxide dismutase and glutathione peroxidase was activated significantly. Furthermore, it reduced fatigue after physical activitie¹⁸.

Red-fleshed pitaya (*Hylocereus polyrhizus*) is a unique fruit with a lot of benefits. The fruit is recently popular among Indonesians and appears to be a natural antioxidant. Several *in vitro* studies have revealed that red-fleshed pitaya extract has the potential to be an antioxidant¹⁹. This research aimed to investigate the antioxidant potential in red-fleshed pitaya extract on HSP70 and cortisol expression in rats that were subjected to strenuous exercise. Red fleshed pitaya contains powerful antioxidants because it consists of various antioxidants, namely flavonoids, and polyphenol^{20,21}. Thus Red fleshed pitaya which has the potential as an antioxidant can reduce ROS and respond to HSP70 expression^{22,23}.

The study about exercise and the potential of red-fleshed pitaya as an antioxidant that affects HSP70 and cortisol expression is still limited. So this research purpose to find out the potential of red-fleshed pitaya as an antioxidant and once to know the effective dose to reduce oxidative stress which is influence on HSP70 and cortisol expression in rats that were subjected strenuous exercise.

Methods**Experimental animals**

The subjects of this research were 32 Sprague Dawley male rats, aged 3 months, with an average weight of 200 g, were obtained from the Animal Holding Unit of the Pharmacy laboratory, University of Sumatera Utara, Indonesia.

All rats were sustained and maintained in groups (four mice per cage) in experimental animal cages of the Pharmacy laboratory, University of Sumatera Utara, Indonesia. The cage is made of plastic (30 x 20 x 10 cm) and covered with fine wire mesh. The base of the cage is covered with rice husk as thick as 0.5 - 1 cm and replaced every day during the research. The room light was controlled to be exactly at 12 hours light and 12 hours dark cycle, while the temperature 25–27°C and humidity of the room were adjusted to a normal range and fed with standard rat pellets 551, and drink (tap water) was given *ad libitum*.

Study design

The research applied a laboratory experiment method with random group posttest-only design. The male rats were obtained from the Pharmacy Laboratory, University of North Sumatra. The experimental animals were simple random sampling divided into four groups: Group 1 was subjected to strenuous exercise and treated with distilled water only; Group 2 was subjected to

strenuous exercise and treated with dosage 100 mg/kg body weight of red-fleshed pitaya extract; Group 3 was subjected to strenuous exercise and treated with dosage 200 mg/kg body weight of red-fleshed pitaya extract; Group 4 was subjected to strenuous exercise and treated with dosage 300 mg/kg body weight of red-fleshed pitaya extract.

Giving 3 doses of 100 mg/kg body weight, 200 mg/kg body weight, and 300 mg/kg body weight to find out what is the optimal dose to neutralize oxidative stress due to strenuous physical exercise. So it is necessary to experiment with 3 doses, in accordance with the purpose of the study to find out the potential of Red-fleshed pitaya as an antioxidant and once to know the optimal dose to reduce oxidative stress.

Red-fleshed pitaya extract

Red-fleshed pitaya fruit, obtained from farmers, in Indonesia, was peeled, washed, cut into small pieces and then dried in a drying cabinet. After that, the fruit was blended using a blender. The fruit extract was isolated through maceration method by using ethanol 96% which has been distilled as much as 10 times the weight of red-fleshed pitaya powder. Red-fleshed pitaya fruit powder in a container had 96% ethanol added to it (ratio 1:7, fruit powder: ethanol), and then was soaked for 3 days then filtered and sealed. The macerates were collected in a container and then processed with rotary evaporator at a temperature of 45°C until the extract was thickened. After that, the same process of were repeated the remaining ethanol 96% for 3 days. The less thickened extract was then evaporated in a water bath until a thick extract was obtained.

100 mg red-fleshed pitaya extract was weighed. Then, it was gently ground using a pestle and mortar. After that, carboxy methyl cellulose (CMC) Na 0.5% solution was slowly added and ground until a homogeneous phase was achieved. Finally, the suspension was added to a 10 mL measuring flask until it reached the mark line. The allocation of red-fleshed pitaya extract, dosage of 100 mg/kg body weight, for instance: weight of 200 g, volume taken: 2 ml extract suspension. Dosage of 200 mg/kg body weight, for instance: weight of 200 g, volume taken: 4 ml extract suspension. Dosage of 300 mg/kg body weight, for instance: weight of 200 g, volume taken: 6 ml extract suspension.

Experimental procedures

Strenuous exercise given to all rats was a morning swim between 8–9 am for 20 minutes/day, 3 days a week over 21 days. The equipment used in this research was a 10-cm length and 25-cm diameter bath as a pool. Group 1, the rats received distilled water only; Group 2, the rats received 2 ml red-fleshed pitaya extract suspension; Group 3, the rats received 4 ml red-fleshed pitaya extract suspension; Group 4, the rats received 6 ml red-fleshed pitaya extract suspension. Administration of red-fleshed pitaya extract suspension and water was performed orally once daily for 21 days.

Testing for HSP70 and cortisol was conducted two days after the rats had completed a strenuous exercise, 3 days a week over 21 days. During the test, the rats were given a maximum training session by swimming as hard as they could until the rats drowned or showed fatigue symptoms such as the entire body

almost dipped into water and limb movements slowed down. After that the rats were sacrificed by placing them in a jar containing cotton which was moistened with 10 ml of chloroform. After that, 2–3 ml blood was taken from the heart.

Blood samples were collected in micro tubes and centrifuged at 3000 rpm for 15 minutes. The serum was separated and stored at a temperature of 20°C until the analysis process would be carried out. Cortisol was measured with enzyme-linked immune sorbent assay (ELISA); Mouse Cortisol Elisa kit (catalog: E1483Mo, Brand Bioassay TL) and UV spectrophotometry at a wavelength of 450 nm. The HSP70 expression was recorded with ELISA; Mouse HSP70 Elisa Kit (catalog: E0302Mo, Brand Bioassay TL); the absorbance was indicated at 405 nm.

Statistical methods

Data was analyzed using SPSS 22 for Windows and displayed in tables and diagrams. Normality test was conducted through Shapiro-Wilk test ($P > 0.05$) in order to determine the average of normal distribution of sample data which is presented as mean \pm SD. The result of the normality test was used for next analysis; parametric analysis was used for normal distribution, otherwise non-parametric analysis was used. The ANOVA statistical analysis was performed to indicate the effects of treatments for each group. If the significant result is obtained, then the procedure is followed by Least Significance Difference or Bonferroni tests.

Ethical approval

The research was performed on the animal subjects were in according with the ethical standards by the Animal Research Ethics Committees/AREC, Faculty of Mathematics and Natural Sciences University of Sumatera Utara, Indonesia (approval number 0011/KEPH-FMIPA/2018).

All efforts were made to reduce any suffering of the rats was during the experiments by following careful procedures and also by anaesthetizing the animal prior to scarifice to prevent experiencing any pain.

Results

A normality test indicated that the data are normally distributed (Table 1). HSP70 expression was decreased across all groups (69.57 vs 46.04 vs 31.47 vs 27.65 pg/mL). Group 4 had the lowest expression compared with the other groups. This research reveals a significant decrease in HSP70 expression ($p=0.000$) between the groups (Table 2).

Cortisol expression was also decreased across all groups (119.02 vs 86.11 vs 62.94 vs 40.86 pg/mL). Group 4 had the lowest expression compared with the other groups. An ANOVA test revealed a significant decrease in cortisol expression ($p=0.000$) between the groups (Table 2).

Figure 1 indicates a significant difference in the average decrease of HSP70 expression between group 4 and either group 1 or 2 ($p=0.000$). However, a significant difference between group 4 and group 3 ($p=0.813$) was not found. This means that group 4, with strenuous exercise and given 300 mg/kg body weight red-fleshed pitaya extract was indicated to be more effective for

Table 1. Normality test for heat shock protein 70 (HSP70) and cortisol expression.

Parameter	Group	Normality test	
		statistic	p-value
HSP70	Group 1	0.969	0.888
	Group 2	0.907	0.332
	Group 3	0.922	0.449
	Group 4	0.875	0.168
Cortisol	Group 1	0.923	0.451
	Group 2	0.962	0.833
	Group 3	0.939	0.603
	Group 4	0.900	0.290

Shapiro-wilk test, P>0.05

Table 2. Average heat-shock protein (HSP70) and cortisol expression in rats who were subjected to strenuous exercise and treated with distilled water (group 1), and 100 mg/ kg body weight (group 2), 200 mg/ kg body weight (group 3) and 300 mg/ kg body weight (group 4) red-fleshed pitaya extract, respectively.

Parameter	Group 1 (n=8)	Group 2 (n=8)	Group 3 (n=8)	Group 4 (n=8)	p-value
HSP70 (pg/ml)	69.57 ± 7.27	46.04 ± 6.45	31.47 ± 1.24	27.65 ± 1.72	0.000*
Cortisol (pg/ml)	119.02 ± 5.56	86.11 ± 11.40	62.94 ± 4.61	40.86 ± 10.94	0.000*

The mean ± SD HSP70 and cortisol expression in shown

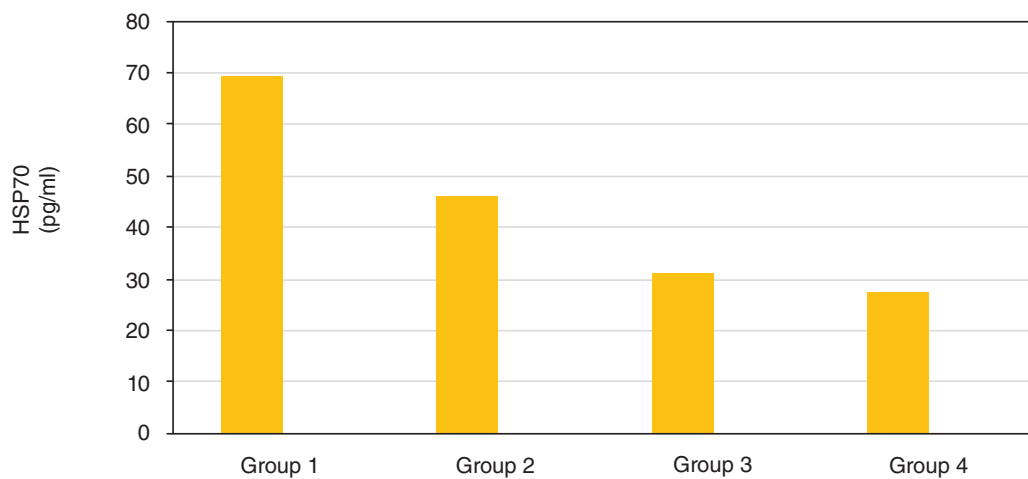


Figure 1. Effect of red-fleshed pitaya extract on HSP70 expression in rats that were given strenuous exercise.

reducing HSP70 expression compared to group 1 and 2, with no big variance from group 3.

Figure 2 confirms that a significant difference is revealed in the average decrease of cortisol expression between groups

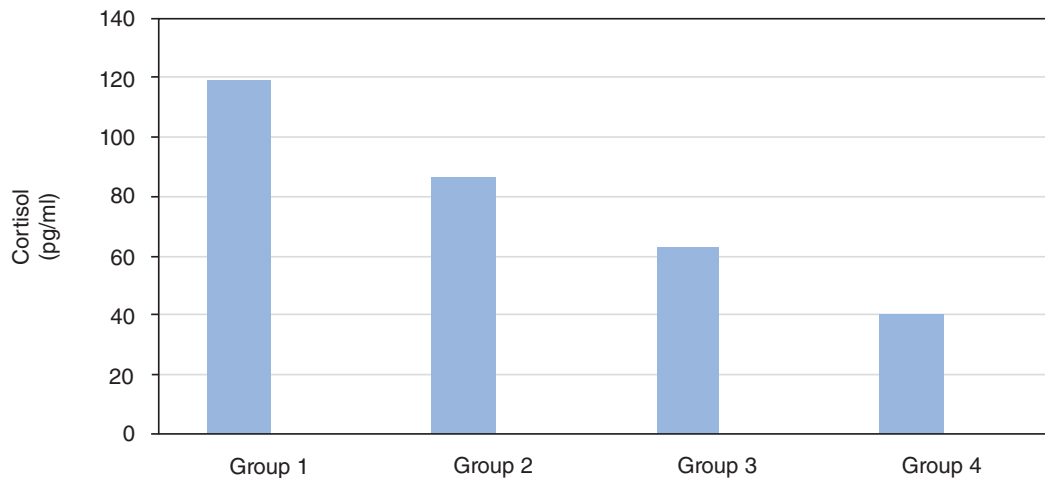


Figure 2. Effect of red-fleshed pitaya extract on cortisol in rats that were given strenuous exercise.

4 and 1 ($p=0.000$), 2 ($p=0.000$), and 3 ($p=0.000$). It can be concluded that group 4 with strenuous exercise and 300 mg/kg body weight red-fleshed pitaya extract was more effective in reducing cortisol expression compared to the other groups.

Discussion

Based on the results of this research, it is found that strenuous exercise combined with daily red-fleshed pitaya extract consumption contributes to a declining expression of HSP70 and cortisol. The dosage of 300 mg/kg body weight red-fleshed pitaya extract was found to be the optimum amount in decreasing cortisol compared to 100 and 200 mg/kg body weight dosage. However, for both 300 mg/kg body weight and 200 mg/kg body weight dosage of red-fleshed pitaya extract could not provide any difference to HSP70 expression. Therefore, the red-fleshed pitaya extract can be categorized as a potential exogenous antioxidant to eliminate free radicals formed during strenuous exercise.

Free radicals are an element that possesses one or more unpaired electrons in its outermost orbital. Consequently, it is very reactive to cells or cell components in its surroundings. Commonly, a reactive element finds its pair by attacking and binding with adjacent electrons. Then, if this element reacts with another radical element, a new radical element will be formed. This will consistently continue to occur leading to an unavoidable chain reaction²⁴. During strenuous exercise, oxygen consumption rises by 20 times. The excess oxygen triggers the formation of free radicals with electrons released from the respiratory chain. Free radicals production from activity, especially superoxides, increases in the mitochondria²⁵. When an imbalance happens due to the excess of free radicals, oxidative stress occurs and damages the DNA. Moreover, proteins will lose their structure and function like enzymes and membrane receptor. Also, there will be damage in the lipid bilayer structure²⁶.

Strenuous exercise triggers the oxidative stress to occur, therefore, the HSP70 expression will elevate and resulting in a

decrease of endogenous antioxidant activity. Strenuous exercise is associated with increased HSP70 expression due to oxidative stress²⁷. HSP70 is an important protein molecule for cell healing and preventing homeostasis and also increased expression as a cyto-protective effect. The HSP70 is induced strongly due to oxidative stress as cyto-protective to prevent the oxidative damages and heal the broken proteins²⁸. The increase of HSP70 expression is aimed to balance between ischemic condition, high temperature and the increased production of free radical²⁹. In this research, it is found that HSP70 expression tends to decrease in a group with strenuous exercise combined with red-fleshed pitaya extract.

The results of this research is in accordance to a research by Kassaf *et al.*, (2003) that antioxidant supplementation is proven to be effective to slow down the HSP70 synthesis caused by high intensity exercise²⁹. Other researches have also by Petiz *et al.* (2017) which discovered that Vitamin A combined with high intensity activities could prevent tissue damage and reduce endogenous antioxidant defense regulation in rats, which also suppressed the HSP70 expression³⁰.

The red-fleshed pitaya extract combined with strenuous exercise is proven to be able to inhibit the increasing of HSP70 expression. Red-fleshed pitaya (*Hylocereus polyrhizus*) is one of the plants that can be used as a source of antioxidants because that red dragon fruit contains the most polyphenols compared to other species³¹. This is occurred due to the potential of red-fleshed pitaya as an antioxidant that is able to balance the increasing amount of free radicals formed and impairs HSP70 synthesis induced by strenuous exercise³². Therefore, the HSP70 expression is lower compared to the groups that are not given by red-fleshed pitaya extract.

Physical activity appears to occur concomitantly with up-regulation of endogenous antioxidant systems and heat shock proteins (HSP) in skeletal muscle. While the main role

of the antioxidant enzymes is to reduce oxidation and prevent oxidative damage, HSPs can prevent and reverse damage to proteins. Intriguingly, the HSPs work with the antioxidant systems, and collectively they have essential roles in cell homeostasis. Up-regulation of these proteins is, therefore, important adaptations for increased protection and recovery capacity in the face of cellular stress and damage induced by strenuous exercise³³.

Physical training combined with low to medium dosage of red-fleshed pitaya extract were measured based on the ability and lead to immunomodulation effect that affect the body immune system and protect the body from cell damages, resulting in an effective condition to reduce the oxidative stress³⁴. Stress resulted from physical activity like oxidative stress is responded by hypothalamus to secrete corticotrophin realizing hormone (CRH) which then delivers a message to pituitary anterior. The pituitary produced adrenocorticotrophic hormone (ACTH) which is useful to activate or affect adrenal cortex where cortisol hormone is secreted. Cortisol contributes a massive influence to immune responses¹⁶ as a sign that the body is suffering from oxidative stress³⁵. In this research, cortisol hormone level tends to decreased in the group with strenuous exercise combined with red-fleshed pitaya extract.

This occurs since the red-fleshed pitaya extract is an effective antioxidant to reduce the risk of oxidative stress and is able to decrease the secretion of cortisol by reducing ACTH secretion in the hypothalamus and CRH in the pituitary gland³⁶.

Increased cortisol levels after physical activity depends on the level of fitness, exercise intensity and exercise program³⁷. Exercise with heavy intensity tends to produce free radicals which can cause the production of the hormone cortisol to increase. Provision of red dragon fruit that contains antioxidant polyphenols can reduce the production of free radicals due to exercise thereby reducing cortisol levels³⁸.

Red-fleshed pitaya is the cactus fruit of the genus *Hylocereus* and *Selenicereus*. Red-fleshed pitaya is very popular and widely planted in Indonesia because it is known by the public as herbal medicine. Research by Widyaningsih *et al.*, (2017) report that red-fleshed pitaya as an alternative treatment for anemia in pregnant women³⁹. The other studies by Irmayanti and Ardiaria (2016) report that *Hylocereus Polyhizus* can reduce cholesterol levels in rats⁴⁰.

Conclusion

Based on this research, it can be concluded that red-fleshed pitaya extract has the potential to be antioxidant with its anthocyanin content, and is able to eliminate oxidative stress due to strenuous exercise. It can be observed by the decreased pattern of HSP70 and cortisol expression in the strenuous exercise combined with red-fleshed pitaya extract group. Furthermore, we find out the optimum result was shown in potential of red-fleshed pitaya as an antioxidant and the dosage of effective dose at 300 mg/kg body weight of red-fleshed pitaya extract. can reduce oxidative stress which is influence on HSP70 and cortisol expression in rats that were subjected strenuous exercise.

given dose at 300 mg/kg body weight of red-fleshed pitaya extract was indicated to be more effective for reducing HSP70 expression.

Data availability

Underlying data

Open Science Framework: The effect of red-fleshed pitaya (*Hylocereus polyrhizus*) on heat shock protein 70 and cortisol expression in strenuous exercise induced rats, <https://doi.org/10.17605/OSF.IO/MGX4K>⁴¹

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

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Yusni Yusni 

Department of Physiology, Syiah Kuala University, Banda Aceh, Indonesia

I have read the details of the author's revision. I decide that I agree with all revisions made by the author. I think this article deserves to be indexed.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Medical Physiology: exercise physiology; endocrine and metabolic physiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 04 March 2020

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Mohd Adzim Khalili Rohin 

Faculty of Health Sciences, Sultan Zainal Abidin University (UniSZA), Kuala Nerus, Malaysia

1. It is an interesting manuscript. However, there are several grammatical issues and some typos that you should check very carefully.

2. My main concern about your manuscript is that you have not mentioned any strengths or limitations in your study.
3. The small number of rats per group is indeed a limitation and should be mentioned and taken into consideration before making conclusions.
4. This is a prospective study on the effect of red-fleshed pitaya (*Hylocereus polyrhizus*) on heat shock protein 70 and cortisol expression in strenuous exercise-induced rats.
5. The conclusion is weak in my opinion.
6. The study could be considered more as a pilot study than a proper study; however the results are interesting and should be indexed.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Functional Food & Nutraceuticals

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 08 November 2019

<https://doi.org/10.5256/f1000research.19175.r55888>

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Yusni Yusni 

Department of Physiology, Syiah Kuala University, Banda Aceh, Indonesia

Introduction:

1. Please explain why red-fleshed pitaya is thought to act as antioxidants that will affect the heat shock protein 70 and the expression of cortisol: include theoretical and other studies that support.
2. The novelty of the article must be clearly explained in the introduction.

Methods:

1. Why use 3 doses, whereas the purpose of the study did not assess the effective dose? In the conclusion, also mention the effective dose.
2. Explain the results of the identification of the chemical content of red-fleshed pitaya and also the results of herbarium analysis because there is a possibility that Indonesia's geographical differences will affect its chemical content.
3. Explain the time of blood-sampling because cortisol production is influenced by circadian.

Discussion:

1. It is important to explain the role or mechanism of action of red-fleshed pitaya on heat shock protein 70 and cortisol expression.

Reference:

1. Use the latest references (from the last 5-10 years).

Note: There are some grammatical errors, you should use the proof-read service.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Medical Physiology: exercise physiology; endocrine and metabolic physiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 26 Nov 2019

Novita Sari Harahap, Faculty of Sports Sciences, Universitas Negeri Medan, Medan, Indonesia

Introduction:

1. Please explain why red-fleshed pitaya is thought to act as antioxidants that will affect the heat shock protein 70 and the expression of cortisol: include theoretical and other studies that support.

*Red fleshed pitaya has antioxidant activity. Red Dragon Fruit contains powerful antioxidants because it consists of various antioxidants, namely flavonoids, vitamin E, vitamin C (Lianiwati, 2011). The results of several studies stated the red dragon fruit (*Hylocereus polyrhizus*) has an anthocyanin content (Kanner et al., 2001) around 26,457 ppm. Dragon fruit contains flavonoids (Damayanti, 2016). Anthocyanin is a pigment that is classified as a type of water-soluble flavonoids. Anthocyanins are anthocyanidin and glucose compounds in organic acids (Shipp and Abdel Aal, 2010). Anthocyanins are believed to have a very good antioxidant effect. A study conducted at the University of Michigan in America showed that anthocyanin can destroy free radicals, more effectively than vitamin E which has been known as a powerful antioxidant. Other studies in the United States prove that anthocyanin is the most powerful antioxidant among other flavonoid classes. The content of anthocyanin is believed to inhibit various free radicals such as superoxide radicals and hydrogen peroxide. Anthocyanins and various forms of their derivatives can inhibit various oxidation reactions by various mechanisms (Astawan and Kasih, 2008). The results of previous studies ripe dragon fruit contains many organic acids (Stinzing, et al., 2004), protein (Le Bellec, et al., 2006), minerals such as potassium, magnesium, calcium and iron and vitamin C (Cal et al, 2003).*

Increased RONS (Reactive Oxygen and Nitrogen Species) production during exercise has potential negative effects, transient increases in RONS seem to be a trigger for many exercise-induced adaptations in skeletal muscle (Powers et al. 2010). Exercise can stimulate an increased concentration of HSP70 in muscles and plasma.

Oxidative stress due to exercise can cause physiological functional changes because physical exercise is considered as a stimulus received by the hypothalamus, then the hypothalamus signals to the HPA axis, the HPA axis responds and gives positive and negative responses to the body (Guilliam & Edward, 2010). Then stimulates the hypothalamus and causes secretion of the hormone corticotrophin-releasing hormone (CRH), which stimulates the hypothalamus for ACTH secretion. Increased ACTH secretion, causes increased cortisol secretion (Usui et al, 2012).

Methods:

1. Why use 3 doses, whereas the purpose of the study did not assess the effective dose? In conclusion, also mention the effective dose.

Giving 3 doses of 100 mg/kg BW, 200 mg/kg BW and 300 mg/kg BW to find out what is the optimal dose to neutralize oxidative stress due to strenuous physical exercise. A dose of 100 mg/kg BW can also reduce oxidative stress but not as well as a dose of 300 mg/kg BW. So it is necessary to experiment with 3 doses, the purpose of the study to find out the potential of Red-fleshed pitaya as an antioxidant and once to know the optimal dose to reduce oxidative stress.

2. Explain the results of the identification of the chemical content of red-fleshed pitaya and also the results of herbarium analysis because there is a possibility that Indonesia's geographical differences will affect its chemical content.

*Red-fleshed pitaya is the cactus fruit of the genera *Hyllocereus* and *Selenicereus*. Dragon fruit is very popular and widely planted in Indonesia because it is known by the public as herbal medicine (Evi, et al., 2007). From the results of previous studies, ripe dragon fruit contains many organic acids (Stinzing, et al., 2004), protein (Le Bellec, et al., 2006), minerals such as potassium, magnesium, calcium and iron and vitamin C (Cal et al., 2003).*

3. Explain the time of blood-sampling because cortisol production is influenced by **circadian**.

Cortisol levels are affected by circadian rhythm. Blood drawn for examination of cortisol is done in the morning because it is a basal state of the body after resting at night. Also besides, our bodies have biological variations according to time, meaning that the analytical levels examined in the morning can give different results if examined in the afternoon. On examination of Cortisol, the levels will increase in the morning and reach the lowest levels in the afternoon. Then the blood draw is done in the morning according to the normal human biological clock, the most efficient and does not result in excessive fatigue.

Discussion:

1. It is important to explain the role or mechanism of action of red-fleshed pitaya on heat shock protein 70 and cortisol expression.

*Research conducted by Rebecca et al (2010) shows that red dragon fruit contains the most polyphenols compared to other species, namely 86.13 ± 17.02 mg in 0.50 g of dried extract of red dragon fruit. Red dragon fruit (*Hyllocereus polyrhizus*) is one of the plants that can be used as a source of antioxidants. Severe physical exercise is associated with increased HSP70 expression due to oxidative stress (Paulsen et al. 2007).*

Oxidative stress causes damage to cells that will cause stress responses in the form of heat shock response (HSR). HSR is regulated at the level of transcription by a mechanism involving heat shock transcription factor (HSF), especially HSF-1. Increased HSF-1 will help in increasing HSP 70 expression in certain amounts/levels in serum and blood plasma (Baird et al., 2006).

Physical activity appears to occur concomitantly with the up-regulation of endogenous antioxidant systems (Powers and Jackson 2008) and heat shock proteins (HSP) in skeletal muscle (Morton et al. 2009b). While the main role of the antioxidant enzymes is to reduce oxidation and prevent oxidative damage, HSPs can prevent and reverse damage to proteins. Intriguingly, the HSPs work with the antioxidant systems, and collectively they have essential roles in cell homeostasis. Up-regulation of these proteins is, therefore, important adaptations for increased protection and recovery capacity in the face of cellular stress and damage induced by high-intensity exercise (Powers and Jackson 2008; Morton et al. 2009b).

Antioxidants have been shown to abolish the acute exercise-induced increases in HSP70 protein levels (Khassaf et al. 2003; Jackson et al. 2004).

Increased cortisol levels after physical activity depends on the level of fitness, exercise intensity and exercise program [Leite et al., 2011]. Exercise with heavy intensity tends to produce free radicals which can cause the production of the hormone cortisol to increase (Stachowicz, 2016). The provision of red dragon fruit that contains antioxidant polyphenols can reduce the production of free radicals due to exercise thereby reducing cortisol levels.

Competing Interests: I declare no competing interests

Author Response 04 Jul 2020

Novita Sari Harahap, Faculty of Sports Sciences, Universitas Negeri Medan, Medan, Indonesia

Thank you for this thorough review of the manuscript and your insightful comments that have improved the clarity and quality of the paper. Below there is a point-by-point response to your inquiries.

The difference between this new version and version 1 is that in this new version, we can explain the antioxidant activity contained in red-fleshed pitaya that can affect heat shock protein 70 and the expression of cortisol. Red fleshed pitaya contains powerful antioxidants because it consists of various antioxidants, namely flavonoids, and polyphenol (Chemah et al. 2010; Adnan, 2011). Thus Red fleshed pitaya which has the potential as an antioxidant can reduce ROS and respond to HSP70 expression.

We can also explain that giving 3 doses of 100 mg/kg body weight, 200 mg/kg body weight, and 300 mg/kg body weight to find out what is the effective dose to neutralize oxidative stress due to strenuous physical exercise. So it is necessary to experiment with 3 doses, in accordance with the purpose of the study to find out the potential of Red-fleshed pitaya as an antioxidant and once to know the effective dose to reduce oxidative stress which is influence on HSP70 and cortisol expression in rats that were subjected strenuous exercise. Red-fleshed pitaya is the cactus fruit of the genera *Hyllocereus* and *Selenicereus*. Red-fleshed pitaya is very popular and widely planted in Indonesia because it is known by the public as herbal medicine. Research by Widyaningsih et al., (2017) report that red-fleshed pitaya as an alternative treatment for anemia in pregnant women. The other studies by Irmayanti and Ardriaria (2016) report that red dragon fruit (*Hylocereus Polyhizus*) can reduce cholesterol levels in rats. This statement explains that red-fleshed pitaya have also affect difference.

Competing Interests: No competing interests were disclosed

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