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ATTENUATION OF SWIMMING-INDUCED OXIDATIVE STRESS BY COMPOSITE HERBAL SUPPLEMENT ON ANTIOXIDATIVE PARAMETERS IN MALE RAT

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ABSTRACT

The present study has been designed to find out the antioxidant potency of composite methanolic extract on swimming-induced oxidative damage on packed erythrocytes in Wistar strain male albino rat. Extract was administered at the dose of 40 mg/100 g body weight/day/rat orally by gavage for 15 days period to experimentation followed by co-treatment of the aforesaid extract at the same dose for 28 days of swimming. In swimming group significant diminution resulted in the activities of catalase, peroxidase, glutathione-S-transferase, superoxide dismutase; whereas, elevation in the level of thiobarbituric acid reactive substances and conjugated dienes in packed erythrocytes in comparison to control. The levels of all these parameters were completely recovered towards the extract treated group. The extract possesses no general toxic effect observed from serum glutamate oxaloacetate transaminase and glutamate pyruvate transaminase activities. Such results conclude that above extract has a significant protective effect on swimming-induced oxidative damage. The antioxidant potency of composite extract was compared with vitamin C.

KEYWORDS: Swimming, Composite herbal extract, Antioxidant enzymes, Lipid peroxidation, Vitamin C, SGOT and SGPT.

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INTRODUCTION

Cellular homeostasis in aerobic organism is extensively threatened by reactive oxygen intermediates and the byproducts generated from oxidative metabolism [1]. Ironically these reactive oxygen species are derived from normal physiological and metabolic processes that are essential to cell [2]. Exercise elevates metabolic rate and increases oxygen consumption in skeletal and cardiac muscles as well as in other tissues. The rate of oxygen uptake by the body during exercise may increase upto 10-15 fold [3]. Oxygen flux in the active peripheral skeletal muscle tissue may, however, increase approximately 100 fold with an ~30 fold increase in blood flow and 3 fold increase in arteriovenous oxygen difference [4]. Enhancement of oxygen consumption during increased metabolic activity increases electron leakage from the mitochondrial transport system. This leads to oxidative stress causing an imbalance between ROS production and antioxidant defense mechanism as well as lipid peroxidation in our body [5]. Regular physical exercise augments antioxidant defense system and reduces exercise-induced oxidative threat in locomotive or exercising muscles [6]. Forced swimming is generally considered as an exhaustive type of exercise [7] in which increased production of free radicals takes place [8] causing polyunsaturated fatty acid (PUFA) peroxidation in membranes. In *in vitro* system, erythrocytes are very sensitive to oxidative stress due to the absence of both nuclei as well as mitochondria in their cell [9].

Cycling, running and swimming have been shown to cause RBC damage [10]. Exercise induced erythrocyte destruction usually leads to 'Sports anaemia' in sedentary or untrained rats [11] but not in exercise trained rats [12]. Supplementation of antioxidants like vitamin-C or vitamin-E in the body from external source decreases hemolysis in sedentary rats and also in exercise trained rats[13]. It has been found that in Ayurvedic medicine and also in Indian system of folk medicine, role of more than one herbal plant in combined way is more effective than single plant extract in the form of tonic for the attenuation of health hazards those result from exhaustive oxidative stress [14]. From such knowledge a composite methanolic extract was prepared from root of *Withania somnifera* (*W. somnifera*), leaf of *Ocimum sanctum* (*O. sanctum*) and rhizome of *Zingiber officinale* (*Z. officinale*).This composite extract was applied on the long term swimming-induced exhausted rats to evaluate its effectiveness on oxidative stress reduction.

O. sanctum Linn commonly known as Tulsi. This herb is a member of the family Lamiaceae and several medicinal properties are attributed in Ayurveda, the Indian system of medicine [15]. *O. sanctum* is also used as anti-stressor and also as an antioxidant [16]. *Z. officinale* belongs to the family Zingiberaceae. Their common names are calamus, sweet ginger. Ginger has a high content of antioxidants [17]. Depending on the medicinal importance *W. somnifera* Dunal (Ashwagandha) is widely used in India and in many other countries. It is used as an antistressor and anti-oxidant agent [18]. The present study may focus on some of the important aspects in the field of management of stress physiology, such as: i) Does exhaustive endurance swimming pose any effect on the hematological parameter like packed cell volume in the non-trained animals? ii) Whether strenuous swimming induces any effect on the corpuscular antioxidant enzyme system in our body. iii) Is there any significant beneficial role of the above mentioned herbal mixture in the management of ROS in the nontrained experimental animals? On the whole, it can be focused that further investigation based on such study may improve the aerobic endurance capacity of athletes.

MATERIALS AND METHODS

Plant materials

The root of *W. somnifera*, leaf of *O. sanctum* and rhizome of *Z. officinale* were collected from Gopali, Indian Institute of Technology, Kharagpur, Paschim Medinipur district of West

Bengal in the month of May and the material was identified by Mr. Ram Kumar Bhakat, plant taxonomist of Botany Department, Vidyasagar University, and voucher specimen number were HPCH No.-3,4 and 5 respectively.

Preparation of composite extract

The specific plant parts were dried in an incubator for 2 days at 40 ^{0}C, crushed in an electrical grinder and then powdered separately. 50 g powder of each plant material was extracted in 250 ml of methanol for 18 h in a soxhlet apparatus at 40 ^{0}C. The deep brown of *Z. officinale,* yellowish brown of *W. somnifera* and deep green of *O. sanctum* extract in methanol were collected. The suspension was then filtered by coarse sieve filter paper. The filtrate was evaporated to dryness under reduced pressure at rotary evaporator. From 50 g of powder 5 g of extract was obtained and stored at $(0\text{-}4)^0$C. These extracts were used for next 7 days of the experiment[19].

Selection of animals and care

Wistar strain healthy male albino rats, scientific name *Rattus norvegicus* (N=40) having body weight of 125±5 g each were selected for this study. They were acclimatized to laboratory condition for 2 weeks prior to experimentation. Animals were housed two per cage in a temperature controlled room (25±2^{0}C) with 12-12 h light-dark cycle at a humidity of 50 ± 10%. They were provided with standard food (wheat, milk powder, dalia, Bengal gram powder) and water *ad libitum*. The present study was conducted in accordance with the internationally accepted 'Principles for Laboratory Animal Use and Care' as found in the US guidelines (NIH publication No. 85-23). The study was approved by our 'Institutional Ethical Committee'.

Experimental design

Forty, adult healthy, male albino rats of Wistar strain were divided equally into 5 groups on the basis of the matching of their body weights of the animals. The treatment schedule of each group was as follows:

Group I (Control group)

Control rats were kept in rat's cage. Rats of this group received olive oil (0.5 ml/100 g body weight/day/rat) for 15 days prior to the experimentation followed by 28 days of experimental period through oral route at 8.00 AM.

Group II (Control + Composite extract treated group)

Animals were subjected to forceful oral administration of methanolic extract of these plant parts at the ratio of 1:2:2 (*W. somnifera* : *O. sanctum*: *Z. officinale*) at the dose of 40 mg/100 g body weight/day/rat in 0.5 ml of olive oil for 15 days prior to starting of experiment followed by next 28 days of experimentation without swimming. This ratio was standardized by trial and error method. The herbal mixture was administered at 8.00 AM of each day by gavage.

Group III (Swimming group)

Rats were subjected to swimming for 8 h/day including rest. The duration of this exercise was fixed for 30 min at a stretch followed by 10 minutes rest. This swimming was continued for 28 days without break. Olive oil was administered through gavage as in group I.

Group IV (Pretreatment followed by swimming and composite extract co-administered group)

Rats were subjected to preconditioning by oral administration of methanolic extract of these plant parts. This extract was dissolved in olive oil for 15 days prior to the starting of swimming at the same ratio as of group II. From 16th day, animals were subjected to swimming for 8 h/day (including rest) at the same protocol like group III for 28 days. Before 2 h of starting the swimming in each day, all the animals of this group were subjected to oral administration of the methanolic extract dissolved in olive oil of these plant parts in composite way at the same dose as per preconditioning period and also as of group II. Food was provided to the animals at least 1 h before forced swimming and it was continued from 10. 00 AM to 18.00 PM including rest every day.

Group V (Swimming + vitamin C treated group)

Animals were subjected to forceful oral administration of vitamin C at the dose of 10 mg/100 g body weight/day/rat in 0.5 ml of distilled water for 15 days prior to staring of experiment followed by next 28 days of experimentation without swimming. To maintain the same physical stress due to handling of animals and forceful ingestion of extract, all the animals of group I, II and III were subjected to olive oil and group V for distilled water treatment by gavage throughout the experimental period at the same time in relation to pre-exercise oral treatment of herbal mixture to group IV.

Forced swimming programme

The forced swimming of rats was performed in acrylic plastic pool (90 cm × 45 cm × 45 cm) filled with water to depth of 37 cm as per design of previous workers [20]. The temperature of the water was maintained at 34 ± 1^0C. The rats were loaded with a steel washer weighing approximately 4% of their body weight attached to the tail. This arrangement forced the rat to maintain continuous rapid leg movement [21]. After completion of 28 days swimming, all the animals were sacrificed serially according to their completion of exercise time after 5 min of post exercise one after another. Blood was collected from dorsal aorta. Blood was centrifuged at 3000 g for 10 min. Plasma sample and packed RBC were separated from one another and used for biochemical assay and plasma fraction was utilized for SGOT and SGPT levels determination.

Biochemical assay of catalase (CAT)

Ctalase activity was measured biochemically [22]. For the evaluation of CAT activity, in packed RBC from each animal, RBCs were homogenized separately in 0.05 M Tris hydrochloric acid buffer solution (pH-7.0) at the concentration of 50 mg/ml. This homogenate was centrifuged at 10000 g at 4^0C for 10 min. In spectrophotometric cuvette, 0.5 ml of hydrogen peroxide and 2.5 ml of distilled water were mixed and reading of absorbance was noted at 240 nm. Packed RBC supernatant was added at the volume of 40 µl and the subsequent 6 readings were noted at 30 sec interval.

Biochemical assay of superoxide dismutase (SOD)

Packed RBC was homogenized in 100 mM Tris-cacodylate buffer to give a concentration of 50 mg/ml and centrifuged at 10000 g for 20 min. The SOD activity of the supernatant was estimated by measuring the percentage inhibition of the pyragallol autooxidation by SOD [23]. The buffer was 50 mM Tris (pH-8.2) containing, 50 nM cacodylic acid (pH-8.2), 1 mM ethylene diamine tetra acetic acid and 10 mM hydrochloric acid. In spectrophotometric cuvette, 2 ml of buffer, 100 µl of 2 mM pyragallol and 10 µl of supernatant were poured and absorbance was noted in a spectrophotometer at 420 nm for 3 min. One unit of SOD was defined as the enzyme activity that inhibited the autooxidation of pyragallol by 50 percent.

Estimation of glutathione-S-transferase (GST)

Activity of GST in the packed RBC was measured spectrophotometrically [24] using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The assay mixture of 3 ml content 0.1 ml of CDNB in ethanol, 0.1 ml of 1M reduced glutathione, 2.7 ml of 100 mM potassium phosphate buffer (pH-6.5) and 0.1 ml supernatant of the packed RBC homogenate. The formation of the adduct of CDNB, S-2,4-dinitrophyneleglutathione was monitored by measuring the net increase in absorbance at 340 nm against blank. Enzyme activity was calculated using the extinction coefficient of 6.9/M/cm and expressed in unit/mg of tissue.

Estimation of lipid peroxidation from the levels of thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD)

Packed RBC was homogenized at the tissue concentration of 50 mg/ml in 0.1 M of ice-cold phosphate buffer (pH-7.4) and the homogenate was centrifuged at 10000 g at 4^0C for 5 min. Each supernatant was used for the estimation of TBARS and CD. For the measurement of TBARS, 0.5 ml homogenate was mixed with 0.5

ml of normal saline (0.9 g%) NaCl and 2 ml of thiobarbituric acid-trichloro acetic acid mixture (0.392 g of TBA in 75 ml of 0.25 N HCl with 15 g TCA). The volume of the mixture was made up to 100 ml by 95 % ethanol and boiled at 100^0C for 10 min. This mixture was then cooled at room temperature and centrifuged at 4000 g for 10 min. The whole supernatant was transferred in spectrophotometer cuvette and read at 535 nm [25]. Quantification of CD was performed by standard method [26]. The lipids were extracted with chloroform-methanol (2:1) mixture followed by centrifugation at 1000 g for 5 min. The chloroform layer was evaporated to dryness under a stream of nitrogen. The lipid residue was dissolved in 1.5 ml of cyclohexane and the absorbance was noted at 233 nm to measure the amount of hydroperoxide formed.

Glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) activities in serum

For the assessment of metabolic toxicity, we measured GOT and GPT activities in serum according to the method of Goel [27].

Statistical analysis

Analysis of variance (ANOVA) followed by multiple two-tail "t" test with Bonferroni modification was used for statistical analysis of the collected data [28]. Difference were considered significantly when P<0.05.

RESULTS

Body weight

Significant diminution in body weight in swimming animals (Group III) was observed as a result of 15 days preconditioning followed by 28 days swimming in comparison to the animals of both control group (Group I) as well as extract treated control group (group II) (Table 1). There was no significant difference in body weight gain between group I and II. After pretreatment followed by co-treatment of the above mentioned composite extract at the dose of 40 mg/0.5 ml olive oil/100 g body weight/day/rat for the aforesaid period to the forced swimming rat (Group IV), or vitamin-E pretreatment cum co-administration (Group V) to the animals of the swimming group a significant elevation in body weight towards the control groups (Group I and II) was recorded.

CAT, Px, GST and SOD activities in packed RBC

In group II animals composite extract showed a partial increment in the packed RBC's antioxidant activities assessed here i.e. CAT, Px, GST and SOD but such elevation insignificantly differed in comparison to group I animals (Table 2). Whereas, all the above mentioned antioxidant enzymes in RBC's in extract untreated swimming (Group III) were decreased significantly compared to group I and II controlled rat. Pretreatment followed by co-treatment of said extract at the aforesaid dose and for the same period to group IV animals resettled all the antioxidative enzymes in packed RBC towards the control group. After co-administration of vitamin C in group V these parameters were resettled towards the control level (Table 2).

Quantification of TBARS and CD levels in packed RBC

In packed RBC a significant increase in the quantification of TBARS and CD levels were noted in group III animals when the data were compared to the group I and group II animals (Table 2). The applied extract as per the previous protocol to the group IV rat attenuated swimming-induced oxidative stress significantly in packed RBC in both TBARS and CD levels in comparison to group III rats. The levels of TBARS and CD in group IV and V attend to group I and group II controlled rat after 15 days preconditioning and next 28 days co-administration of the extract and vitamin in forced swimming animals (Table 3)

Assessment of plasma GOT and GPT levels

The activities of GOT and GPT in plasma were not altered significantly among the groups studied here (Table 4).

Table 1

Effect of composite methanolic extract of root of W. somnifera, leaf of O. sanctum and rhizome of Z. officinale on body weight in swimming-induced oxidative stress.

Group	Initial body weight (g)	Final body weight (g)	Weight gain (%)
Control (Group-I)	120.4±4.1[a]	156.3±4.96[a]	29.8
Extract treated control (Group-II)	118.9±4.0[a]	160.2±4.29[a]	34.7
Swimming (Group-III)	121.2±3.9[a]	130.3±4.30[b]	7.5
Extract treated swimming (Group-IV)	120.8±3.5[a]	150.4±3.98[a]	24.5
Vitamin C treated swimming (Group-V)	119.6±3.4[a]	149.9±3.48[a]	25.3

Data expressed as Mean±SE (n=8). ANOVA followed by multiple two-tail t-test. In vertical column mean values with different superscripts (a,b) differ from each other significantly (P<0.05).

Table 2

Effect of composite methanolic extract of root of W. somnifera, leaf of O. sanctum and rhizome of Z. officinale on CAT, Px, GST and SOD in packed RBC in swimming-induced oxidative stress.

Group	CAT (mM of H_2O_2 consumption/mg of tissue/min)	Px (Unit/mg of tissue)	GST (Unit/mg of tissue)	SOD (Unit/mg of tissue)
Control (Group I)	11.18±0.3[a]	1.68±0.03[a]	0.276±0.04 [a]	1.754±0.03[a]
Extract treated Control (Group-II)	12.02±0.02[a]	1.85±0.02[a]	0.285±0.05 [a]	1.875±0.02[a]
Swimming (Group-III)	9.42±0.4[b]	1.21±0.06[b]	0.171±0.01[b]	1.457±0.03[b]
Extract treated Swimming (Group-IV)	11.82±0.5[a]	1.81±0.04[a]	0.270±0.01[a]	1.7491±0.03[a]
Vitamin C treated swimming (Group-V)	11.99±0.5[a]	1.79±0.03[a]	0.276±0.02[a]	1.7471±0.04[a]

Data expressed as Mean±SE (n=8). ANOVA followed by multiple two-tail t-test. In vertical column mean values with different superscripts (a,b) differ from each other significantly (P<0.05).

Table 3

Effect of composite methanolic extract of root of W. somnifera, leaf of O. sanctum and rhizome of Z. officinale on TBARS and CD levels in packed RBC in swimming-induced oxidative stress.

Group	TBARS (nM/mg of tissue)	CD (nM hydroperoxide/mg of tissue)
Control (Grooup-1)	11.28±0.30[a]	214.74±3.52[a]
Extract treated control (Group-2)	12.60±0.50[a]	225.82±3.24[a]
Swimming (Group-3)	16.93±0.77[b]	267.36±4.19[b]
Extract treated swimming (Group-4)	12.83±0.92[a]	228.57±4.04[a]
Vitamin C treated swimming (Group-V)	11.94±0.85[a]	220.87±3.97[a]

Data expressed as Mean±SE (n=8). ANOVA followed by multiple two-tail t-test. In vertical column mean values with different superscripts (a,b) differ from each other significantly (P<0.05).

Table 4

Effect of composite methanolic extract of root of W. somnifera, leaf of O. sanctum and rhizome of Z. officinale on GOT and GPT levels in serum in swimming-induced oxidative stress.

Group	SGOT (Unit/ml of serum)	SGPT (Unit/ml of serum)
Control (Grooup-1)	15.20±0.50[a]	16.10±0.60[a]
Extract treated control (Group-2)	14.90±0.45[a]	15.90±0.55[a]
Swimming (Group-3)	16.03±0.47[a]	16.13±0.58[a]
Extract treated swimming (Group-4)	15.03±0.62[a]	15.93±0.54[a]
Vitamin C treated swimming (Group-V)	14.84±0.55[a]	15.64±0.52[a]

Data expressed as Mean±SE (n=8). ANOVA followed by multiple two-tail t-test. Values in same column with same superscript (a) did not differ from each other significantly (P<0.05).

DISCUSSION

This is well known that there is a benefit of exercise in promoting good health and preventing various diseases. Exercise causes decrease in levels of antioxidants and antioxidant enzymes in the muscle [29] and blood [30]. Increases in oxidative damage biomarkers [31] as well as effects on mitochondrial function [32] have been reported by several investigators. In nontrained animals the above reflections have been found more prominent and attain much quickly when the animal perform exercise. Body weight is one of the important indicators of general toxicity assessment [33]. In this experiment strenuous exercise resulted a significant diminution in final body weights in forced swimming animals. Exercise-induced oxidative stress has been completely overcome by co-administration of the said extract to the group IV animals. This suggest that the composite herbal extract as tonic has some remedial measures on oxidative threat that may protect the body weight by preventing general tissue degeneration in associating with oxidative stress threat. Decreased antioxidative enzyme concentrations of erythrocytes have been noted after acute submaximal exercise. The present study revealed that there is significant reduction in the levels of antioxidative enzymes like CAT, SOD, Px and GST in extract untreated forced swimming animals. Such results may probably due to an increase in the usage and destruction of them in intracellular defense system. Strenuous swimming-induced diminution in the activities of the aforesaid important biomarker for scavenging free radicals in nervous tissues as well as muscles have been reported in previous publication [34] and such findings have been strengthen by several workers [35]. Consequence of low antioxidant enzyme activity, an increased susceptibility to lipid peroxidation is facilitated [36]. Reduction of hydrogen peroxide and hydro peroxide to nontoxic products are catalyzed by GST and Px. Strenuous physical exercise decreases plasma level of testosterone [37]. This may also be another possibility for the low levels of important antioxidative biomarkers assessed in this experiment.

Elevations in the levels of products of free radicals like TBARS and CD in packed RBC in forced swimming group of animals also support the low antioxidant enzymes activity that elevate lipid peroxidation while TBARS and CD are the products of lipid peroxidation. Ischaemia-reperfusion injury may be another causative response for such elevation in TBARS and CD in forced swimming animals [38]. It is well established fact that in stressful exercise catecholamin is secreted in excess which augments free radicals production in the

vital organs allied to exercise stress either through autooxidation or through metal ion or superoxide catalyzed oxidation [39]. From this study, co-treatment of composite extract to the forced swimming rats protected effectively in the management of forced swimming-induced oxidative damages in packed RCS's without leading to stress related anaemia. During the period of strenuous exercise, when the body suffers from its in-house antioxidant defense machinery, an exogenous co-administration has been confirmed by several workers [40]. The above mentioned plant parts contain a good number of antioxidative phytochemical ingredients like flavonoids [41], flavones, oxymethyl phenols or glycowithanolides [42], etc. can collectively combat on the oxidative damage as well as these compounds are well established potent antioxidant in our body system. Supplementation of vitamin C to the forced swimming animals resulted resettlement of antioxidant enzymes and lipid peroxidation to the control level. Vitamin C suppresses the propagation of lipid peroxidation, inhibits hydroperoxide formation. The aforesaid extract possesses no general toxic effect proved by SGOT and SGPT activities, as these enzymes are themselves important markers for toxicity assessment [43].

CONCLUSION

From this study it may be stated that the antioxidative property of composite extract functions through an indirect protective mechanism in the body of forced swimming-induced oxidative stressed rat. To explain the above mentioned report, further research is required but it may be due to some adaptive changes in the haemopoitic system during the preconditioning followed by forced swimming period.

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