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The role of cortisol and interleukin-10 gene expression patterns in exhaustive exercise

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ABSTRACT

Cortisol is a steroid hormone produced by the zona fasciculate of the adrenal cortex in response to stress, whereas interleukin-10 is an important immunoregulatory cytokine which increases with exercise. Cortisol most often is viewed as having a counter-productive role in exercise. Therefore, the objective of this study was to establish the critical role of cortisol during exercise. Moreover, the immunoregulatory role of interleukin-10 in limiting host immune response to stress was also investigated. The longitudinal study randomly selected twenty-five young apparently healthy students from the Faculty of Health Sciences and Technology, Nnamdi Azikiwe University, Nnewi Campus, aged 24.3 ± 3 years. The participating male undergraduate students were placed under resting and relaxed conditions for 48 hours prior to the test. Their blood pressures and pulse rates were taken and fasting blood sugar determined as a prerequisite screening before enlisting in the study. The subjects took part in an endurance race using the Bruce treadmill protocol for sub-maximal exercise for a maximum of 21 minutes. The target heart rate on the treadmill was 60 – 80 percent of the heart rate reserve. Blood samples were collected from the participants before commencement of the study, at 1 hour, 4 hours and 24 hours post exercise. Serum cortisol (nmol/L), troponin I (ng/ml), creatine kinase MM (ng/ml) was determined using enzyme-linked immunosorbent assay methods. Interleukin-10 genes expression patterns were detected using reverse transcriptase polymerase chain reaction method. There was a significant increase in cortisol, troponin and creatine kinase MM level at 1 hour post exhaustive exercise when compared with pre-exercise stage ($F = 6.032$, $P = 0.000$), ($F = 4.551$, $P = 0.000$) and ($F = 10.282$, $P = 0.000$) respectively. The expression patterns of interleukin-10 genes were up-regulated at 4 hours post exercise and sustained till 24 hours post exercise ($\chi^2 = 50$, $P = 0.000$). Post exercise stress activates the release of cortisol, and interleukin-10 genes to reinstate homeostasis through modulation of the immune response.

Keywords: Homeostasis, immune response, interleukin-10 genes, cortisol, stress.

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INTRODUCTION

Cortisol is a steroid hormone produced by the zona fasciculate of the adrenal cortex in the adrenal gland, this hormone plays a vital role in the body's defense mechanism when dealing with stress as well as being important in blood glucose regulation (Hackney and Walz, 2013). Interleukin 10 is known to play a significant role in immune regulation involving both T helper 2 (Th2) and T helper 1 (Th1), thereby enhancing normal tissue homeostasis (Stenberg et al., 2000). Physical exercise is important for maintaining physical fitness and strengthening the immune system (Stampfer et al., 2000; Hu et al., 2001; Gosker and Schol, 2008).

Cortisol most often is viewed as having a counter-productive role in physical exercise. Therefore, the objective of this article was to establish the critical role of cortisol during exercise. Moreover, the immunoregulatory role of interleukin-10 in limiting host immune response to stress was also investigated. The emphasis is specifically to correct several false impressions about the counter-productive roles of cortisol by some exercise specialists. The view by some exercise specialist is that the elevated cortisol level during exercise can lead to a predominance of catabolism in the body (Hackney and Walz, 2013). This is an oversimplification of the hormonal action of cortisol.

MATERIALS AND METHODS

Study area

The study was carried out in the Faculty of Health Sciences and Technology, Nnamdi Azikiwe University, Nnewi Campus, Nigeria.

Subjects

Twenty five (25) healthy young male undergraduate students with an average age of 24.3± 3 years and body mass index of 22.7± 1.8 (kg/m^2) participated fully in the study (Table 1). Patient consent was obtained from the subjects.

Inclusion criteria

This study was limited to apparently healthy young male undergraduate students of the Faculty of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus, Nigeria, within 18 and 35 years of age who are willing to participate in the study.

Exclusion criteria

Young male with an underlying history of illness, e.g. Hypertension, irregular heart rate, glucose utilization disorders, asthmatics, sickle cell anemia and other forms of anemia were excluded. Those currently undertaking examination were excluded. Those that engage in strenuous activities such as (professional athletes, welders etc) were excluded. Subjects currently on antioxidant supplementation, alcohol and any antimicrobial agents were excluded.

Research design

The research design was the longitudinal experimental design.

Study design

The subjects were encouraged to eat balance diet two hours prior to the endurance race and avoid any strenuous activity during the course of the research. Upon arrival at the venue of the research, their height (H) and weight (W) was measured and recorded and they were allowed to rest for at least ten minutes. The subjects took part in an endurance race using the Bruce treadmill

protocol for sub maximal exercise. The exercise protocol started at 2.7 km/hr and a 10% grade and increased by 2% every 3 minutes in a step-like manner to a final stage at 9.6 km/hr with a 22% grade as described by Vanessa and Elizabeth (2000). The target heart rate on the treadmill was 60-80 percent of the heart rate reserve (HRR).The difference between maximal heart rate (MHR) and resting heart rate (RHR).The HRR was calculated using the formula:

HRR=MHR-RHR.

MHR=220-age in years. As described by Ogwumike et al. (2011). The subjects continued this for twenty-one minutes or stopped when they are tired. Fourteen out twenty-five subjects were exhausted before the twenty-one minutes. Blood sample was collected at four different time points: before, one hour, four hours and twenty-four hours post exercise stages.

Collection of blood samples

Six milliliters of venous blood sample were collected from the medial cubital vein using vacutainer and needle from each of the subjects shared equally into plain containers and RNAgard vacutainer for total lymphocyte RNA isolation.

Determination of serum cortisol level

Enzyme-linked immunosorbent assay was used in the determination of the level of serum cortisol in the serum as previously been described by Ehiaghe et al. (2014). In brief, 20 µl of standard or sample(s) was added per microplates. 200 µl cortisol hormone conjugate was added to the standard or sample(s) and covered with a sealing tape. It was incubated at room temperature for 1 hour. The solution was discarded and microplates washed three times with 300 µl of 1X wash solution. 100 µl of tetramethyl benzidine one step substrate was added to each micro plate and incubated for 15 minutes at room temperature in the dark with gentle shaking. 100 µl of stop solution was added to each micro plate. The intensity of the color developed was measured at 450 nm wavelength using stat fax® 4700 micro strip reader.

Determination of serum troponin inhibitory level

Enzyme-linked immunosorbent assay (ELISA) was used in the determination of serum troponin inhibitory level as previously been described by Goett et al. (2011). In brief, 100 µl volume of standards, specimens, and controls were dispensed into appropriate wells. It will be gently mixed for 10 seconds. A 100 µl volume of enzyme conjugate reagent was added into each well. It was thoroughly mixed for 30 seconds and incubated at room temperature for 90 minutes. The incubation mixture was removed by flicking plate contents into a waste container. The wells were rinsed five times with wash solution. The wells sharply strike onto absorbent paper to remove all residual water droplets. A 100 µl volume of TMB reagent dispense into each well and incubated at room temperature for 20 minutes. The reaction was stopped by adding 100 µl volume of stop solution to each well. It was gently mixed for 30 seconds. The absorbance was read at 450 nm with a microtitre well reader within 15 minutes.

Determination of serum creatine kinase-MM level

Enzyme-linked immunosorbent assay (ELISA) was used in the determination of serum creatine level as previously been described by Marianne et al. (2012). In brief, 20 µl volume of standard or sample(s) was added per microplates. A 200 µl volume hormone conjugate was added to the standard

or sample(s) and covered with a sealing tape. It was incubated at room temperature for one hour. The solution was discarded and microplates washed three times with 300 µl volume of 1X wash solution. A 100 µl volume of tetraethyl benzidine one step substrate was added to each micro plate and incubated for 15 minutes at room temperature in the dark with gentle shaking. A 100 µl volume of stop solution was added to each micro plate. The intensity of the color developed was read at 450 nm wavelength using stat fax® 4700 micro strip reader.

Polymerase chain reaction methods

Total RNA Extraction using the ZYMO RESEARCH Whole-Blood RNA MiniPrep

Total RNA was extracted using the ZR Whole –Blood RNA MiniPrep with catalog number R1020 and R1021 by ZYMO RESEARCH CORPORATION according to manufacturer's specification at the Lahor.

Research Laboratory and Medical centre, 121 Old Benin- Agbor Road, Benin City, Edo State, Nigeria. 70 µl of the Total RNA extracted was transferred into an RNA. Stable tube supplied by Biomatrica with catalog number 93221-001 for storage of Total RNA at room temperature after proper drying.

One Taq One-Step reverse transcriptase polymerase chain reaction

The extracted Total RNA was retro-transcribed and amplified using One Taq One Step RT-PCR kit with catalog number NEB E5315S by NEW ENGLAND BioLabs incorporation according to the manufacturer's specification. Interleukin 10 genes forward and reverse primers (ATGCACAGCTCAGCACTGC; TCAGTTTCGTATCTTCATTGTC) were used to target lymphocyte template using Peltier thermal cycler polymerase chain reaction machine at the Lahor Research Laboratory and Medical Centre, 121, Old Benin-Agbor Road, Benin City, Edo state, Nigeria. The system components were thaw and mixed by inverting ten times. The PCR was performed in a 50 µl reaction mixture containing 25 µl One Taq one-step reaction master mix (2x), 2 µl One Taq one-step enzyme mix (2x), 2 µl of each gene-specific forward primer (10 µM), 2 µl of each gene-specific reverse primer (10 µM), 9 µl of nuclease-free water and 10 µl of the RNA template(s) was added last. The PCR was started immediately as follows: Reverse transcriptase at 48 ^{0} C for 30 minutes, initial denaturation at 94 °C for 1 minute, denaturation at 94 °C for 15 seconds, annealing at 54 °C for 30 seconds, extension at 68 °C for 1 minute, Go to the denaturation step for 39 cycles, final extension at 68 °C for 5 minutes and final holding at 4 °C forever. Five micro liters of the amplified PCR products were analyzed on 1% agarose gel containing ethidium bromide in 1X Tris EDTA buffer. Electrophoresis was performed at 90 volt for 30 minutes with the EDVOTEK tetra source electrophoresis machine, Bethesda, USA. The targeted genes were visualized by Wealtec Dolphin-Doc UV transilluminator and photographed. Molecular weights were calculated using molecular weight standard of the marker. The graphical analysis was done on a bar chart.

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reaction machine at the Lahor Research Laboratory and Medical Centre, 121, Old Benin-Agbor Road, Benin City, Edo state, Nigeria. The system components were thaw and mixed by inverting ten times. The PCR was performed in a 50 µl reaction mixture containing 25 µl One Taq one-step reaction master mix (2x), 2 µl One Taq one- step enzyme mix (2x), 2 µl of each gene-specific forward primer (10 µM), 2 µl of each gene- at 68 °C for 1 minute, Go to the denaturation step for 39 cycles, final extension at 68 °C for 5 minutes and final holding at 4 °C forever. Five micro liters of the amplified PCR products were analyzed on 1% agarose gel containing ethidium bromide in 1X Tris EDTA buffer. Electrophoresis was performed at 90 volt for 30 minutes with the EDVOTEK tetra source electrophoresis machine, Bethesda, USA. The targeted genes were visualized by Wealtec Dolphin-Doc UV transilluminator and photographed. Molecular weights were calculated using molecular weight standard of the marker. The graphical analysis was done on a bar chart.

Specific reverse primer (10 µM), 9 µl of nuclease-free water and 10 µl of the RNA template(s) was added last. The PCR was started immediately as follows: Reverse transcriptase at 48 ^{0}C for 30 minutes, initial denaturation at 94 ^{0}C for 1 minute, denaturation at 94 ^{0}C for 15 seconds, annealing at 54 ^{0}C for 30 seconds, extension

Statistical analysis

Values were expressed as mean ± standard deviation (SD). All numerical results were analyzed with one-way ANOVA with post hoc multiple comparisons test while student's t- test was used to compare independent variables. Gene expression results were analyzed with Chi-Square test using SPSS for windows version 20.0 statistical program. P values < 0.05 were considered statistically significant.

Ethics

Ethical approval were obtained from the Ethics Committee of the Faculty of Health Science and Technology, Nnamdi Azikiwe University, Nnewi Campus, Nigeria and Lahor Research Laboratory and Medical centre in Benin City, Edo State, Nigeria with reference number LRL/ 005/014.

Table 1: Baseline physiological characteristics of the subjects.

Variables	All subjects (N=25)	Subjects that were exhausted before 21 minutes (x ± S.D) (N=14)	Subjects that were exhausted at 21 minutes (x ± S.D) (N=11)	t- values	P-values
Age (yrs)	24.3 ± 3.0	23.50 ± 3.0	25.36 ± 3.0	0.191	0.666
Height (m)	1.7 ± 0.07	1.73 ± 0.07	1.72 ± 0.06	0.177	0.678
Weight (kg)	67.6 ± 6.2	68.6 ± 6.67	66.3 ± 6.55	1.083	0.309
Body mass index (kg/m^2)	22.7 ± 1.8	22.90 ±1.8	22.43 ± 1.8	0.002	0.968

SD: = Standard deviation
X = Mean value
P = P < 0.05 were considered significant
N = Number of subjects
Note: All values are presented as mean ± SD

RESULTS

The study revealed that the mean ± S.D of cortisol level of subjects who were exhausted before the end of the exercise bout (21 minutes) were 293.1 ± 150.86 nmol/L pre-exercise, 411.0 ± 169.71 nmol/L one hour post exercise, 246.0 ± 155.12 nmol/L four hours post exercise and 183.4 ± 72.83 nmol/L twenty-four hours post exercise. The cortisol level were significantly higher at one hour post exercise when compared with pre-exercise (P = 0.000). However, there was no significant difference when compared with the four and twenty-four hours post exercise (P = 0.222). The mean ± S.D of cortisol level of subjects who were exhausted at twenty-one minutes of the exercise bout were 226.4 ± 67.95 nmol/L pre-exercise, 355.6 ± 153.62 nmol/L one hour post exercise, 190.1 ± 64.05 nmol/L four hours post exercise and 183.6 ± 58.11 nmol/L twenty-four hours post exercise. The cortisol level were significantly higher at one hour post exercise when compared with pre exercise (P = 0.000). Similarly, there was no significant difference when compared with the four hours post exercise (P = 0.494) and twenty-four hours post exercise (P = 0.420) (Table 2).

The mean ± S.D of troponin I level of subjects who were exhausted before the end of the exercise bout were 1.7 ± 1.83 ng/ml pre-exercise, 5.7 ± 3.04 ng/ml one hour post exercise, 4.5 ± 3.00 ng/ml four hours post exercise and 3.9 ± 2.89 ng/ml twenty-four hours post exercise, the troponin I level were significantly higher at one hour post exercise, four hours post exercise and twenty-four hours post exercise when compared with pre-exercise (P= 0.000). The mean ± S.D of troponin I level of subjects who were exhausted at the end of the exercise bout were 3.4 ± 1.87 ng/ml pre-exercise, 6.7 ± 2.91 ng/ml one hour post exercise, 4.2 ± 2.48 ng/ml four hours post exercise and 3.2 ± 2.02 ng/ml twenty-four hours post exercise. The troponin level were significantly higher at one hour post exercise when compared with pre-exercise (P = 0.000) (Table 2).

The mean ± S.D of creatine kinase MM level of subjects who were exhausted before the end of the exercise bout were 3.7 ± 1.12 ng/ml pre-exercise, 6.4 ± 1.55 ng/ml one hour post exercise, 4.4 ± 1.77 ng/ml four hours post exercise and 3.3 ± 1.18 ng/ml twenty-four hours post exercise bout. The creatine kinase MM level were significantly higher at one hour post exercise when compared with pre-exercise (P = 0.000). The mean ± S.D of creatine kinase MM level of subjects who were exhausted at the end of the exercise bout were 3.5 ± 1.20 ng/ml pre-exercise, 5.9 ± 1.73 ng/ml one hour post exercise, 4.0 ± 1.57 ng/ml four hours post exercise and 2.7 ± 1.13 ng/ml twenty-four hours post exercise. The creatine kinase MM level were significantly higher at one hour post exercise when compared with pre-exercise (F = 10.28, P = 0.000) (Table 2).

Moreover, the reverse transcriptase polymerase chain reaction results for interleukin 10 genes revealed that the genes were up-regulated at 4 hours post exercise and sustained till 24 hours post exercise at 250 bp on a 1% agarose gel electrophoresis stained with ethidium bromide in all the exercised subjects (Plate 1 and Figure 1).

Table 2: Mean (±SD) values of the cortisol (nmol/l), troponin I (ng/ml) and creatine kinase – MM (ng/ml) of the exercised subjects.

Time intervals	Cortisol		Troponin I		Creatine kinase MM	
	Subjects exhausted before 21 minutes (n =14)	Subjects exhausted at 21 minutes (n =11)	Subjects exhausted before 21 minutes (n =14)	Subjects exhausted at 21 minutes (n =11)	Subjects exhausted before 21 minute (n =14)	Subjects exhausted at 21 minutes (n =11)
Pre-exercise(A)	293.1± 150.86	226.4 ± 67.95	1.7 ± 1.83	3.4 ± 1.87	3.7 ± 1.12	3.5 ± 1.20
1hour post exercise(B)	411.0± 169.71	355.6 ± 153.62	6.8 ± 3.04	6.7 ± 2.91	6.4 ± 1.55	5.9 ±1.73
4 hours post exercise(C)	246.0± 155.12	190.1 ± 64.05	4.5 ± 3.00	4.2 ± 2.48	4.4 ± 1.77	4.0 ± 1.57
24 hours post exercise(D)	183.4 ± 72.83	183.6 ± 58.11	3.0 ± 2.89	3.2 ± 2.02	3.3 ± 1.18	2.7 ± 1.13
F Value	6.032		4.551		10.282	
P Value	0.000*		0.000*		0.000*	
A vs. B	0.013*	0.016*	0.000*	0.003*	0.000*	0.000*
A vs. C	0.316 (ns)	0.494 (ns)	0.555(ns)	0.468 (ns)	0.206 (ns)	0.414 (ns)
A vs. D	0.222(ns)	0.420 (ns)	0.223(ns)	0.856 (ns)	0.453 (ns)	0.198 (ns)

ns = non-significant; * = significant.

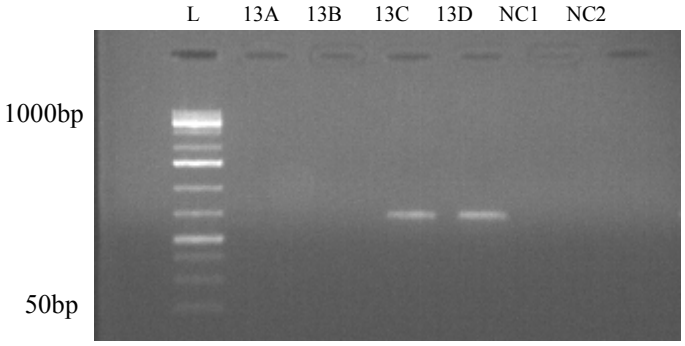

Plate 1: Reverse transcriptase PCR results for interleukin 10 genes detected in exercise-induced stress subjects analyzed on a 1.0% agarose gel electrophoresis stained with ethidium bromide. L is a 50 bp-1000 bp DNA ladder (molecular marker). Lanes 13C and 13D are positive bands for the expressed interleukin 10 genes at 250 bp from exercised subject. Lanes 13A and 13B are negative bands from the pre stage and 1 hour post exercise respectively while NC1 and NC2 are no template control.
A = pre-exercise
B = 1-hour post exercise
C = 4-hours post exercise
D = 24-hours post exercise

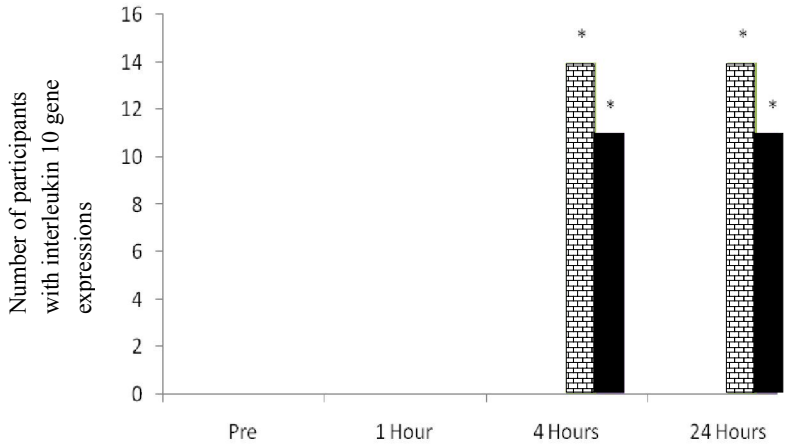

Figure 1: An overall multiple bar chart representation of the up-regulation of IL-10 gene detected in exercised subjects at different time intervals. The expression patterns were up-regulated at 4 hours post exercise and sustained till 24 hours post exercise when (χ^2 = 50, P = 0.000) respectively. Pre = Gene not expressed in exercised participants; Bricks = Gene expression in subjects who were exhausted before 21 minutes at different time intervals; Black = Gene expression in subjects who were exhausted at 21 minutes at different time intervals; *= Significant P < 0.05

DISCUSSION

This study observed that the cortisol level of the exercised subjects was significantly higher at 1 hour post exhaustive exercise stage (approximately 9:30 am) when compared with pre-exercise stage (approximately 8: 30 am) (Table 2). These results support the fact that stress of any origin, whether physical or mental can greatly enhance the secretion of adrenocorticotrophic hormone and consequently cortisol. It has been reported that elevated cortisol inhibits the action of insulin thereby encouraging higher blood glucose level via gluconeogenesis and glycogenolysis (Carl and Edward, 2006; Raddatz and Ramadori, 2007). Thus, it is possible that the 1 hour post exhaustive exercise cortisol elevation observed in this study inhibits the action of insulin thereby enhancing the production of blood glucose via gluconeogenesis and glycogenolysis in an attempt to regain glucose homeostasis. Tortora et al. (2006) observed that post exercise cortisol elevation enhances the production of blood glucose from non-carbohydrate substrates such as amino acids and glycerol. This is known as gluconeogenesis.

According to Sang-Hoon et al. (2007) post exercise stress increases blood glucose utilization by enhancing both glycogenolysis and gluconeogenesis in the cell of the liver and skeletal muscles. It has been reported that glycogenolysis and gluconeogenesis enhances the release of glucose into the bloodstream during physical activity thereby ensuring that blood glucose levels do not drop below the fasting blood glucose level (Fox, 2009). Also, Holloszy (2003) reported that gluconeogenesis accounts for approximately 20% of blood glucose production during low to moderate intensity exercise in human subjects. Furthermore, post exercise cortisol elevation has been reported to stimulate the breakdown of triglycerides in adipocytes thereby enhancing the release of free fatty acid which are needed for the generation of adenosine triphosphate via the beta-oxidative pathway for muscle contractions (Brooks et al., 2005). However, there was a significant decrease of cortisol level at 4 hour post exhaustive exercise stage (approximately 1: 30 pm) and 24 hours post exercise stage (approximately 8: 30 am) when compared with the pre exercise stage (approximately 8: 30 am). The significant decrease in cortisol

level could be attributed to a restored homeostasis as the subjects feel relieved of the stress induced by the exhaustive exercise bout.

Humans respond to stress by activating cytokines producing cell to release both pro and anti inflammatory cytokines, the pro-inflammatory cytokines such as tumor necrosis factor alpha activate the hypothalamus–pituitary–adrenal to release cortisol into the blood-Stream in attempt to regain cellular homeostasis (Wingfield et al., 2002). The observed up-regulation in the expression patterns of the interleukin 10 genes at 4 hours post exercise and sustained till 24 hours post exhaustive exercise at 250 bp (Plates 1 and Figure 1) might be an indication that there is an effective control of the immune responses to stress within these periods. Stenberg et al. (2000) reported that humans respond to post exercise stress by activating cytokine producing cells to induce the production of cytokine genes such as tumor necrosis factor alpha, interferon gamma; it simultaneously releases interleukin-10 to effectively control immune response to stress. Bente (2006) and Rodrigo et al. (2012) in their studies reported that post exercise stress enhances the up-regulation of interleukin-10 genes in attempt to regain cellular homeostasis during muscle contractions. However, other work demonstrated that interleukin-10 levels are elevated during strenuous exercise (Ostrowski et al., 2000). Although Peake et al. (2005) reported that exercise induced muscle fatigue can up-regulate interleukin-10 genes. Nieman et al. (2005) also observed that 30 minutes' walk increases the level of interleukin-10 genes in young male students.

It has been reported that adenosine triphosphate generated from anaerobic metabolism (phosphocreatine, stored adenosine triphosphate and anaerobic glycolysis) and aerobic metabolism modulates the sliding of actin along the length of myosin to achieve muscle contraction (Kenney et al., 2012). The essential role of anaerobic glycolysis during muscle contraction is manifested in the elevation of blood lactate level from 1.6 to 8.3 mM in actively contracting muscles (Jeremy et al., 2001). Moreover, aerobic metabolism is the major source for adenosine triphosphate production from blood glucose, glycogen and triglycerides during exercise that last longer than 2 to 3 minutes. Unlike anaerobic adenosine triphosphate production, the aerobic metabolism system is slow to turn on; but it has a much larger adenosine triphosphate producing capacity, this places considerable demands on the cardiovascular and respiratory system to deliver oxygen to the active muscles (Kenney et al., 2012). These changes are essential for producing adenosine triphosphate required for supporting muscle contractions. Goett et al. (2011) and Marianne et al. (2012) reported in their studies that an increased mechanical stress on the contracting muscles during exhaustive exercise can significantly elevate the level of troponin inhibitory and creatine kinase. Thus, it is probable that the 1 hour post exercise elevations of troponin inhibitory and creatine kinase are indicative of an increased mechanical stress on the contracting muscle during the exhaustive exercise bout. However, there were no significant differences observed at 4 hours and 24 hours post exercise elevation of troponin inhibitory and creatine kinase-3 when compared with the pre-exercise stage (Table 2). This is suggestive of a restored homeostasis as the subjects feel relieved of the mechanical stress induced by the exhaustive exercise bout.

Conclusion

The post exercise elevation of creatine kinase-3 and troponin I level are indicative of an increased mechanical stress on the contracting muscles during the exhaustive exercise bout. Furthermore, post exercise stress activates the release of cortisol, and interleukin-10 genes to reinstate homeostasis through modulation of the immune response.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration with all the authors. Authors EFA, OCC and ACO designed the study,

conducted the exercise protocol and performed the statistical analysis. Authors EFA, ADE and EIJ conducted and managed the Laboratory analysis. All the authors read and approved the final manuscript.

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