BENEFICIAL EFFECT OF PROCESSED SHILAJIT ON SWIMMING EXERCISE INDUCED IMPAIRED ENERGY STATUS OF MICE

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Summary

Oral supplementation of a processed shilajit formulation significantly improved physiological energy status in albino mice in a model of forced swimming test (FST). There was a significant fall of adenosine tri phosphate (ATP) concentration in muscle by 82%, in brain by 33% and in blood by 35% in exercised control animals on the 7th day of a 7-day swimming regime. Post exercise shilajit treatment retrieved loss of the energy currency (ATP) in different tissues/cells in mice. The fall of ATP was attenuated to 65% in muscle, 22% in brain and 14% in blood on the 7th day of similar exercise, when the animals were treated orally with shilajit (30 mg/Kg body weight, *p.o.*) for the last 4 days of the swimming regime. About 18% rise in the inosine mono phosphate (IMP) concentration, a marker for energy depletion in muscle, was observed in the exercised control animals. This rise of IMP was only 5% on oral administration of shilajit. Improved status of some energy related indices, such as Adenylate Energy Charge (AEC) and Total Adenine Nucleotide (TAN), was also observed following shilajit treatment. The energy augmenting effects of shilajit were at par with those of coenzyme Q_{10} (CoQ_{10}), administered orally to the animals as a positive control (15 mg/Kg body weight, *p.o.* x 4 days). A synergistic effect in the improvement of the energy related parameters was observed when the animals were treated with a combination of shilajit (15 mg/Kg body weight, *p.o.* x 4 days) and CoQ_{10} (7.5 mg/Kg body weight, *p.o.* x 4 days). Yet another improvement of shilajit treatment constituted of the CoQ status in muscle and blood of the treated animals. The FST-induced impairment of CoQ status in mice was manifested by a fall of CoQ concentration by 75% in blood and a rise in CoQ by 68% in muscle in exercised control animals on the 7th day of the swimming regime. The fall in CoQ concentration in blood was attenuated to 50% and its rise was arrested in muscle, when the animals were treated orally with shilajit (30 mg/Kg body weight, *p.o.* x 4 days). Effect of shilajit on blood and muscle CoQ status was at par with those of orally administered CoQ_{10} (15 mg/Kg body weight x 4 days).

Keywords: Shilajit, coenzyme Q_{10} (CoQ_{10}), Forced swimming test (FST), Adenylate energy charge (AEC), Total adenine nucleotides (TAN),

Introduction

Shilajit is described in *Ayurveda* as a *maharas* (super-vitalizer), which restores systemic homeostatic balance when impaired under adverse situations [1]. It is a strong antioxidant and is also used as a performance enhancer [2]. A typical good quality shilajit should comprise of oxygenated dibenzo-α-pyrones (DBPs, 0.3-0.5%) and dibenzo-α-pyrone chromoproteins (DCPs, 10-20%) as the major bioactives, and low and medium molecular weight fulvic acids (FAs, 50-60%), as the delivery system (*yogabahi*) [3]. The major bioactives of shilajit, viz. the DBPs and DCPs, are naturally occurring in human and animal bodies as systemic metabolites [3,4]. Hence, their depletion due to stress would need supplementation to restore their systemic balance and function.

Extensive exercise produces reactive oxygen species in muscles, which ultimately causes damage to mitochondria [5]. Since ATP is the main energy currency in the cell, production of ATP in mitochondria is vital for muscle contraction, chemiosmotic homeostasis and normal cellular function [6]. Severe fatigue and energy depletion can occur in experimental animals if the animals are engaged in forced swimming. For this reason, forced swimming test was used as a model for energy depletion by earlier workers in several studies [7].

Oral supplementation of CoQ_{10} was found to produce performance-enhancing activities [8]. CoQ_{10} is an essential component of the mitochondrial respiratory chain, which carries electrons in mitochondrial electron transport to facilitate production of ATP [9-10]. However, CoQ is known to suffer from a number of attendant deficiencies, e.g. pro-oxidant effect and susceptibility to systemic degradation by reactive oxygen species (ROS). Processed shilajit, on the other hand, is devoid of any adverse pharmacological effects and toxicity, and, by virtue of its strong antioxidant activity, can be used as an effective agent against muscle damage.

The purpose of this study was to determine the effect of shilajit, after oral administration, on the systemic synthesis and metabolism of ATP and to assess whether the effect is comparable to that of CoQ_{10}, a well known energy restoring supplement, in a mouse model of forced swimming exercise. Concurrent administration of CoQ_{10} and shilajit may overcome the deficiencies of oral supplementation of CoQ_{10} due to the potent vitalizing and antioxidant effects of shilajit.

Materials and methods

Test Sample: Authenticated Shilajit powder was obtained from Indian herbs Ltd, Saharanpur (U.P.). A specimen has been preserved in our file as reference.

Extraction Procedure: Processed shilajit (viz. PrimaVie) was prepared from raw shilajit powder by an established procedure [3].

Chemicals and Reagents: Fine chemicals (viz. ATP, ADP, AMP, IMP, CoQ_{10}) were of analytical grade (SRL, India) and the solvents used were of HPLC grade (Merck, India).

Animals: Male Swiss albino mice weighing 20-24 g procured from Central Research Institute (Ayurveda), Govt. of India, Salt Lake City, Kolkata, were used. The animals were housed in an animal room with alternating light-dark cycle of 12 hr each. The animals were acclimatized for at least 7 days to the laboratory conditions before conducting experiments. Experiments were carried out between 0900 h and 1700 h. The study was conducted in accordance with Good Laboratory Practice (GLP) Regulations of WHO (WHO Document, 1998).

The "Principles of laboratory animal care" (NIH Publication # 85-23, 1985) were also followed in the study. The 'Institutional Animal Ethics Committee' (IAEC) approved the experimental protocol.

Forced swimming test [11]: The mice were placed individually into glass cylinder (height 25 cm, diameter 20 cm) containing water at 23±2^{o}C. The swim test was performed for 7 days, once each day for 90 minutes and two hours after administration of the drugs. On the 7th day, immediately after the swimming was completed, the animals were sacrificed by cervical dislocation and muscle (soleus and gastroc-nemius of hind legs) and brain from each animal collected. Blood was collected from retro-orbital plexus prior to sacrifice.

Treatment protocol:
Animals were divided into following five groups comprising 6 animals in each group.
Control: Control mice that received 0.8% (w/v) carboxymethyl cellulose (CMC) in water as vehicle, for 7 days. These animals were not engaged in any type of experimental activity.
Swim-exercise (SE): Mice that received 0.8% (w/v) CMC in water as vehicle and subjected to forced swimming exercise for 7 days.
SE+CoQ: Mice subjected to forced swimming exercise exercise for 7 days, administered CoQ_{10} (15 mg/Kg body weight, *p.o.*) suspended in 0.8% CMC, once daily, for the last 4 days of the exercise.
SE+shilajit: Mice subjected to forced swimming exercise exercise for 7 days, administered shilajit (30 mg/Kg body weight, *p.o.*) suspended in 0.8% CMC, once daily, for the last 4 days of the exercise.
SE+shilajit+CoQ: Mice subjected to forced swimming exercise for 7 days, concurrently administered shilajit and CoQ_{10} (respectively, 15 and 7.5 mg/Kg body weight, *p.o.*) suspended in 0.8% CMC, once daily, for the last 4 days of the exercise.

Determination of purine nucleotides and energy related indices: Muscle and brain samples were homogenized with 1.3 M perchloric acid. The homogenates were then centrifuged at 10000 rpm for 5 minutes and the supernatants were neutralized with saturated K_2CO_3 solution. The product was used for the determination of contents of purine nucleotides by HPLC [12-13]. Likewise, whole blood was mixed with 1.3 M perchloric acid (1:1, v/v), centrifuged and neutralized by saturated K_2CO_3 solution for the determination of contents of purine nucleotides by HPLC [14].

Determination of coenzyme Q [15]: Muscle and/or brain homogenate, and blood, was mixed with ethyl alcohol after addition of 8% aqueous solution of SDS (Sodium dodecyl sulfate). To the mixture, after thorough vortexing, *n*-hexane was added and vortexed again. The mixture was then centrifuged at 6000 rpm for 5 minutes when the hexane layer separated out. After collection of the hexane layer, hexane extraction was repeated again, pooled together and the total hexane fraction was evaporated by nitrogen flush. Hexane extractives were analyzed for total coenzyme Q (CoQ_{10} and CoQ_9) content. CoQ_9, the prevalent form of CoQ in rodents, was quantitated using standard curve of CoQ_{10}.

HPLC of purine nucleotides: The samples were injected in a μBondaPak RP C_{18} [300 x 3.9 mm; 10 μm] column from Waters, with a mobile phase of 30 mM aqueous phosphate buffer [pH 6.00] at a flow rate (isocratic) of 0.7 ml/min. The HPLC system was consisted of a Waters 2996 Photodiode array (PDA) detector, Waters 515 pump and Rheodyne 7725i injector. AEC was calculated according to the method of Atkinson and Walton [16] and TAN was calculated according to that of Spencer and Katz [17].

HPLC of coenzyme Q: The hexane extractives were injected in a XTerra RP C_{18} [250 x 4.6 mm, 5 µm] column from Waters, with a mobile phase consisted of Methanol:Acetonitrile:Ethanol 30:30:40 (v/v/v) at a flow rate (isocratic) of 1 ml/min. The HPLC system was consisted of a Waters 2996 Photodiode array (PDA) detector, Waters 515 pump and Rheodyne 7725i injector.

Statistical analysis: The results were expressed as mean ± standard deviation of six animals. The level of significance was calculated using one-way ANOVA followed by Tukey's post-hoc test for multiple comparison, using the software – GraphPad Prism 4.0 (Graph Pad Inc., USA). The values at $p < 0.05$ or less were considered as significant.

Results

Effect of oral supplementation of shilajit and CoQ_{10} on energy status in mice muscle after swimming exercise:

After exhaustive swimming exercise, a significant deterioration of all the energy related parameters, in muscle of animals, were observed. Due to exhaustive swimming, ATP, ADP, AEC, TAN and ATP/ADP ratio found to be lowered significantly with a concomitant increase in IMP content. After administration of shilajit, a significant improvement of all the parameters was achieved, which was comparable to that of CoQ_{10} treated animals. Improvement of all the energy related parameters were further achieved by administration of a combination of shilajit and CoQ_{10}. The results are incorporated in Table 1.

Table 1. Effect of oral supplementation of shilajit and CoQ_{10} treatment on energy status in mice muscle after forced swimming exercise

Treatment groups	ATP (µmol/g)	IMP (µmol/g)	AEC	TAN (µmol/g)	ATP/ADP
Control	1.39±0.36	4.42±0.45	0.76±0.04	2.22±0.40	2.45±0.68
SE	0.25±0.05$^{\#}$	5.21±0.90$^{\#}$	0.52±0.04$^{\#}$	0.84±0.31$^{\#}$	0.77±0.42$^{\#}$
SE+CoQ	0.48±0.04***	3.52±0.82	0.55±0.09	1.12±0.41	1.52±0.44*
SE+shilajit	0.49±0.05***	4.66±0.93	0.62±0.06*	1.11±0.10	1.19±0.37
SE+shilajit+CoQ	0.61±0.06***	3.01±0.62*	0.70±0.04***	1.22±0.29	1.52±0.48*

Values are mean ± SD for 6 animals in each group

$^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$; in comparison to control mice treated with vehicle and fed with normal diet.

$^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; in comparison to SE mice treated with vehicle.

Effect of oral supplementation of shilajit and CoQ_{10} on energy status in mice brain after swimming exercise:

After exhaustive swimming exercise, a significant deterioration of different energy related parameters, in brain of animals, was observed. Due to exhaustive swimming, ATP, AEC, TAN and ATP/ADP ratio were found to be lowered with a concomitant increase in IMP content. The ADP content remained unchanged after SE. After administration of shilajit, a significant improvement of all the parameters was achieved, which was comparable to that of CoQ_{10} treated animals. Better results in all of the energy related parameters were obtained by administration of a combination of shilajit and CoQ_{10}. The results are incorporated in Table 2.

Table 2. Effect of oral supplementation of shilajit and CoQ_{10} treatment on energy status in mice brain after forced swimming exercise

Treatment groups	ATP (µmol/g)	IMP (µmol/g)	AEC	TAN (µmol/g)	ATP/ADP
Control	0.27±0.02	0.05±0.02	0.74±0.20	0.44±0.03	19.80±6.10
SE	0.18±0.05$^{\#}$	0.12±0.03	0.45±0.05$^{\#}$	0.39±0.06	16.60±6.47
SE+CoQ	0.20±0.04	0.11±0.02	0.47±0.10	0.46±0.03	13.60±6.60
SE+shilajit	0.21±0.02	0.08±0.06	0.54±0.03*	0.42±0.02	14.60±8.42
SE+shilajit+CoQ	0.28±0.03**	0.07±0.03	0.55±0.03*	0.52±0.05***	20.30±11.30*

Values are mean ± SD for 6 animals in each group
$^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$; in comparison to control mice treated with vehicle and fed with normal diet.
$^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; in comparison to SE mice treated with vehicle.

Effect of oral supplementation of shilajit and CoQ_{10} on energy status in mice blood after swimming exercise:

After exhaustive swimming exercise, a significant deterioration of different energy related parameters, in blood of animals, was observed. Due to exhaustive swimming, ATP, AEC, TAN and ATP/ADP ratio were found to be lowered with a concomitant increase in IMP content. The ADP content remained unchanged after SE. After administration of shilajit, a significant improvement of all the parameters was achieved, which was comparable to that of CoQ_{10} treated animals. Further improvement in all of the energy related parameters was observed by administration of a combination of shilajit and CoQ_{10}. The results are incorporated in Table 3.

Table 3. Effect of oral supplementation of shilajit and CoQ_{10} treatment on energy status in mice blood after forced swimming exercise

Treatment groups	ATP (mmol/l)	AEC	TAN (mmol/l)	ATP/ADP
Control	0.69±0.11	0.89±0.02	0.81±0.12	6.29±1.43
SE	0.45±0.06	0.80±0.04	0.68±0.10	3.53±1.28
SE+CoQ	0.57±0.11	0.89±0.02***	0.72±0.09	5.60±1.53
SE+shilajit	0.59±0.11	0.89±0.02***	0.71±0.08	6.03±0.75*
SE+shilajit+CoQ	0.62±0.06*	0.90±0.02***	0.76±0.08	5.69±1.80

Values are mean ± SD for 6 animals in each group
$^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$; in comparison to control mice treated with vehicle and fed with normal diet.
$^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; in comparison to SE mice treated with vehicle.

Synergism in energy related parameters in muscle, brain and blood:
Administration of shilajit in conjunction with CoQ_{10} imparted synergistic effects in the ATP level and energy related parameters (viz. AEC, TAN and ATP-ADP ratio). Synergism was further manifested in muscle and brain (Fig. 1). However, in blood, the synergism was not appreciable (Fig. 1).

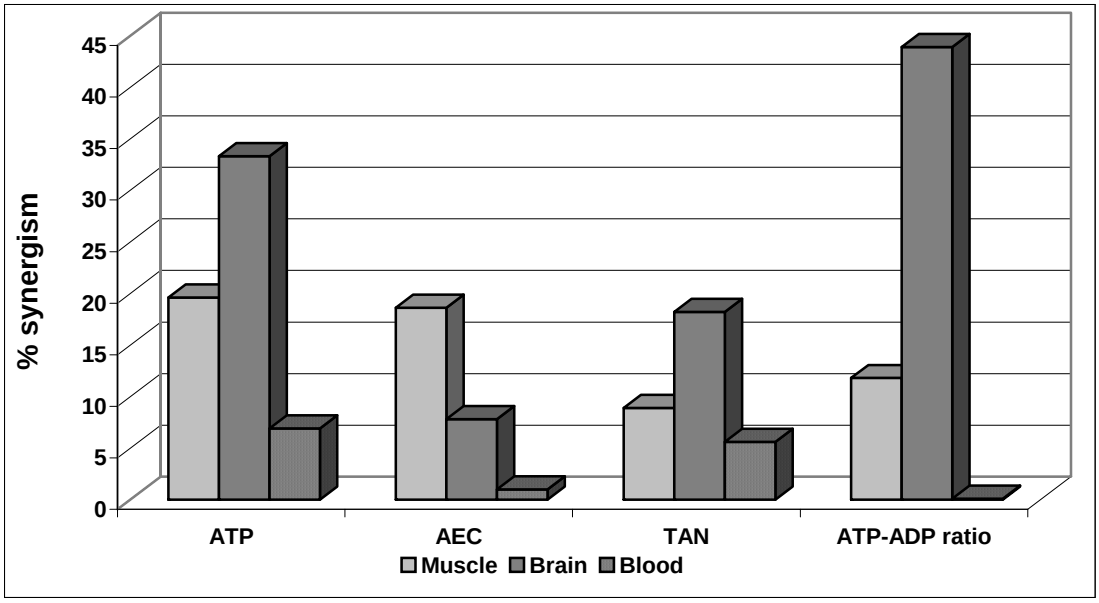

Fig. 1- Synergistic effect of shilajit and CoQ_{10} on some energy related parameters in mice

Effect of oral supplementation of shilajit and CoQ_{10} on total coenzyme Q content in muscle and blood:
There was a sharp fall in the total CoQ concentration (CoQ_9 and CoQ_{10}) (nearly 75%) in blood of SE group animals, compared to that of the control - from 2.4 mmol/l to 0.6 mmol/l (Fig. 2) after swimming. In contrast, in muscle, CoQ content was increased by 68% (from 14.36 nmol/g to 24.14 nmol/g) (Fig. 2). In brain, there was no change in CoQ content, before and after swimming, with or without the test compound treatment. The normalcy in respect of CoQ-content in muscle and blood was restored (Fig. 2) by oral administration of shilajit to SE+shilajit group of animals. The increase in CoQ content in muscle was also reversed by shilajit treatment (Fig. 2) and the effect was comparable to that of CoQ_{10} treatment. Moreover, the fall in CoQ concentration in blood of animals was also reduced to 50% (from 75%) after shilajit treatment. Co-administration of shilajit and CoQ_{10} also showed marked improvement, as the depletion in CoQ content in blood was only 15% (marked reduction from 75%); while in muscle, the CoQ level remained unchanged with respect to the control, indicating that shilajit treatment prevented muscle damage, caused by FST. In case of brain, no significant change in total CoQ content was observed before and after swimming, as well as before and after shilajit treatment (hence, data not shown).

Discussion

Most of the energy required for muscle contraction is generated at the expense of ATP. Energy status of cells can be determined by the relative concentrations of the adenine nucleotides (ATP, ADP and AMP) since the nucleotides act as allosteric modifiers of several key enzymes of energy metabolism [18].

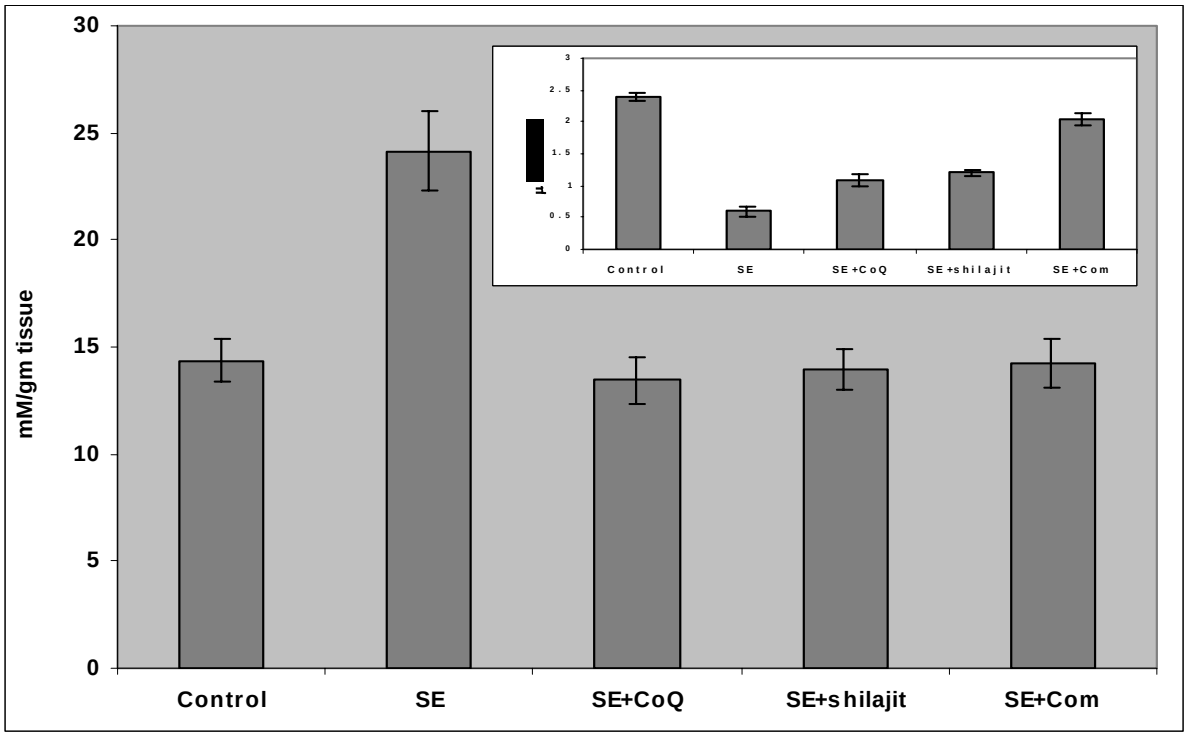

Fig. 2 – Effect of oral supplementation of shilajit and CoQ_{10} on total coenzyme Q content in muscle and blood (inset) of experimental animals engaged in ES exercise. Values are expressed as mean ± SD of 6 animals

In the present study, it was observed that oral supplementation of shilajit upregulated ATP synthesis in muscle, where the need of ATP is maximum during exhaustive exercise. It was also observed that shilajit was equi-active to CoQ_{10}, a popular energy booster, in respect of ATP synthesis. In most tissues, the mitochondrial ATP synthase plays a central role by synthesizing the bulk of ATP [19]. Shilajit might be augmenting functions of mitochondrial electron transport chain for efficient ATP synthesis, conceivably, by its potent electron transfer capacity and antioxidant activity [3]. This postulate is further supported by the fact that exercise induces ROS (reactive oxygen species) production in rodents, which damages the mitochondrial respiratory chain [20]. The energy currency effect of shilajit was no less prominent than that of the popular energy booster, CoQ_{10}. The latter is known to restore affected mitochondrial functions in animals [21].

Many reactions in metabolism are controlled by the overall energy status of the cell. One index of energy status is adenylate energy charge (AEC), which depends on the relative concentrations of ATP, ADP and AMP [13]. The AEC can have values ranging from 0 (all AMP) to 1 (all ATP). The ATP generating pathways are inhibited and the ATP utilizing pathways are stimulated by high AEC. Normally, the AEC value lies between 0.80 and 0.95 in blood and tissues of unstressed animals [22]. However, during exercise, ATP utilizing pathways are stimulated, ATP is hydrolyzed to ADP or AMP and, concomitantly, the AEC decreases [23]. This energy index in albino mice, exposed to swimming exercise, was markedly restored after oral administration of shilajit. This finding lends credence to the billing that shilajit has the attributes of a *maharasa* (super vitalizer), as described in the Ayurveda [1].

At the time of intensive exercise, the oxygen consumption in the tissues increases, and a need for oxygen is developed. Since extracellular ATP and ADP trigger, respectively, vasodilatatory and prothrombic signaling events in the vasculature [24], higher

concentrations of circulating ATP in blood helps in vasodilatation and augments oxygen supply to tissues. Exercise decreases ATP production in mitochondria. Consequently, ATP concentration in blood decreases, resulting in diminished oxygen supply to tissues. In the present study, it was observed that administration of shilajit during exercise augmented ATP concentration in blood in the SE+shilajit group, compared to SE group. This might happen by improving systemic ATP synthesis, which would result in increasing oxygen supply to the tissues, especially in muscle, to cope up with the loss of energy.

Elevated blood levels of ADP significantly augment platelet activity during strenuous exercise, which leads to prothrombic responses and ultimately to lethal consequences [24]. In the present study, it was observed that ADP level was elevated, albeit to a small extent, in blood, after exercise, and treatment with shilajit attenuated the ADP level to reduce the risk of prothrombic responses. However, free ADP in tissues is the rate-limiting factor [6] in mitochondrial ATP synthesis. In the present study, it was observed that ADP concentration in muscle of the exercised animals increased after shilajit treatment (SE+shilajit group), compared to the SE group, indicating a balancing effect to maintain the optimum ADP concentration for efficient ATP synthesis in the shilajit treated animals.

Intense muscle contraction during exercise is associated with degradation of adenine nucleotides to IMP, leading to decrease in the total adenine nucleotide pool (TAN) with a concomitant increase in IMP concentration [16]. In the present study, the control animals showed reduced TAN and elevated IMP concentrations after the exercise. Oral supplementation of shilajit produced perceptible improvement from these adverse conditions. TAN contents were increased in muscle, brain and blood of mice, after swimming, when shilajit was administered. Rise in IMP concentration in muscle and brain due to swimming exercise markedly decreased after treatment with shilajit, with concomitant increase in TAN contents. This finding suggested that shilajit inhibited the degradation of adenine nucleotides to IMP during stressful exercise.

The primary biochemical function of coenzyme Q_{10} (CoQ_{10}) is to synthesize ATP in mitochondria in a series of oxidation-reduction reaction [8]. During swimming exercise, ROS is generated which might induce degradation of mitochondrial membrane, thereby releasing CoQ in the muscle and the muscle content of CoQ increased. Loss of CoQ was reflected in the diminished CoQ content in blood after the swimming exercise. Shilajit treatment attenuated that loss, and when used concurrently with CoQ_{10}, even in sub-optimal doses, significantly restored the CoQ level in blood and normalized its content in muscle (Fig. 2). In addition to decrease in the biosynthesis of CoQ_{10} during exercise, other factors may affect systemic levels of CoQ (e.g. in blood). These include oxidative degradation or changes in the integrity of lipids, which impede the quinone (CoQ) movement. Both these factors seemed to be improved by shilajit treatment concurrently with CoQ_{10} (Fig. 2).

CoQ_{10} is well-known for its use in energy restoration in humans in energy deficient situations. The observed synergistic effect of shilajit and CoQ_{10}, in relation to the systemic ATP production, may prompt further detailed studies in this direction.

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