



## Ethanol extract of black grapes (*Vitis Vinifera*) ameliorates overtraining-induced pancreatic $\beta$ -cells and muscle damage

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### Abstract

*Overtraining is a high-volume, high-intensity, long-duration, and high-frequency training. Increasing physical training burden followed by oxygen demand required for aerobic metabolism. Increasing oxygen usage causes an elevation of electron leakage in mitochondria; thus, produce a higher amount of reactive oxygen species (ROS). Antioxidant inhibits oxidative damage in a target molecule. Grapes contain a lot of antioxidants, such as polyphenols and anthocyanins. The purpose of this study was to examine the effect of ethanol extract of Balinese grapes (*Vitis vinifera*) on  $\beta$ -cells and muscle damage in overtraining-induced rats. This study was a completely randomized experimental study using a posttest only control group design. Samples were 36 male albino rats (*Rattus norvegicus*), aged 2.5-3 months, divided randomly into two groups. The control group (P0), 18 rats, were given overtraining and placebo of 2 ml distilled water; the treatment group (P1), 18 rats, were given overtraining and 25g/kg.BW ethanol extract of Balinese grapes. The result showed the number of pancreatic  $\beta$ -cells in the P1 group was significantly higher than the P0 group ( $p < 0.001$ ). In contrast, the average level of total creatine kinase was significantly lower in the P1 group than those of the P0 group ( $p < 0.001$ ). It can be concluded that the administration of ethanolic extract of Balinese grapes mitigates the damage on pancreatic  $\beta$ -cells and muscle cells induced with overtraining.*

**Keywords:** Balinese grapes, pancreatic  $\beta$  cells, total creatine kinase, overtraining.

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### Introduction

Physical activity has been widely known to inhibit the aging process (Haryanto, Pangkahila, Aman, & Siswanto, 2019; Siswanto & Pangkahila, 2014; Zenitalia, Pangkahila, Pangkahila, & Siswanto, 2018). However, the lack of physical activity or excessive physical activity will cause

a hormonal imbalance that causes cell damage, leading to a premature aging process (Kartiko & Siswanto, 2015; Siswanto & Pangkahila, 2015). Research showed that regular exercise can reduce the risk of death by 17% for every 1-metabolic equivalents of exercise capacity increase (Gulati et al., 2003). On the other hand, excessive exercise will cause adverse effects. It commonly occurs, as research on athletes who died suddenly while competing indicated that the cause of death due to excessive physical training-induced hypertrophic cardiomyopathy (Wasfy, Hutter, & Weiner, 2016). Many studies show that excessive physical training can lead to oxidative stress (Kartiko & Siswanto, 2018; Nyandra, Kartiko, Arunngam, Pangkahila, & Siswanto, 2018). This is due to increased oxygen consumption up to 100-200 times. Generally, 2-5% of the oxygen used in metabolic processes will become superoxide ions, so that heavy physical activity increased production of free radicals that would trigger oxidative stress and eventually damage a lot of cells in the body (Pangkahila, Linawati, Sugiritama, & Siswanto, 2019), including pancreatic  $\beta$ -cells (Siswanto, Yenniastuti, Putra, & Kardena, 2015) and skeletal muscle cells (Pereira et al., 2013).

The levels of the creatine kinase in serum is a potential marker to determine the functional status of muscle tissue. Increased serum level of creatine kinase describes cellular necrosis index and damage of muscle tissue both in an acute or chronic injury. Changes in muscle enzyme levels can also be found in normal subjects and in athletes after strenuous training. The creatine kinase (CK) is a typical enzyme found in muscle (Baird, Graham, Baker, & Bickerstaff, 2012). In order to prevent oxidative stress-related pathology, the administration of antioxidants is necessary. A well known non-enzymatic antioxidant is a flavonoid that acts as a free radicals chain-breaking (Nimse & Pal, 2015). The grape (*Vitis vinifera*) peel and seed abundantly contain flavonoids (Doshi, Adsule, Banerjee, & Oulkar, 2015). It also contains anthocyanins, which are antioxidants that have a high potential as a free radical scavenger and is protective against oxidative stress, and also inhibit lipid peroxidation in the body (Khoo, Azlan, Tang, & Lim, 2017). The grape seed extract is also known to have a strong antioxidant action, especially the content of proanthocyanidins which have a better antioxidant property compared to vitamin C, vitamin E and  $\beta$ -carotene in protecting cells from DNA damage and lipid peroxidation due to free radical (Yang et al., 2018).

Despite the well-established antioxidant content of grape, a study on its effect to ameliorate overtraining-induced pathology is limited. Hence, we aimed to examine the effect of ethanol extract of Balinese grapes (*Vitis vinifera*) on  $\beta$ -cells and muscle damage in overtraining-induced rats.

## Materials and Methods

This study was true experimental research using a completely randomized posttest only control group design. The samples were male albino rats (*Rattus norvegicus*), wistar strain, aged 2.5-3 months, with around 180-200 grams weight. The number of samples was 18 rats/group, therefore a total of 36 rats were used.

Rats were adapted for a week and were divided into two groups: The control group (P0), 18 rats, were given overtraining and placebo of 2 ml distilled water; the treatment group (P1) 18 rats, were given overtraining and 25g/kg.BW ethanol extract of Balinese grapes. The Balinese grapes extract was prepared according to the previously described method (Widhiantara, Arunngam, & Siswanto, 2018). In summary, the dried whole-fruit was coarsely powdered using a grinder and then extracted with 95% ethanol in the Soxhlet apparatus, filtered by Whatman paper and the solvent was dried by vacuum rotary evaporator at 50 °C.

After 14 days of treatment, blood sample was drawn through *medial canthus sinus orbitalis* for the total creatine kinase levels measurement. The rats were then euthanized to collect the pancreas for pancreatic  $\beta$ -cells examination. The pancreas of each animal was sliced in the size of about  $1 \times 1 \times 1 \text{ cm}^3$  and immersed in a fixative solution of 10% neutral buffered formalin, prior to further processing for histopathological preparations.

Serum creatine kinase levels were measured by Creatine Kinase (CK) Colorimetric Assay Kit (BioVision, San Francisco, CA, USA) according to the manufacturer's instruction that has been described previously (Han, Wu, & Wang, 2019). The method in tissue preparations for histopathology were adapted from Kiernan's method as described previously (Widhiantara et al., 2018). Gomori Chrome Hematoxylin Phloxine B-Staining staining was used in this study (Siswanto et al., 2015).

The data were analyzed statistically using the Statistical Package for Social Sciences (SPSS) version 22.0. Data analysis was performed using an independent sample *T*-test.

## Results and Discussion

### *Pancreatic $\beta$ -Cells Count*

Pancreatic  $\beta$ -cells stained by Gomori Chrome Phloxine Hematoxylin B-Staining will appear brown, pancreatic  $\alpha$  cells will appear red, and pancreatic  $\delta$  cells will be pink (Figure 1). The mean of pancreatic  $\beta$ -cells count of the P1 group was significantly higher than those observed in P0 group ( $43.63 \pm 7.74$  vs  $30.00 \pm 3.56$ ,  $p < 0,001$ ) (Table 1).

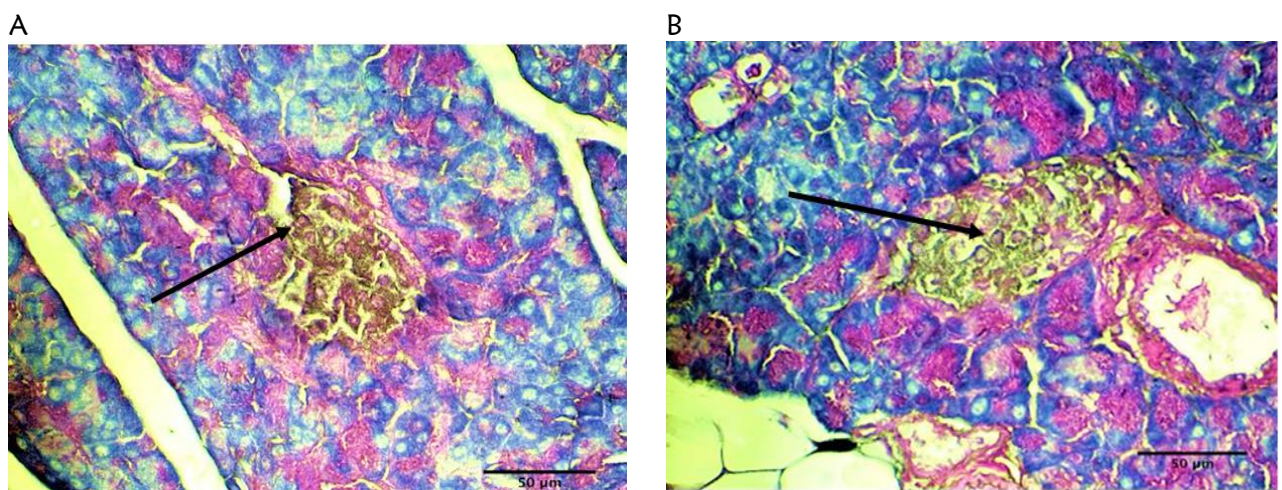


Fig 1. Histopathological examination of the Langerhans of Pancreatic Tissue. (A) Control Group (P0), and (B) Treatment Group (P1). Arrows show a single pancreatic  $\beta$ -cells. Stained with Gomori Chrome Phloxine Hematoxylin B-Staining, 400 times magnification.

The results of this study showed that the ethanol extract of Balinese grapes can prevent a decrease in the number of pancreatic  $\beta$ -cells induced by excessive physical training. In this study, rats were swum until almost drowned in order to obtain conditions of oxidative stress (Siswanto et al., 2015). During overtraining, increased oxygen supply are often unable to meet the oxygen demand. The sudden supply of high oxygen will increase the formation of oxygen free radicals could even achieve 10 times, which was called as reperfusion (González-Montero, Brito, Gajardo, & Rodrigo, 2018).

Table 1. Pancreatic  $\beta$ -Cells Count and Total Creatine Kinase level Comparison

| Variables   | Group                | N  | Mean   | SD     | <i>t</i> | <i>p</i> -value |
|---|----------------------|----|--------|--------|----------|-----------------|
| Pancreatic $\beta$ -Cells Count (cells/field of view) | Control group (P0)   | 18 | 30.00  | 3.559  | 6.40     | 0.000           |
|   | Treatment group (P1) | 18 | 43.63  | 7.736  |          |                 |
| Total Creatine Kinase level (mU/mL)                   | Control group (P0)   | 18 | 289.56 | 18.822 | 205.626  | 0.000           |
|   | Treatment group (P1) | 18 | 243.56 | 19.304 |          |                 |

N= Number of Sample; SD= Standard Deviation, *t*= t-value.

The imbalances between reactive oxygen species (ROS) and antioxidants can initiate oxidative stress, which without proper treatment, can manifest into disease. In type 1 diabetes mellitus, T-cell-mediated autoimmune destruction of pancreatic  $\beta$ -cells is secondary to the primary invasion of macrophages and dendritic cells (DCs) into the islets. Macrophages/DCs, however, are activated by intercellular ROS from resident pancreatic phagocytes and intracellular ROS formed after receptor-ligand interactions via redox-dependent transcription factors such as NF- $\kappa$ B. Activated macrophages/DCs ferry  $\beta$ -cells antigens specifically to pancreatic lymph nodes, where they trigger reactive T cells through synapse formation and secretion of proinflammatory cytokines and more ROS. ROS generation, therefore, is pivotal in formulating both innate and adaptive immune responses accountable for islet cell autoimmunity (Delmastro & Piganelli, 2011).

Ethanol extract of Balinese grapes may help prevent pancreatic  $\beta$ -cells damage since grapes are a source of antioxidants with high polyphenol content. Flavonoid is one of polyphenol which abundantly contains in grapes. Effects of flavonoids against ROS occurs through two mechanisms: by increasing endogenous antioxidant and direct scavenger activity. Increased endogenous antioxidant by flavonoids has been proven in vitro studies through an increased Nrf2 transcription factor that increases the expression of proteins HO-1 (Velagapudi, El-Bakoush, & Olajide, 2018). Flavonoids can activate the ERK, JNK, and p38 then activates Nrf2 resulting in increased gene expression of endogenous antioxidants (Mansuri, Parihar, Solanki, & Parihar, 2014).

Flavonoids have been known as a natural antioxidant that protects pancreatic  $\beta$ -cells from free radical damage. Furthermore, previous studies reported that besides protecting pancreatic  $\beta$ -cells from damage by oxidative stress, it also triggers the proliferation of  $\beta$ -cells within pancreatic islet (Pinent et al., 2008). Flavonoids play a role in controlling blood glucose by increasing insulin secretion through elevating metabolism of  $Ca^{2+}$  and regenerate pancreatic  $\beta$ -cells (Dias Soares, Pereira Leal, Silva, Almeida, & de Oliveira, 2017; Ghorbani, Rashidi, & Shafiee-Nick, 2019). Through interaction with the ATP-sensitive K channels in the membrane of beta cells causing membrane depolarization and this situation will open Sodium gate channels. The opening of sodium gate channels caused  $Ca^{2+}$  entering beta cells and then stimulate granules containing insulin secretion (Zhong & Jiang, 2019).

The content of resveratrol in Balinese grapes can effectively reduce oxidative damage to the pancreas, and thus can protect pancreatic  $\beta$ -cell function (Lee et al., 2012). Quercetin is a flavonoid polyphenol naturally found in a wide variety of plant-based food sources including grape, which has anti-diabetic activity. Studies showed quercetin can protect the function and keep the number of pancreatic  $\beta$ -cells from oxidative damage induced by  $H_2O_2$  (Youl et al., 2010). This effect is mediated by phosphorylation of extracellular signal-regulated kinase (ERK-1/ERK-2) (Maurya & Vinayak, 2016). Quercetin also reduces the concentration of thiobarbituric acid and lipid hydroperoxide in the pancreas and increase the activity of antioxidant enzymes (Boots, Drent, de Boer, Bast, & Haenen, 2011). These results indicate that the antioxidative activity of quercetin may protect pancreatic beta-cells from damage by

reducing oxidative stress caused by various exogenous and endogenous sources such as excessive physical training.

### ***Total Creatine Kinase Level***

The mean level of total creatine kinase of the control group (P0) was  $289.56 \pm 18.82$  mU/mL, and the treatment group (P1) was  $243.56 \pm 19.30$  mU/mL ( $p < 0.001$ ; Table 1). Creatine kinase (CK) is an enzyme typically found in muscle. CK activity as measured by muscle biopsy showed a different level before and after training (Brancaccio, Maffulli, & Limongelli, 2007). In addition to pancreatic  $\beta$ -cells damage, excessive physical training can also result in damage to skeletal muscle. Increased production of ROS that occurs during physical exercise can cause oxidative damage to muscle, liver, blood and other tissues, as proven by increasing CK in this study. Excessive physical training has proved contribute to an increase in oxygen consumption in skeletal muscle (Jones et al., 2017), an increase in lipid peroxidation, and inhibition of mitochondrial enzymes such as citrate synthase and malate dehydrogenase (Zoppi & Macedo, 2007). In this study, the level of total CK in P1 group is significantly lower than those observed in P0 group, suggesting that the ethanolic extract of Balinese grape reduced the muscle damage caused by excessive physical exercise.

Serum CK levels can increase due to damaged muscle tissue as a consequence of the training with the high frequency and intensity level. This may be a result of metabolic processes and mechanical causes. Metabolic processes underlying muscle damage due to excessive physical training is a decrease in membrane resistance which was followed by an increase in intracellular free calcium ions, which then induce the activation of potassium channels. Other mechanisms that cause local tissue damage muscle degeneration sarcomere Z-disk. CK is a good marker for muscle necrosis due to metabolic or mechanical processes, which will increase along with the increased burden of physical activity (Brancaccio et al., 2007).

### **Conclusion**

Based on the results of this study, it can be concluded that the administration of 25g/kg.BW ethanolic extract of Balinese grapes can prevent the pancreatic  $\beta$ -cells and skeletal muscle cells damage on overtraining-induced wistar rat (*Rattus norvegicus*). Further study is required to dissect the molecular mechanism underlying the phytochemical compound-specific mechanism on anti-oxidative damage of Balinese grapes extract.

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