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Overtraining Induces Oxidative Stress Mediated Renal Damage in Male Wistar Rats

[1] Made Nyandra, [1] Bambang Hadi Kartiko, [2] Pakajiraporn Arunngam, [3] Alex Pangkahila and [*1, 2] Ferbian Milas Siswanto

[1] Faculty of Health, Science and Technology, Dhyana Pura University, Badung, Indonesia, [2] Department of Biomedical Chemistry, School of Science and Technologi, Kwansei Gakuin, University, Sanda, Japan and [3] Department of Sports Physiology, Faculty of Medicine, Udayana University, Denpasar, Indonesia

Abstract

Background: Highly competitive athletes usually experience overtraining, which causes an increased production of ROS which can lead to oxidative stress and oxidative damage in several organs such as the kidney. The purpose of this study was to prove the involvement of oxidative stress in overtraining-related renal damage.
Methods: Thirty experimentally naïve male Wistar albino rats (2.5–3 months old, weighing 150–200 g) were divided equally into three groups: control (C) group, over trained (OT) group, and over trained + N-acetylcysteine (OTN) group.
Results: In this study, we found that no apoptotic cell was observed in kidneys of the C group, while it was observed in OT group. This apoptosis was followed by increasing blood urea nitrogen and creatinine level in OT group compare to C group ($p < 0.01$). We further found that this apoptosis is mediated by oxidative stress, as the antioxidant defenses were decreased and the level of MDA was elevated ($p< 0.01$ for all). The treatment of N-acetylcysteine (NAC) which alleviated oxidative stress and oxidative damage compared to OT group ($p< 0.01$), also ameliorated the apoptosis-induced overtraining ($p< 0.01$).
Conclusion: In summary, our research indicates that overtraining induces renal damage in male Wistar rats (Rattus norvegicus) via oxidative stress.

Keywords: Overtraining, oxidative stress, oxidative damage, apoptosis, kidney, rats.

* corresponding author: Faculty of Health, Science and Technology, Dhyana Pura University, Badung, Indonesia.

Introduction

Physical exercise is important to maintain physical fitness, prevent degenerative diseases and extend life expectancy. In order to obtain the health benefit of exercise, the frequency, intensity, time and type of exercise should be monitored tightly (Thompson *et al.*, 2013). However, many highly competitive athletes tend to increase the training loads and undergone excessive physical training which can cause adverse health effects (Carfagno and Hendrix, 2014, Kreher and Schwartz, 2012). In addition, many noncompetitive exercisers experience overtraining (OT) as a result of inadequate rest/recovery because of busy work lives, family, and health stressors, meal skipping, and poor sleep. The overload exercise and/or not enough recovery results in both physiological and psychological impairment that limit performance (Kreher and Schwartz, 2012). It has been widely established that overtraining causes the elevation of free radical production within the cell leading to oxidative damage. Silvestre *et al.* (2017) and Nigro *et al.* (2016) stated that overtraining induces stress oxidative in both mice and human respectively as indicated by increasing lactate dehydrogenase (LDH). Excessive physical activity elevates oxygen consumption (Townsend *et al.*, 2013). Generally, about 2 - 5% of the oxygen used in the metabolism process will become superoxide ions. Therefore, during overtraining, the superoxide ions and other free radicals production will be elevated and causes multi-organs damage (Powers and Jackson, 2008, Souza Jr. *et al.*, 2005). Oxidative stress mediates apoptosis through both intrinsic and extrinsic pathway, by decreasing Bcl-2 level and increased FAS protein expression respectively (Redza Dutordoir and Averill Bates, 2016). In addition, a research found that overtraining causes oxidative damage in DNA of blood cells especially leukocytes (Krüger and Mooren, 2014) and skeletal muscle cells (Pereira *et al.*, 2013) that lead to the induction of apoptosis. Several studies also showed that excessive physical training can cause damage to the renal kidney (Wu *et al.*, 2011, Wu and Huang, 2009a).The kidney is an interesting organ due to its important roles and major impact on health when it is damaged, as well as the high frequency of end-stage renal disease (Robson, 2014). Oxidative stress in renal tubular cells elevates the Bax to Bcl2 protein ratio and Bax to Caspase 3 protein ratio that would induce apoptosis (Wu *et al.*, 2011). In contrast, another independent study showed that oxidative stress induces apoptosis via caspase-independent pathway but depends on the Bax/Bcl-2 ratio and the depolarization of mitochondrial membrane potential (Tor *et al.*, 2015). Another study showed that renal cell apoptosis caused by overtraining is partly involving the inflammatory signal pathway characterized by elevated levels of TNF-α and NFκB (Wu and Huang, 2009b). Previously we have shown that overtraining induced renal apoptosis in parallel with the elevation of serum protease level and increasing renal p16INK4α gene expression (Kartiko and Siswanto, 2018). However, the involvement of oxidative stress in overtraining-induces renal apoptosis remains to be investigated. Thus, the aims of this study is to prove the involvement of oxidative stress in overtraining-related renal damage.

Materials and Methods

Animals

Thirty male Wistar albino rats, 2.5–3 months old, weighing 180 ± 16 g at the beginning of the experiment, were used. The animals were housed in cages, under environmentally controlled condition (22 ± 2 °C on a 12-h light/12-h dark cycle, lights at 7:00 a.m.), with food and water available ad libitum throughout the experiment. Animals were allowed to adjust to a new condition for 7 days. All efforts were done to minimize animal suffering. All the experimental protocols and treatments were approved by the committee on the ethics of animal experiments in Faculty of Veterinary Medicine, Udayana University that was completely in agreement with the "NIH Guide for the Care and Use of Laboratory Animals".

Experimental Design

Thirty, experimentally naïve male Wistar albino rats (2.5–3 months old, weighing 180 ± 16 g) were divided randomly into three groups: control (C) group (n= 10, without any training program), over trained (OT) group (n= 10), and over trained + N-acetylcysteine (OTN) group (n= 10). Over trained (OT) groups undertook a 60 min until the rats showed a sign of drowning, seven times a week for 3 weeks. The over trained + N-acetylcysteine (OTN) group undertook same protocol as OT group and was given N-acetylcysteine (NAC, Sigma, St. Louis, MO) of 1200 mg/kg/day for 3 weeks. The swimming apparatus was 80 cm in length, 50 cm in width and 90 cm in depth. The water level was adjusted to 70 ± 5 cm and the water temperature was maintained at 33–35 °C. Rats exercised once a day, 7 days per week for 3 weeks. The training periods were about 60 min until the rats showed a sign of drowning (Kartiko and Siswanto, 2018).

Sample Collection

At the end of the study, animals were anesthetized with intra-peritoneal injection of a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine, peripheral

blood was collected from the retro-orbital sinus according to previously described method (Wellington *et al.*, 2013). After allowing blood to clot on the ice, a serum sample was separated by centrifuging at 3000 rpm for 10 min. Serum was collected and stored at -20°C for blood urea nitrogen (BUN) and creatinine examination. The necropsy was performed afterward according to Parkinson *et al.*, (2011) method. In brief, kidneys were collected and were washed (for MDA examination) or fixed with a transport medium buffered formalin 10% (for apoptotic index examination) and with RNA later (for superoxide dismutase, catalase, and glutathione peroxidase mRNA examination).

Detection of Apoptosis

Apoptosis index was examined in the histological section using a commercially available in situ Apoptosis Detection Kit according to the manufacturer's instruction. In brief, paraffin-embedded kidney sections were treated with proteinase K (20 µg/ml, for 20 min, room temperature). After being washed, slides were incubated with a TUNEL reaction mixture (ID Labs, London, Ontario, Canada) containing terminal deoxynucleotidyl transferase. Under a high-power microscope (400× magnification), five visions were randomly collected and analyzed by pathological image software. The positive cells were counted, with the average value of five visions as the index of cell apoptosis.

Biochemical Analysis

Blood urea nitrogen (BUN) concentration and serum creatinine (Cr) level were measured by enzymatic kinetic UV assay and a kinetic colorimetric assay based on the Jaffé method on Cobas c701 (Roche Diagnostics, Mannheim) according to the manufacturer's instructions. Blood was sampled at 24 hours after the last treatment. MDA level was examined by thiobarbituric acid reactive substances assay (TBRAS) according to the previously described method (Wasowicz *et al.*, 1993). In brief, 1 g of renal tissue was homogenized in9 mL of 1.15% KCl and centrifuged at 1000 g for 10 min. The precipitate was discarded and the suspension used to measure renal MDA level by the TBA method. A working solution containing 15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25 N hydrochloric acid was prepared. For each sample, 250 µL serum or renal tissue homogenate and 500 µL working solution were mixed and placed in boiling water for 10 min. After the samples cooled, 25 µL of 5 mol/L HCl was added (final pH 1.6-1.7), and the reaction mixture was extracted by agitation for 5 min with 3.5 mL of n-butanol, and butanol phase was separated by centrifugation at 1500 x g for 10 min. The precipitate was discarded and the suspension used to measure the absorbance at 525 nm for excitation and 547 nm for emission. Protein concentration was measured using BSA as a standard by the method according to Lowry *et al.* (1951).

Isolation of RNA and Quantitative Real Time-PCR (Qrt-PCR)

Total RNA was extracted from renal tissue using Trizol (Roche, Mannheim, Germany) according to the manufacturer's instructions and stored at -80 °C. RNA was converted to cDNA by reverse transcription as follows (Kartiko and Siswanto, 2018): A reaction mixture containing 1 µg of RNA and 200 units of reverse transcriptase (Thermo Scientific, Waltham, MA) was incubated according to the manufacturer's instructions as follows: 10 min at 25 °C followed by 60 min at 42 °C and then 10 min at 70 °C to stop the reaction. Quantitative real-time PCR was performed using a Thermal Cycler Dice Real Time System Single TP850 (Takara Bio Inc., Shiga, Japan). SYBR Primer Ex TagII, 10 pmol of forward and reverse primers, and 1 µg of cDNA were mixed, and qRT-PCR was then performed according to the manufacturer's instructions. The PCR was carried out at 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. The PCR primer sequences used are as follows: Rat GAPDH, 5′-CAACTACATGGTTTACATGTTC-3′ (forward) and 5′-GCCAGTGGACTCCACGAC-3′ (reverse), rat SOD1, 5′-AATGTGTCCATTGAAGATCGTGTGA-3′ (forward) and 5′- GCTTCCAGCATTTCCAGTCTTTGTA-3′ (reverse), rat GPx1, 5′-GAAGCCACGTGATCTCAGCC-3′ (forward) and 5′- CTTGGGGTCGGTCATGAGCGC-3′ (reverse), rat Cat, 5′-CCCAAGCAACATGCCCCCTGGCAT-3′ (forward) and 5′- AAGAGCCTGGACTCGGGCCCCG-3′ (reverse).

Statistical Analysis

Data are presented as mean ± SD. Statistical differences were determined by one way ANOVA with Turkey post-hoc test. $P< 0.05$ was considered statistically significant.

Results

Effect of OT on Kidney Damage

In this study, we showed that there was no apoptosis in renal tubular epithelial cells isolated from the control (C) group (Figure 1A). In contrast, the over trained (OT) group exhibited some tubular epithelial cells in the kidney that underwent apoptosis with an apoptotic index of 58.53 ± 12.31% (Figure 1B). This result indicates that overtraining lead to injury to the kidney. To further confirm overtraining-induced kidney damage clinically, we measured the level of blood urea nitrogen (BUN) and serum creatinine (Cr), which are widely used screening tests of renal function.

Expectedly, both BUN and Cr levels of OT group were higher than the C group ($p< 0.01$ for both, Table 1).

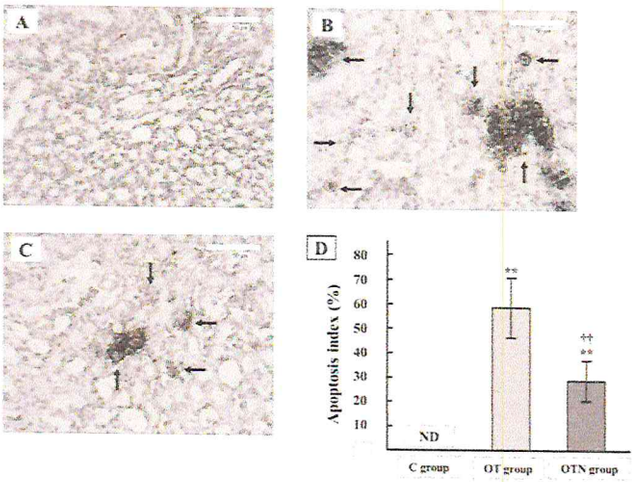

Figure 1: Detection of apoptotic renal tubular epithelial cells using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). (A) The apoptotic cell was not observed in C group. In contrast, apoptotic cell death was observed in both OT group (B) and OTN group (C). TUNEL-positive cells occasionally appeared in tubules and interstitium of tissue. Arrows indicate apoptotic cells. Scale bar, 50μm. (D) The proportion of apoptotic cells, values are mean ± SD (n = 10 rats per group, **p < 0.01 versus the C group, ††p < 0.01 versus the OT group, ND not detectable, one way ANOVA with Turkey post-hoc test).

Table 1: Effects of three-weeks overtraining with or without N-acetylcysteine

	C Group	OT Group	OTN Group
Blood urea nitrogen (mg/dL)	24.72±2.48	34.24±4.48 **	29.44 ± 1.18 ** †
Serum creatinin (mg/dL)	0.26±0.05	0.42±0.08 **	0.34 ± 0.06 * ††
Renal MDA (nmol/gr.prot)	459.33± 34.81	573.80 ± 23.44 *	480.17± 21.44 †
Final body weights (gr)	184.28 ± 16.71	161.13 ± 14.82 *	163.45±10.7 *

Values are mean ± SD, *p< 0.05 vs. C group, **p< 0.01 vs. C group, †p< 0.05 vs. OT group, ††p< 0.01 vs. OT group

Effect of OT on Renal Antioxidant Defences

As it is widely showed that overtraining causes oxidative stress (Nigro *et al.*, 2016, Silvestre *et al.*, 2017), we are intrigued whether it also causes oxidative stress in renal tissue. Under physiological conditions, the production of ROS and the capacity of the antioxidant enzyme are maintained in balance level. However, under several conditions such as overtraining, the production of ROS surpass the antioxidant defences and exert toxic effects. Superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (Cat) are considered as the classical and antioxidant enzymes (Birben *et al.*, 2012). In this study, we showed that the expression levels of SOD, GPx, and Cat were significantly higher in OT group compared to C group (Figure 2). These results indicate that overtraining cause's oxidative stress and induces the antioxidant defences in renal tissue.

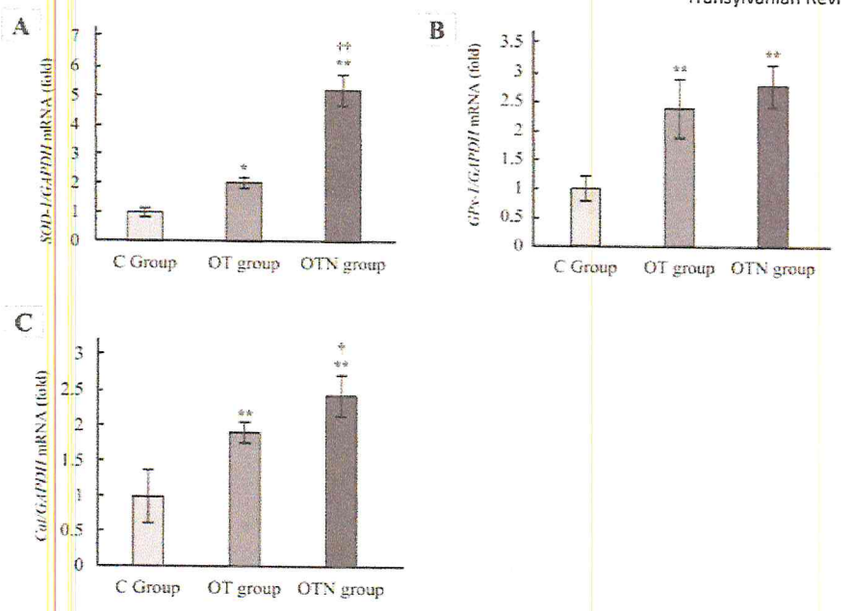

Figure 2: The antioxidant defences expression level in the kidney tissue. Quantitative determination of mRNA expression of superoxide dismutase (A), glutathione peroxidase (B), and catalase (C) was performed using a pair of primers specific to the investigated genes as described. The ratio of SOD-1, GPx-1, and Cat to GAPDH mRNA was normalized as 1. The data are presented as the mean ± standard deviation (n= 10 rats per group, *p< 0.05, **p< 0.01 versus the C group, †p< 0.05, ††p< 0.01 versus the OT group, one way ANOVA with Turkey post-hoc test).

Effect of OT on MDA Level

Malondialdehyde (MDA) is the most frequently used biomarker of oxidative stress-related tissue and organ damage. MDA is generated in vivo via peroxidation of polyunsaturated fatty acids (Ayala *et al.*, 2014). In support of the apoptosis results, overtraining also induces oxidative stress, shown by an elevation of renal MDA level (p< 0.05, Table 1).

Effect of NAC on OT-Induced Renal Damage

In order to confirm that overtraining induced renal damage is mediated by oxidative stress, we treated the over trained rats with a widely used chemical that act as both in vitro and in vivo antioxidant, N-acetylcysteine (NAC). As expected, the apoptosis index of over trained + N-acetylcysteine 1200 mg/kg/day (OTN) group was lower than the OT group (p< 0.01, Figure 1). In addition, both BUN and Cr levels were decreased by NAC treatment (p< 0.05 for both, Table 1). The NAC treatment resulted in the improvement of renal antioxidant defences as indicated by higher expression of SOD and Cat in OTN group compared to OT group, but not GPx expression (Figure 2). The oxidative damage effect of overtraining was diminished by NAC treatment, as the renal MDA level was lower in OTN group than the OT group (p< 0.05) and the MDA of OTN group was comparable to C group (p> 0.05, Table 1). Together, these results suggest that NAC alleviates overtraining-induced oxidative stress and renal damage.

Discussion

Overtraining-induces oxidative stress has been widely reported (Nigro *et al.*, 2016, Silvestre *et al.*, 2017), and we previously showed that overtraining induces renal apoptosis (Kartiko and Siswanto, 2018). However, to the best of our knowledge, no study has specifically shown the overtraining-induces renal apoptosis is mediated by oxidative stress. In this study, we showed that overtraining for 3 weeks results in renal tubular epithelial cells apoptosis. This apoptosis also lead to impairment of physiological performance of kidney in general, as both BUN and creatinine levels were increased. Overtraining cause's apoptosis is likely mediated by oxidative stress, as the antioxidant defences expression are elevated by overtraining and followed by increasing MDA levels. Oxygen consumption is greatly increased during physical training. Because the mitochondria as an energy-producing intracellular organelle, are also a major source of reactive oxygen species (ROS), overtraining lead to increase production of ROS and further cause oxidative stress (Laforgia *et al.*, 2006, Souza Jr. *et al.*, 2005, Turrens, 2003). Moreover, excessive physical training lead to ischemia where the adenosine triphosphate (ATP) will be converted to adenosine diphosphate, adenosine monophosphate, inosine, and eventually hypoxanthine, with the superoxide anion and H_2O_2 formation, a process known as xanthine oxidase (XO) pathway. XO pathway has been

considered to be responsible for the production of ROS (Vergeade *et al.*, 2012) and tissue damage during intensive exercise. The inhibition of XO by several compounds such febuxostat (Malik *et al.*, 2011, Nomura *et al.*, 2015) or allopurinol (Zajączkowski *et al.*, 2018) has been proven to reduce the production of ROS. Oxidative stress causes apoptosis of renal cell through several molecular pathways. It can cause a shift of the Bax/Bcl-2 and Bax/caspase-3 ratios (Alarifi *et al.*, 2017, Wu *et al.*, 2014), increases the levels of TNF-α and NFκB (Almeida *et al.*, 2010, Voltan *et al.*, 2016), and mitochondrial permeability transition (MPT) leakage of cytochrome C (Ishihara and Shimamoto, 2006). In addition, ROS pro-apoptotic proteins such as protein-1 activator (AP-1) and p53 (Mashayekhi *et al.*, 2014, Morgan and Liu, 2011). Recently, we also found that oxidative stress-induced overtraining causes apoptosis by improving the activity of protease and p16INK4α expression (Kartiko and Siswanto, 2018). Next, our findings indicated that the apoptosis of renal tissue is attenuated by the treatment of antioxidant, N-acetylcysteine (NAC). The NAC has been proved to attenuated oxidative stress-related ischemia-reperfusion injury in several organs such as heart (Bartekova *et al.*, 2018), liver (Lee *et al.*, 2012), intestine (Ayvaz *et al.*, 2009), lung and muscle (Sotoudeh *et al.*, 2012) through its antioxidant activity. Several studies established that antioxidant activity of NAC is nonspecific. It inhibits activation of the c-Jun N-terminal kinase, p38 MAP kinase and redox-sensitive activating protein-1 and nuclear factor kappa B transcription factor activities (Cotgreave, 1997, Zafarullah *et al.*, 2003). This study showed that NAC increased the SOD, GPx, and Cat expression, which in agreement with previous research that showed NAC maintains intracellular glutathione levels in exercise-induced intestinal lymphocyte apoptosis (Quadrilatero and Hoffman-Goetz, 2004).

Conclusion

In conclusion, we showed that a renal tubular epithelial cells apoptosis occurs following 3 weeks overtraining in rats relative to the sedentary rats. This apoptosis was preceded by impairment physiological performance of kidney in general, as both BUN and creatinine levels were increased. This apoptosis is likely mediated by oxidative stress, as the antioxidant defences were up-regulated and MDA level was increased. It is further confirmed by the treatment of NAC that is able to diminish the renal apoptosis and improves the antioxidant defences. Together, these results suggest that overtraining-induces renal apoptosis is mediated by oxidative stress.

Conflict Of Interest

The authors declare there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Ethical Approval

All procedures performed in this studies that involving rats as animal models were conducted in accordance to the guideline on the welfare of experimental animals and with the approval of the Ethics Committee on the use of animals of Faculty of Veterinary Medicine, Udayana University, and Denpasar, Indonesia.

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