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Solar Dried Natural Ingredients based Antioxidant Rich Nutritional Supplements for Service Personnel stationed at High Altitudes II – Efficacy of the antioxidant rich nutritive supplements in moderating the Oxidative cum Physical stress in experimental animals

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Abstract: Service personnel deployed in an alien hostile environment of high altitude regions are distraught both psychologically and physically due to hypobaric hypoxia, physical exercise and the allied intensified metabolic rate causing oxidative / reductive stress due to the activation of reactive oxygen and nitrogen species [RONS] generating systems, causing oxidative damage to lipids, proteins, and DNA. With a view to mitigate the effects of RONS through nutritional means, two types of ready-to-eat chocolate coated antioxidant rich nutrient [CC-ARN] bars have been successfully developed lately using cereals, pulses, solar dried fruits and vegetables etc., which are known to be rich in vitamin C, β - carotene, vitamin E, iron and selenium. Consumption of a bar of 30g daily, as a snack, by a soldier in addition to the normal recommended ration has been assessed / projected to provide the constituent antioxidant nutrients like vitamin C, vitamin E, selenium, zinc, iron, carotenoids besides potent polyphenols and flavonoids in quantities not exceeding fifty percent of the RDA levels. In order to evaluate the efficacy of these CC-ARN bars in moderating the oxidative cum physical stress

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in a biological system, the present study has been carried out using adult male albino rats subjected to physical stress induced by swimming exercise. Five groups of animals of ten each were fed diets prepared using the authorized ration scale for service personnel stationed at high altitudes, either with or without additional antioxidants derived from CC-ARN bars. Since the high-altitude situations could not be simulated due to non-availability of the necessary infrastructure and certain inherent limitations, the trials were carried out at normal conditions of temperature and atmospheric pressure. During the three weeks of experimental period the blood lactic acid and serum lactic dehydrogenase were evaluated periodically before and after exercise. At the end of the experiment the animals were humanely sacrificed and various tissues were harvested for evaluation of the changes associated with antioxidant supplementation vis-à-vis tissue levels of antioxidant components like glutathione, malonal di aldehyde, superoxide dismutase and catalase. The results evidently indicated that antioxidants supplemented through CC-ARN bars considerably influenced alleviation of the oxidative stress induced due to swimming exercise.

Keywords: Green Energy, Fruits & Vegetables, Antioxidant Capacity, Blood Lactic Acid, Lactic Dehydrogenase, Superoxide Dismutase, Catalase, Malonaldehyde, Swimming Exercise.

1. Introduction

Service personnel stationed in high altitude (HA) regions are predisposed to hypobaric hypoxia (HH), a condition wherein the bioavailability of oxygen is reduced due to the decreased partial pressure of oxygen, causing alterations in the oxygen transport system from the air to the mitochondrial membrane resulting in the stimulation of compensatory physiological mechanisms encompassing natural enzymatic and non-enzymatic antioxidant defenses to help the individuals tolerate and acclimatize to the situation (Radak *et al.*, 2014). Depending on the degree of altitude, the associated lower partial pressure of oxygen and the level of physical activity the HH exacerbates the normal metabolic rate causing oxidative / reductive stress, triggering a wide range of reactive oxygen and nitrogen species [RONS] generating systems including the mitochondrial electron transport chain, xanthine oxidase, and nitric oxide synthase leading to oxidative damage of lipids, proteins, and DNA (Sauberlich 1946; Machlin and Bendich, 1987; Alessio, 1993; Aruoma, 1994; Yu, 1994; X-Ji

et al., 1995; Tiidus and Houston, 1995; Maxwell, 1995; Sen, 1995; Dekkers *et al.,* 1996; Packer, 1997; Ji *et al.,* 1998; Ashton *et al.,* 1998; Kanter, 1998). These RONS / free radicals have been well documented to cause a large number of diseases like ulcerative colitis (Ramakrishna *et al.,* 1997), Parkinson's disease (Bolton *et al.,* 2000), Alzheimer's disease (Smith *et al.,* 2000), cancer (Kinnula and Crapo, 2004), cardiovascular diseases (Singh and Jialal, 2006; Penaloza and Arias-Stella, 2007; León-Velarde *et al.,* 2010), mild cognitive impairment (Guidi *et al.,* 2006), neural disorders (Sas *et al.,* 2007) etc. Several studies have shown that exposure of animals to different levels of simulated HA conditions caused oxidative stress resulting in an enfeebled capacity of enzymatic and non-enzymatic antioxidant systems interrelated with the changes in the enzymatic pattern of various tissues as exemplified by increased levels of immune reactive mitochondrial superoxide dismutase in serum, decline in glutathione peroxidase (GPX) activity in the liver (Sauberlich, 1946; Yu, 1994; Ji *et al.,* 1998; Ashton *et al.,* 1998; Kanter, 1998), decreased levels of reduced glutathione (GSH) and increased oxidized glutathione concentration (Gleeson *et al.,* 1987; Robertson *et al.,* 1990). Exposure of rats intermittently (12h /day) to simulated altitude of 4000m, showed increased lipid peroxidation in the skeletal muscle (Simon-Schnass, 1996) with a concomitant increased oxidative damage of proteins (Fischer-Wellman and Bloomer, 2009), while short term exposure (5 days) to an altitude of 7576m caused increased lipid peroxidation in plasma of rats (Patil *et al.,* 2012).

Healthy human subjects exposed to HA (2700m) and cold thereof revealed a significant increase in the level of urinary lipid peroxidation products and DNA damage (Dekkers *et al.,* 1996). Increased levels of H_2O_2 and lipid peroxidation products have been reported in exhaled breath condensate of mountain bikers performing a maximal cyclo - ergo metric exercise at 670m / 2160m and in soldiers climbing to 6125m in the Andes Mountains of Northern Chile (Pfeiffer *et al.,* 1999). Acclimatization studies with humans exposed to an altitude of 4500m for a period of 3 and 13M revealed that a 3M exposure caused increased lipid peroxidation and decreased enzymatic and non-enzymatic defence, while a 13M sojourn normalized the redox balance (Machlin and Bendich, 1987). Another study lasting 3-weeks (5,250-7,161m), with six acclimatized Himalayan mountaineers revealed a systemic oxidative stress resulting in deleterious consequences including assessable changes in erythrocyte antioxidant enzyme activity and membrane fatty-acid profile (Sauberlich, 1946; Dillard *et al.,* 1978).

Both human and animal studies corroborate the fact that HH coupled with heavy and sustained exercise causes oxidative stress due to the formation

of large quantities of RONS. Under such circumstances the RONS produced outstrips the biologically synthesized antioxidants like glutathione, catalase and superoxide dismutase to quench the free radicals for the protection of healthy cells from allied damage (Olson, 1996) causing impairment of lipids, proteins and DNA, striking an adverse bearing on the overall health (Aruoma, 1994; Ji, 1995).

Extensive studies accentuate on the effective role of antioxidant supplementation vis-à-vis the body's response to HA (Simon-Schnass, 1996) via the beneficial effects induced either by attenuating and or preventing the causative oxidative damage (Mariott and Carlson, 1996) like preclusion of "worsening" blood flow and reduced physical performance due to the damage of cellular antioxidant defence systems [Sauberlick, 1946; Machlin and Bendich, 1987; Gleeson *et al.,* 1987; Aruoma and Halliwell, 1987; Robertson *et al.,* 1990; Simon-Schnass, 1996; Olson, 1996; Fisher-Wellman and Bloomer, 2009; Patil, 2012). Consumption of an antioxidant mixture, containing vitamin E, β-carotene, ascorbic acid, selenium, α-lipoic acid, N-acetyl-1-cysteine, catechin, lutein, and lycopene has been found to effectively reduce the degree of oxidative damage caused by oxidative stress at HA (Simon-Schnass, 1996). Antioxidant supplementation mitigated the HA induced ventilatory threshold in exercising humans (Askew, 1995). Oral supplementation of vitamin E (40 mg rat^{-1} d^{-1}) for 5 days prior to and during the period of hypoxic exposure to simulated altitude condition (7576 m) significantly reduced the induced upsurge in lipid peroxidation (Gleeson *et a.,l* 1987; Sumida *et al.,* 1989; Robertson *et al.,* 1990). Exposure of male albino rats maintained on normal diet to hypoxia showed a significant increase in hematocrit and hemoglobin with a decrease in RBC deformability index, while the group of animals receiving vitamin E (40 mg $rat^{-1}d^{-1}$ orally) supplement, 5 d prior to and during the period of hypoxic exposure maintained normal levels, as indicated by a significantly lower rise in MDA levels and increased GSH concentration corroborating the potential role of vitamin E in preventing HA related oxidative damage (Ilavazhagan *et al.,* 2001).

On the contrary a few studies do not substantiate the positive role of antioxidant supplements against HA related oxidative stress. A daily dose of a supplemental antioxidant mixture of β-carotene 20,000 IU, vitamin E 400 IU, vitamin C 500 mg, selenium 100 mg, and zinc 30 mg, did not appear to counteract the oxidative damage to macromolecules (Wei-Hsun *et al.,* 1999). Yet, another study with humans exposed to a cold environment at moderate altitude coupled with high levels of physical exertion of training and receiving

an antioxidant mixture containing carotene, ascorbic acid, tocopherols selenium, catechin (green tea), lutein (*Tagetes erecta*), lycopene (tomato), N-acetyl-1-cysteine, pomegranate extract (5mg) plus a blended vegetable concentrate did not lessen the mean oxidative stress levels in the entire group of test subjects (Schmidt *et al.,* 2002).

Barring these few reports, the majority of the studies have emphasized the positive effect of supplementation of the regular diets at high altitude regions with vitamins A, C and E in rendering protection against the concerns arising due to oxidative stress. Consumption of a wide variety of plant based foods including fruits and vegetables help in maintaining optimal antioxidant status due to their contained antioxidant vitamins such as vitamin C, β-carotene and other potent polyphenolic compounds. Herein two types of chocolate coated antioxidant rich nutritive (CC-ARN) bars have been efficaciously developed lately by blending various food items such as cereals, pulses, solar dried fruits and vegetables etc., for use by the service personnel stationed at HA, to moderate the oxidative stress. The products in the ready-to-eat compact form, packaged in metalized polyester (12μ) LD/ LLD (MP, 75 μ) pouches have been extensively tested for their microbiological quality, sensory attributes, antioxidant potential and storage stability (Sagar *et al., 2023*). The organoleptic studies showed that the products are shelf stable for 16 weeks at ambient conditions.

In order to establish the efficacy of these products in reducing the oxidative cum physical stress in a biological system, the present study was conducted with adult albino rats as experimental models. Since hypobaric conditions encompassing both temperature and pressure could not be simulated due to the non-availability of the necessary infrastructure either at SEED or elsewhere in the vicinity of Hyderabad, the study has been confined only to the induced physical stress at normal temperature and pressure.

Experimental

Animal Experimentation Facility

Since SEED did not have the facility to carry out the animal experiments, other competent commercial sources were explored for the purpose, keeping in view the economic feasibility, expertise of the personnel involved and availability of the required infrastructure. M/s Sanzyme Research Centre, Hyderabad, Telangana State, India, was identified to possess the facility and expertise of the desired quality of R&D to carry out the entailed experiments under our constant

monitoring and guidance. The work was entrusted after suitably entering into a memorandum of understanding. The associated scientists / technicians were distinctly explained the protocol of our study and stern instructions to strictly comply with the scheduled plan of work.

2. Materials and Methods

2.1. Chocolate coated antioxidant rich nutrient [CC-ARN] Bars

The ragi (*Elucine coracana*) and moong (*Phaseolus aureus Roxb*) based CC-ARN bars (A bar of 30g person^{1} day^{1}) formulated and developed exclusively at SEED, as antioxidants supplement, to soldiers stationed at HA regions for use as a snack food is proposed / intended to satisfactorily combat the oxidative cum physical stress therein. The calculated amount of both ragi and moong based CC-ARN bars required for the entire period of experimentation to feed the animals and for chemical analysis was prepared in a single batch using freshly processed ingredients, as described earlier (Sagar *et al.,* 2023). The thoroughly mixed processed ingredients for each of the types were used without moulding into bars, in order to uphold homogeneity of the diets prepared for feeding the animals.

2.2. Method of preparation of Diets employed for the study

The authorized ration scale (Babusha *et al.,* 2008) used lately by Defence Institute of Physiology and Allied Sciences (DIPAS), Delhi & Defence Food Research Laboratory (DFRL), Mysuru, in their experiments with soldiers stationed at HA region (9000 to 15000 ft) was employed for the preparation of various diets. Several common items arising due to alternatives contained in the ration scale have been pooled and both control and experimental diets required for the entire experimental period were individually prepared as described below. The proportionately up - scaled quantities of various ration items used for the purpose are given in table 1.

2.2.1. Control diet A

The ingredients used were as per column 3 of table – 1 and the method of preparation was as described earlier (Saraswathy *et al.,* 1974). In brief the method of preparation is as follows. Atta, skimmed and whole milk powder, salt and about 80% of oil hydrogenated was kneaded into a dough of required consistency using potable water, covered with a layer of muslin cloth and set aside for about 15 to 20 min. Nicely washed red gram dhal, bengal gram

Table 1: Ingredients of the authorized ration scale for army personnel stationed at high altitudes herein used for the preparation of control* and experimental diets

Sl. No.	*Items*	*Diet A** Qty. [g]*	***Diet B Qty. [g]***	***Diet C Qty. [g]***
1	Atta [whole wheat flour]	5130	2280	2280
2	Dal (split legume) [Red gram]	765	340	340
3	Dal / Besan [bengal gram dal]	270	120	120
4	Oil hydrogenated [80+14]	846	376	376
6	Sugar [140 + 9]	1341	596	596
7	Tea	126	56	56
8	Condiments : [powdered mix of chilli 5 g + dhania 5 g + turmeric 2 g + cloves 2 g + cinnamon 2 g]	45+45+ 18+18+18	20+20+ 8+8+8	20+20+ 8+8+8
9	Salt evaporated	180	84	84
10	Skimmed milk powder	261	116	116
11.	Whole milk powder	711	316	316
12.	Onion fresh	540	240	240
13.	Potato fresh	1260	560	560
14.	Dehydrated carrot in lieu of fresh	180	80	80
15.	Fruits dried	135	60	60
16.	Cashew nut	72	32	32
17.	Pickles	105	60	60
18.	Vitamin C tablet 100mg 1 tablet	9	4	4
19.	Processed ingredients of CC-ARN bar – ragi based	NIL	equivalent to 4 Bars@	NIL
20.	Processed ingredients of CC- ARN bar – moong based	NIL	NIL	equivalent to 4 Bars@

*The quantities of ingredients expressed in column 3 represent the authorized scale upgraded four folds for preparation of the diets required for the entire period of experimentation.Note: Since SEED regularly processes dehydrated carrot, the same has been used in lieu of fresh (Item 14 above).

@ As per our project proposal, One ARN bar per day is to be provided to a soldier stationed at high altitude to combat the oxidative / physical stress. Hence quantity equivalent to four bars has been included in the diet being prepared which corresponds to the ration scale of a soldier for four days.

*The Army Ration - Control diet A provides: total protein: 143 g OR 572 Cals. [12.3 % of total energy]; total fat: 144 g OR 1296 Cals. (27.7 % of total energy); total carbohydrates: 699 g OR 2796 Cals. (60.0 % of total energy); total energy value: 4664 kcals / man / day (Babusha *et al* 2008).

dhal and peeled potatoes together with dehydrated carrot, condiments mix and salt were pressure cooked using minimum quantity of water, cooled and macerated. Dressed and chopped onion was fried in the remaining 20% of oil hydrogenated to a golden brown colour. Set aside for cooling.

Chapattis of suitable size were baked with the kneaded dough and spread on large trays to cool. The cooled product was then manually made into small bits and mixed well in a suitable stainless steel vessel along with macerated dhals, fried onion, powdered sugar, pulverized dry fruits, cashew nuts, pickles and finely powdered vitamin C tablets. The tea leaves were boiled separately in a minimum quantity of water, cooled and strained completely into this mixture. The entire concoction was thoroughly mixed and passed through a meat mincer twice to obtain a homogeneous blend of the diet. This diet in quantities equivalent to feed ten animals per day (about~ 500g) was then packaged, labeled and sealed in polypropylene pouches. The packages were stored in a deep freezer (at -18^{o} C) at the storage facility of M/s Sanzyme Research Centre, Hyderabad, TS, India.

2.2.2. Test diets B and C

Diets B & C were also prepared as described above but with the following additions. Calculated quantities of uniformly mixed processed ingredients of CC- ANR bars – ragi based for diet B and moong based for diet C - were added in the final stage of manual mixing and then homogenized and stored as above. One pouch each of all the three diets A, B & C was stored in a refrigerator at SEED for the analysis of nutrients and antioxidants.

2.2.3. Analysis of the Diets

All the three diets A, B & C were analyzed for their proximal score and various antioxidant components, as described earlier (Sagar *et al., 2023*).

2.3. *Animals and Grouping*

Albino rats of Wistar strain (Species *Rattus novergicus*) have not only been established to be the most suitable rodent species for the present type of studies but also are recommended and accepted by various Regulatory Guidelines & Agencies, for the present type of experiments since the data generated with these animals can easily be extrapolated to humans. Hence these animals were preferred herein for use after obtaining the clearance from the Committee for the Purpose of Control and Supervision of Experiments on Animals [CPCSEA], Ministry of Environment and Forest, Government of India, and subsequent

approval from the Institutional Animal Ethical Committee of the M/s. Sanzyme Research Centre, Animal housing, bedding, environmental conditions etc., were strictly in accordance with the standards laid down by the competent authorities, as is routinely followed by M/s. Sanzyme Research Centre. All tests and procedures described in this study were performed according to the Organization for Economic Co-Operation and Development (OECD, 1998), principles of the Good Laboratory Practice (GLP) as specified by National GLP Compliance Monitoring Authority, Department of Science and Technology, India and International OECD (OECD, 1997).

Fifty healthy, young adult (8 to 10 weeks old ± 5days) male albino rats (Wistar Strain, species *Rattus novergicus*), of body weight 190 ± 10 g were randomly divided into five groups of ten each. Care was taken to maintain uniformity with zero variance statistically, in their average body weights between the groups. The animals were housed individually in well ventilated cages under controlled conditions of light / dark and humidity. In order to acclimatize the animals to the test conditions, all the groups were maintained on control diet A for seven days. On the eighth day blood samples were drawn from all the animals via retro orbital plexuses for generating the basal data on blood indices such as lactic acid, lactic dehydrogenase (LDH), glycogen and glucose levels. Lactic acid levels were evaluated by the method of Barker & Summerson (Barker & Summerson, 1941), glycogen by the method of Roe & Dailey (Roe & Dailey, 1966) and glucose by the method of Barham & Trinder (Barham & Trinder, 1972) using the Kit Spin react SA (Marketed by Euro Diagnostic Systems Pvt. Ltd., Chennai). LDH was determined by the method as described by Burtis & Ashwood (Burtis & Ashwood, 1999).

The various groups were then assigned different test diets as given below for a period of 21 days. Diets and drinking water were provided *ad libittum* with a periodical recording of food consumption.

Group 1	***Group 2***	***Group 3***	***Group 4***	***Group 5***
Fed Control Diet (A) throughout. ***Sedentary***. Sacrificed on 23rd day	Fed Control Diet (A) Made to ***Swim for*** 15 to 20' daily until 20thday. On 21st day the animals were made to Swim until **EXHAUSTION.** Rest on 22nd day. Sacrificed on 23rd day	Fed Test Diet (B) Made to ***Swim for*** 15 to 20' daily until 20th day. On 21st day the animals were made to Swim until **EXHAUSTION.** Rest on 22nd day. Sacrificed on 23rd day	Fed Test Diet © Made to ***Swim for*** 15 to 20' daily until 20th day. On 21st day the animals were made to Swim until **EXHAUSTION.** Rest on 22nd day. Sacrificed on 23rd day	Fed Test Diet (B / C) on alternate days ***Sedentary***. Sacrificed on 23rd day.

Experimental Protocol

2.4. Swimming Exercise

Animals of groups 2, 3 & 4 were made to swim daily (Fig.1) for 20 min. in a swimming tank designed for the purpose. On 7^{th}, 14^{th} & 19^{th} day blood samples were drawn immediately after swimming exercise as above for assessing the changes in lactic acid, LDH, glycogen and glucose levels consequent to exercise for comparison with the basal levels, vis-à-vis effect of diet consumed by various groups. Samples were analyzed without any delay, for the said parameters, as indicated above. On the 21st day all the animals, except those of sedentary groups 1 & 5, were made to swim until exhaustion**.** The time of exhaustion was recorded in all the cases and the blood samples were immediately drawn and analyzed for the various traits as described above.

Figure 1: Animals being made to swim to induce stress

2.5. Energy Expenditure / Whole Animal Metabolic Rate

Energy expenditure was measured on the 18th day**,** by indirect Calorimetry using Comprehensive Laboratory Animal Monitoring System [CLAMS (Columbus Instruments)]. Animals were accustomed to the chambers for a day before collecting the data. While the animals were on *ad libittum* access to food and water, VO_2 consumption & VCO_2 exhalation was measured for a period of 24 hrs in order to calculate the Respiratory Quotient [RQ].

2.6. Collection of Urine Samples

On the 19th and 20^{th} day, the animals were housed individually in metabolic cages and 24 hours urine samples were collected over a drop of toluene for evaluating total volume, specific gravity & pH.

2.7. Sacrificing of animals

All the groups of animals were allowed to rest on 22nd day and on the 23rd day after an overnight fast (with free access to drinking water), the individual body weights were recorded and they were humanely sacrificed using CO_2. The animals were subjected to necropsy and detailed gross pathology evaluation. The body was incised and a maximum quantity of blood was drawn with a suitable syringe into appropriately numbered centrifuge tubes for obtaining serum. Organs such as brain, liver, heart, kidney etc., were quickly excised, trimmed off the connective tissue, weighed and preserved in 10 % neutral buffered formalin for subsequent analysis. However the excised eyes and testes were preserved using Modified Davidson fluid.

2.8. Serum and Tissue Analysis

2.8.1. Antioxidants

The liver tissue and serum were analyzed for antioxidant components viz., glutathione reductase (GSH) (Ithyaraja, 2011), superoxide dismutase (SOD) (Mc Cord & Fridovich, 1969), catalase, malonal-di-aldehyde (MDA) (Ohkawa *et al*., 1979) and hydro peroxides (Wolff, 1994). However the potential biomarkers viz., brain monoamines (5-HT, NA & DA) & GABA) could not be evaluated due to certain limitations beyond our control.

3. Statistical Analysis

All the data generated and expressed as mean ± standard error (SE) for each experimental group was statistically analyzed by one–way Anova followed by post hoc Tukey test for comparing the various traits between the different groups ($P \leq 0.05$) using Graphphad Prism Version

4. Results and Discussions

Facilities for simulation of high altitude conditions were since not available; the present investigation was carried out at normal temperature and pressure conditions prevailing at Hyderabad, Telangana State, India, during the months of July and September.

The ragi and moong based CC-ARN bars provided respectively - total protein 14.01, 15.15 %; total fat 6.81, 6.86%; total energy 363, 362 Cals % (Sagar *et al., 2023*). Consumption of a bar of 30g is calculated to cater about 100 Cals of energy and many additional antioxidants from natural sources. The ragi

and moong based CC-ARN bars have been found to contain respectively: vitamin C 100.1 *vs.* 127.6 mg/100g; vitamin E 1.59 *vs.* 2.93 mg/100g; β carotene 10.28 *vs.*11.69 mg/100g; total carotenoids 29.91*vs.*33.99 mg /100g; and total polyphenols 269.88 *vs.*194.45 mg/100g. The marginal variations between the two types of bars in their constituent nutrients / antioxidants, is attributable only to the composition of ragi or moong in the product.

The ration scale for the army personnel stationed at HA region is listed in table - 1, under the column titled, diet A. It may be noted herein that the quantities of the ingredients specified represent entitlement for four days (vide footnote of table -1). Some of the important antioxidants were analyzed in triplicate and these values being similar to the average values have been reported. The constituent antioxidants present in the control diet A and experimental diets B & C (expressed as mg constituent antioxidant present in an individual's ration per day) are respectively:

Total polyphenols as gallic acid equivalents 357.35 *vs.* 442.25 *vs*. 456.27;

Total carotenoids: 23.22 *vs*. 31.58 *vs.* 34.02;

Total flavonoids as quercetin equivalents 518.11 *vs.* 587.23 *vs.* 618.35;

Total antioxidant activity as vitamin C equivalents 659.05 *vs.* 759.53 *vs.* 769.85;

Ascorbic acid 212.93 *vs.* 240.58 *vs.* 249.23;

Vitamin E 3.11 *vs.* 3.59 *vs.* 3.79.

It can be seen that the consumption of one antioxidant rich bar daily increases the intake of each of the antioxidant components significantly and this surge is proposed to be enough to combat the induced oxidative stress.

4.1. Body weight and food consumption

The mean body weights of animals of various groups at different periods of experimentation together with their corresponding food intake data are given in table - 2. The initial body weights of animals of various groups are nearly identical and are not different significantly. Similarly the final body weights of animals on the 19th day of experimentation too did not show any significant difference with the values ranging between 202.1 ± 3.6g and 198.4 ± 4.8g. The weight gain and food consumption pattern also did not show any significant difference between the various groups fed either the control diets or test diets supplemented with CC-ARN bars or either sedentary or exercised. The average total weight gain by the animals of different groups during the entire experimental period ranged between 11.9 and 16.2 g. Since the animals used

to be adults, the observed lesser weight gain in a span of 19 days does not bear much significance. The sedentary group 1 showed the maximum weight gain of 16.2 g. The difference is apparent with respect to group - 2 and group - 5 it does not differ significantly from other groups [P≤0.05]. The slightly lower body weights of groups - 2, 3 and 4 on the day of sacrifice could be attributed to the induced exercise until exhaustion on the previous day coupled with overnight fasting. Likewise the food intake values of animals of various groups at different periods of experimentation (table–2) are nearly identical throughout the period of experimentation, irrespective of the diet fed. Although all the groups showed similar pattern of food consumption regardless of whether they were sedentary or stressed, the intake of antioxidant nutrients remain higher in case of groups - 3, 4 and 5, due to the inclusion of CC- ARN bars.

Table 2: Body weights of animals and their corresponding food intake values* of various groups at different periods of experimentation [Values are Mean ± SE]

Group / Diet	*Day – 1*	*Day – 5*	*Day – 9*	*Day – 15*	*Day – 19*	*Necropsy Day*
Group 1 Diet A control Sedentary	185.9 ± 4.0 (17.4 ± 5.9)	194.9 ± 3.4 (18.6 ± 5.5)	198.6 ± 3.7 (19.4 ± 5.1)	200.5 ± 3.4 (20.0 ± 4.3)	202.1 ± 3.6 (20.3 ± 3.7)	200.6 ±3.2 (17.4±5.9)
Group 2 Diet A + exercise	186.5 ± 3.4 (19.8 ± 1.4)	194.2 ± 4.6 (21.3 ± 0.9)	195.4 ± 5.5 (21.6 ± 0.7)	197.0 ± 5.3 (21.8 ± 0.7)	198.4 ± 4.8 (21.9 ± 0.7)	196.6 ±4.7 (19.8±1.4)
Group 3 Diet B + exercise	184.8 ± 3.6 (20.6 ± 1.0)	194.2 ± 5.8 (21.6 ± 0.9)	197.0 ± 4.9 (22.0 ± 0.8)	199.0 ± 5.0 (22.40± 0.6)	200.2 ± 5.0 (22.6 ± 0.6)	198.2±4.5 (22.6 ± 0.5)
Group 4 Diet C+ exercise	186.0 ± 3.6 (19.8 ± 1.5)	194.8 ± 4.5 (21.0 ± 1.1)	197.2 ± 4.1 (21.2 ± 0.9)	200.0 ± 4.2 (21.76± 1.0)	201.1 ± 4.1 (21.9 ±1.1)	198.9±3.7 (19.8±1.5)
Group 5 Diets B&C Sedentary	186.7 ± 4.1 (19.5 ± 1.6)	190.5 ± 6.5 (20.3 ± 1.1)	194.0 ± 6.4 (20.7 ± 1.0)	197.4 ± 5.8 (21.1± 0.9)	198.9 ± 6.0 (21.2 ± 0.9)	197.3±5.9 (19.5±1.6)

*Values given in parenthesis represent the particular days' mean food consumed/rat/day

4.2. Swimming exercise

4.2.1. Blood lactic acid and serum lactic dehydrogenase

Blood lactic acid and serum lactic dehydrogenase data are given in table - 3. The zero day values represent the data prior to exercise while the 7th and 19th day values are for post exercise. No significant difference was found between the basal values of all the five groups. The blood lactic acid concentrations of both the sedentary groups -1 and 5 are similar throughout the experimental period despite group - 5 receiving supplemental antioxidants through CCARN bars. The experimental group - 2 which received the control diet with no added

antioxidants but regularly exercised showed a significant rise in the blood lactate levels compared to the basal values. Consequent to exercise the lactic acid levels of groups - 3 and 4 receiving diets B & C supplemented with CC-ARN bars, interestingly remained almost similar and significantly lower than the values of group - 2 indicating negligible effect of induced stress. Evidently this is attributed to the effect of supplemental antioxidants. Ostensibly these values did not show any difference in comparison with group - 5. Lactate a normal end product formed only from pyruvate mediated by LDH generally gets higher in conditions of strenuous exercises such as sprinting / swimming, due to utilization of more oxygen by the body than the lungs / heart can supply. Oxidation of pyruvate and gluconeogenesis require the presence of oxygen and in conditions of reduced oxygen levels the glucose is mainly converted to lactate. The significant rise in lactate levels consequent to exercise in case of animals of group - 2 can be attributed to the stress experienced and the effort applied therein by the body to inhale enough oxygen during swimming, thus triggering the conversion of glucose to lactate. However it is surprising to note that although these animals were regularly exercised, the rise in lactate levels due to exercise has not shown any reversal even at the end of 19 days of experimentation, which is normally expected on acclimatization. On the other hand the higher intakes of antioxidants in case of animals of groups - 3 and 4, the resultant higher cellular oxygen levels might have been favourable in stimulating pyruvate to acetyl CoA pathway moving towards Krebs cycle in energy production. Hence our results strongly indicate that the additional intake of antioxidants, in the form of CC-ARN bars, has mitigated the induced oxidative stress due to exercise.

Lactate and LDH being inter-related, the LDH levels too showed a similar pattern with no difference either in the basal values or between the two sedentary control groups - 1 and 5. LDH values are significantly higher in animals of group - 2 compared to all other groups. When the lactate concentration is high LDH exhibits feedback inhibition to decrease the rate of conversion of pyruvate to lactate. In the present study the LDH levels are significantly higher in group - 2 animals compared to all other groups in a similar pattern as lactate. Lactate levels should have exhibited a lower trend since the LDH levels are higher, but this correlation heretofore remains inscrutable warranting a need for auxiliary in-depth investigations.

4.3. Respiratory Quotient [RQ] and Heat Production (HP)

The RQ (VCO_2/VO_2) and the HP (Kcal/ b.w / hr.) in the animals of groups - 1, 2 and 5 are nearly identical - (RQ 0.827 *vs.* 0.848 *vs.* 0.871) *vs.* (HP 6.72 *vs.* 6.69

Table 3: Changes in blood lactate and serum lactic dehydrogenase [LDH] levels of various groups of animals fed diets with and without supplemental CC-ARN Bars vis-à-vis exercise.

[Values are Mean ± SE]*

Blood lactate values in mmol/L			
Group / Diet	Zero Day	7th Day	19th Day
Group 1 - Diet A control Sedentary	3.77 ± 1.75	3.32 ± 1.65	3.78 ± 1.24
Group 2 - Diet A + exercise	3.67 ± 1.33	6.73 ± 1.16[a]	8.62 ± 0.86 [a]
Group 3 - Diet B + exercise	3.40 ± 1.31	3.92 ± 0.69 [b]	3.65 ± 1.05[bc]
Group 4 - Diet C+ exercise	3.47 ± 0.49	3.90 ± 0.52 [b]	3.95 ± 0.66[bc]
Group 5 - Diets B & C Sedentary	3.78 ± 0.51	3.25 ± 0.64 [b]	3.47 ± 0.96[bc]
Serum Lactic dehydrogenase [LDH] values in U/L			
Group 1- Diet A control Sedentary	219.67 ± 5.47	221.17 ± 3.13	221.17 ± 07.06
Group 2 - Diet A + exercise	223.17 ± 7.57	240.50 ± 0.02[a]	258.50 ± 05.89 [a]
Group 3- Diet B + exercise	223.67 ± 5.43	215.00 ± 8.39	211.67 ± 10.71
Group 4 - Diet C+ exercise	222.50 ± 7.29	221.33 ± 8.41	214.83 ± 11.66
Group 5 -Diets B & C Sedentary	224.83 ± 8.01	219.33 ± 7.66	220.67 ± 09.52

Values carrying the same superscript in a row are significantly different from the basal value. Values carrying a different superscript in a column are significantly different from the corresponding value for group 2

vs. 6.69) and amazingly these values are slightly higher in case of regularly exercised animals of groups - 3 and 4 [RQ (0.923 *vs.*0.952) and (HP 7.35 *vs.* 7.42)] receiving diets supplemented with antioxidants.

4.4. Glycogen Levels in various muscle fractions

The glycogen concentration has been expressed as Mean ± SE, mg/100g tissue:

Liver: Glycogen concentration in samples of sedentary animals of group -1 and group - 5 are similar but are significantly (P≤ 0.05) higher than all the other groups; **group - 1; 4.55 ± 0.48** *vs* group - 2; 2.63 ± 0.38: group - 3; 3.68 ± 0.72: group - 4; 3.65 ± 0.56: **group - 5; 4.50 ± 0.51.**

Soleus muscle: Likewise the glycogen values are similar with groups -1 and group - 5 with other groups remaining significantly (P≤ 0.05) lower; **group - 1; 0.472 ± 0.121** *vs* group - 2; 0.261 ± 0.082: group - 3; 0.324 ± 0.063: group - 4; 0.333 ± 0.075: **group - 5; 0.453 ± 0.099**.

Gastrocnemius muscle: The glycogen values exhibited similarity between groups 1,3, 4 and 5 while the group 2 values were found to be significantly (P≤ 0.05) lower: group - 1; 0.472 ± 0.094) *vs* **group - 2; 0.393 ± 0.072**: group - 3; 0.432 ± 0.062: group - 4; 0.445 ± 0.086: group - 5; 0.460 ± 0.075.

As seen the effect observed in case of sedentary experimental controls of group - 5 does not differ appreciably from group - 1. The animals of groups

- 2, 3 and 4 subjected to exercise showed significantly lower concentrations of glycogen in all the muscle fractions. Due to the fact that the liver and gastrocnemius muscle play an important role during exercise, group - 2 which did not receive any antioxidant supplement showed a significant depletion of glycogen stores compared to groups - 3 and 4, the latter two being similar it further corroborates that the supplementation of the diet with antioxidants does exert mitigating effect on induced oxidative stress.

4.5. Urine volume, specific gravity and p^H

The mean total volume (ml/day) of urine excreted by the animals of all the groups is similar; group - 1; 9.75 ± 2.13: group - 2; 9.75 ± 1.53: group - 3; 9.30 ± 1.40: group - 4; 8.95 ± 0.69: group - 5; 9.35 ± 1.49. The volume of urine excreted by group - 4 appears to be slightly lower which might be due to lower water consumption. The specific gravity of the urine of all the groups was found to be 1.03± 0.01. No change is observed in pH values between various groups (9.8 *vs.* 8.3 *vs.* 8.2 *vs.* 8.5 *vs.* 8.4) except the control group - 1 which showed more alkalinity.

4.6. Gross / Relative Organ weights

The mean gross and relative weights of various organs are given in table - 4. No significant difference between the various groups indicates a normal pattern which is expected because all the animals received similar diets made from natural ingredients with the only difference being either sedentary / exercised vis-à-vis with / without antioxidants supplementation, which is again from natural source.

Table 4: Gross [GW] & Relative [RW] organ weights of various groups of animals fed diets with and without supplemental CC-ARN Bars vis-à-vis exercise [Values are Mean ± SE]

Tissues	*Gross / Relative Wt.*	*Group 1 Diet A Control Sedentary [g]*	*Group 2 Diet A With Exercise [g]*	*Group 3 Diet B With Exercise [g]*	*Group 4 Diet C With Exercise [g]*	*Group 5 Diets B & C Sedentary [g]*
Brain	GW	2.548 ±0.141	2.487 ± 0.141	2.546 ± 0.101	2.583 ± 0.134	2.746 ± 0.162
	RW*	1.262±0.072	1.226±0.067	1.235±0.059	1.283±0.061	1.333±0.082
Adrenals	GW	0.102 ±0.006	0.102 ±0.007	0.101 ± 0.006	0.102 ±0.006	0.102 ±0.008
	RW	0.050 ±0.003	0.050±0.003	0.049±0.003	0.051±0.003	0.049±0.004
Testes	GW	4.430 ±0.326	4.503 ±0.248	4.517 ± 0.180	4.654 ±0.191	4.722 ±0.111
	RW	2.194±0.149	2.220±0.138	2.192±0.122	2.312±0.100	2.293±0.064

Epididymis	GW	1.827 ±0.071	1.852 ±0.062	1.856 ± 0.061	1.850 ±0.038	1.895 ±0.049
	RW	0.906±0.051	0.912±0.032	0.900±0.026	0.919±0.027	0.920±0.030
Heart	GW	1.730 ±0.077	1.733 ±0.069	1.777 ± 0.047	1.779 ±0.033	1.845 ±0.043
	RW	0.857±0.036	0.854±0.040	0.862±0.036	0.884±0.023	0.896±0.020
Liver	GW	13.101± 0.69	13.189 ± 0.61	13.303 ± 0.56	13.432 ± 0.54	13.809 ± 0.35
	RW	6.490±0.324	6.497±0.256	6.450±0.245	6.674±0.266	6.703±0.075
Kidneys	GW	2.593 ±0.099	2.682 ±0.133	2.701 ±0.120	2.730 ±0.162	2.766 ±0.202
	RW	1.285±0.065	1.322±0.066	1.309±0.053	1.356±0.078	1.343±0.102
Spleen	GW	0.853 ±0.079	0.864 ±0.051	0.867 ±0.045	0.836 ±0.043	0.845 ±0.034
	RW	0.423±0.044	0.426±0.031	0.421±0.023	0.416±0.023	0.410±0.013

*RW – Relative weight – Wt. of tissue expressed as wt./100g body wt.

4.7. Serum and Liver Antioxidant Components

4.7.1. Glutathione (GSH)

Glutathione, the most prevalent intracellular thiol, known for its function as an antioxidant, is produced biologically in the liver to quench ROS and peroxides, thus preventing damage to important cellular components. As seen from table-5 the serum GSH levels in animals of groups - 1, 2 and 5 are significantly ($P \leq 0.01$) higher than the values for groups - 3 and 4 receiving CC-ARN bars. The animals of group - 5 showed highest GSH levels which could be due to non-utilization of metabolic GSH because of sedentary state coupled with higher intakes of antioxidants. The animals of groups - 3 and 4 which received supplemental antioxidants and subjected to exercise had similar serum GSH levels. Lower levels of GSH herein indicate mitigation of oxidative stress induced due to exercise by the supplemental antioxidants while sparing the endogenously synthesized GSH.

On the other hand the liver GSH value of animals of group - 1 is significantly ($P \leq 0.05$) lower compared to all the other four groups. GSH in liver of animals of group - 2 is higher than the sedentary controls of group - 1 indicating increased synthesis due to stress. The sedentary experimental controls of group - 5 showed a much greater GSH levels than the rest of the groups, which can be attributed to the higher intakes of antioxidants and their lesser utilization because of lack in physical activity. However animals of group - 2 had similar GSH levels as is with groups - 3 and 4. Glutathione, a major antioxidant in the cells exists both in the reduced (GSH) as well as in the oxidized state (GSSG). In healthy cells under normal conditions, more than 90% of the glutathione

pool comprises reduced form (GSH) and remaining in the disulphide form. The ratio of GSH: GSSG decreases with increased oxidative stress (Asensi *et al.,*1999). In our present experiment the oxidative stress induced by exercise in animals of groups - 3 and 4 fed diets with added CC-ARN bars, might have improved the antioxidant status resulting in retarded conversion of GSH to GSSG and accumulation thereof followed by an increase in the ratio of GSH: GSSG. Increase in this ratio indicates the beneficial effects induced by the CC-ARN bars.

Table 5: Changes in Antioxidant Components in Serum and Liver of various groups of animals fed diets with and without supplemental CC-ARN Bars vis-à-vis exercise. [Values are Mean ± SE]*

Parameters	*Tissue*	*Group 1 Diet A Control Sedentary [g]*	*Group 2 Diet A With Exercise [g]*	*Group 3 Diet B With Exercise [g]*	*Group 4 Diet C With Exercise [g]*	*Group 5 Diets B & C Sedentary [g]*
Glutathione nmol / mg Protein	Serum	8.6 ± 0.3 ^{a}	8.2 ± 1.2 ^{b}	5.5 ± 0.5 ^{b}	5.9 ± 0.8 ^{c}	10.0 ± 0.6 ^{a}
	Liver	44.1 ± 3.9 ^{a}	59.9 ± 4.0 ^{b}	60.6 ± 3.5 ^{b}	66.9 ± 2.4 ^{c}	79.0 ± 2.1 ^{c}
Malonadi-aldehyde nmol MDA/ mg Protein	Serum	21.7 ± 3.4 ^{a}	22.8 ± 1.6 ^{a}	22.6 ± 3.9 ^{a}	21.7 ± 2.5 ^{a}	22.5 ± 1.4 ^{a}
	Liver	50.7 ± 1.5 ^{a}	55.2 ± 3.6 ^{a}	65.8 ± 3.2 ^{b}	60.2 ± 2.7 ^{c}	71.7 ± 4.7 ^{b}
Hydro peroxides nmol/ mg Protein/min	Serum	16.0 ± 1.1 ^{a}	14.0 ± 0.7 ^{b}	12.9 ± 0.5 ^{b}	12.9 ± 0.4 ^{b}	14.9 ± 0.7 ^{a}
	Liver	27.0 ± 2.9 ^{a}	34.3 ± 2.5 ^{b}	37.3 ± 2.0 ^{b}	35.6 ± 1.1 ^{b}	38.9 ± 0.8 ^{b}
SOD Activity Units/ mg Protein	Serum	120.9 ± 13.2 ^{a}	152.5 ± 14.7 ^{b}	316.0 ± 32.8 ^{c}	348.4 ± 55.2 ^{a}	372.7 ± 40.4 ^{d}
	Liver	74.1 ± 2.6 ^{a}	61.9 ± 8.8 ^{a}	56.5 ± 8.0 ^{b}	59.3 ± 3.9 ^{a}	83.0 ± 8.4 ^{c}
Catalase umol/ mg Protein/ min	Serum	126.6 ± 8.9 ^{a}	139.8 ± 8.1 ^{a}	110.6 ± 37.8 ^{b}	123.5 ± 21.9 ^{a}	142.9 ± 17.8 ^{a}
	Liver	1.12 ± .2 ^{a}	0.39 ± 0.1 ^{b}	0.29 ± 0.0 ^{b}	0.37 ± 0.0 ^{b}	0.23 ± 0.0 ^{b}

* Values carrying different superscripts in a row are significantly different at $P \leq 0.05$

4.7.2. Malonal-di-aldehyde [MDA] and Hydro peroxides [HP]

Malonal-di-aldehyde, a product of lipid peroxidation, is considered to be a sensitive and reliable biomarker of oxidative tissue damage (Gutteridge, 1995). Oxidative stress is a state of imbalance between ROS and the ability of the antioxidant defenses that are constantly formed in the biological system to readily detoxify the free radicals and other ROS (Alessio, 1993, X Ji *et al,* 1995; Sies *et al*, 2017). Lipid peroxidation happening during oxidative stress, leads to the formation of lipid peroxides which, being highly unstable, readily decompose to form a series of complex compounds, including MDA,

a mutagenic, tumorigenic and highly reactive three carbon di aldehyde. MDA thus produced serves as a reliable biomarker to monitor and predict oxidative stress. Strenuous physical exercise induces oxidative stress (Sen *et al*., 1994), both in animals (Davis *et al*., 1982), and humans (Sen, 1995). A measure of the plasma MDA and serum HP levels are considered indicative of the degree of lipid peroxidation (Lovlin *et al*., 1987).

The MDA and HP levels observed in serum and liver are given in table - 5. As seen the change in pattern of these parameters both in serum and liver show a similar trend between the different groups with no significant difference in the serum MDA levels, irrespective of the treatments espoused. On the other hand, liver MDA values of animals of group - 1 are marginally lower than group - 2 counterparts subjected to exercise but significantly lower than groups - 3, 4 and 5. The levels are the highest with a sedentary group - 5. The values in case of animals of groups - 3 and 4 although appear slightly varying does not differ statistically. The hydro peroxide levels are higher in serum samples of group - 1animals compared to all the other groups. These values for groups - 3 and 4 while being similar are significantly lower than groups - 1, 2 and 5. The liver HP levels of group - 1 are significantly lower than all the other groups. Incidentally the values of group - 5 are significantly higher than groups - 2, 3 and 4. However the observed differences in both MDA and HP values between the serum and liver samples between the different groups subjected to varied treatments do not appear to correlate adequately and need further studies.

4.7.3. Superoxide dismutase [SOD] and Catalase

SOD and catalase form an integral part of the natural antioxidant system that help the body to remove the superoxide radicals by converting them into hydrogen peroxide (H_2O_2) which the catalase present in most of the aerobic cells rapidly catalyzes the conversion of hydrogen peroxide to water and oxygen. Glutathione peroxidases are also involved in the removal of H_2O_2. The SOD and catalase levels observed in the present investigation are also given in tables –5. The serum SOD levels in case of group - 1 the sedentary controls, are significantly lower than the animals of group - 2 exposed to exercise. These values show an opposite trend in liver indicating more storage in liver in case of group - 1 to meet exigencies and more mobilization in group - 2 to meet the stringent need arising due to exercise. Serum levels of SOD in animals of groups - 3 and 4 are highest indicating that the induced oxidative stress needed an effective counteract. However the catalase activity showed comparatively lower levels. Both SOD and catalase enzyme activities in serum samples of group - 5, the sedentary experimental controls remained the highest.

The changes observed in the concentration of various antioxidant enzymes studied heretofore in relation to the induced oxidative stress vis-à-vis supplementation of antioxidants through CC-ARN bars provide substantial evidence that additional antioxidants do have a considerable defensive impact on the induced oxidative stress. The germane infrastructure for carrying out the animal studies strictly under simulated high altitude conditions being not available the present experiment although has been carried out under normal conditions of temperature and pressure. However the results obtained strongly suggest that providing one CC-ARN bar (30g man^{-1} day^{-1}) as a snack to the soldiers stationed in HA regions would considerably influence the beneficial effects on their antioxidant status.

5. Conclusion

Service personnel stationed at HA regions are normally exposed to oxidative stress due to extreme climatic conditions like low temperatures, low atmospheric pressures etc., coupled with strenuous routine activities including exercise. In order to combat the oxidative stress through nutritional route, two types of CC-ARN bars were developed lately using solar dried natural products. The solar drying technology standardized and patented hitherto at Society for Energy, Environment and Development (SEED) Hyderabad - 500033, Telangana State, India (SEED) has been employed. A bar of 30g each when given to a soldier per day will provide him additional antioxidant nutrients / components like vitamin C, E, selenium, zinc, iron, carotenoids, polyphenols and flavonoids in quantities not exceeding fifty percent of the RDA levels when consumed along with the normal recommended ration. Heretofore the animal experiments have been carried out at normal conditions of temperature and pressure since the high altitude situations could not be simulated for the purpose. The results of the present animal study infer that both the types of bars tested are considerably effective in mitigating the oxidative stress resulting due to swimming exercise. The findings would have been more encouraging if the animal experiments had been carried out under simulated conditions of high altitude.

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