

Spirulina Supplementation: A Protective Strategy Against Overtraining-Induced Physiological Stress and Anemia in Rats

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ABSTRACT

Physical training is essential for improving athletic performance, but excessive training can have negative consequences. This study examined the potential protective effects of Spirulina supplementation against the impacts of overtraining and anemia in rats. The experiment involved thirty male Wistar rats, which were categorized into five groups: Control, Moderately Trained (MT), Overtrained (OT), Moderately Trained with Spirulina (MTS), and Overtrained with Spirulina (OTS). The training protocol involved daily swimming sessions for either 1 hour (MT and MTS) or 4 hours (OT and OTS) over a 4-week period. Biochemical assessments encompassed indicators such as creatine phosphokinase (CPK), lactate dehydrogenase (LDH), Testosterone, Cortisol, and parameters related to iron levels (hemoglobin, ferritin, iron, Total Iron Binding Capacity). Results showed that overtraining induced significant increases in CPK and LDH levels, indicative of muscle damage. Moreover, overtrained rats exhibited lower hemoglobin and ferritin levels, suggesting iron deficiency anemia. Spirulina significantly attenuated these effects, particularly in the OTS group, which showed reduced CPK and LDH levels and improved iron profile compared to the OT group. Spirulina's antioxidant and anti-inflammatory properties likely contributed to these protective effects. Spirulina supplementation appears to mitigate the adverse effects of overtraining and anemia in rats, potentially enhancing performance outcomes and underscoring the importance of proper nutrient supplementation in athletic training.

Keywords: Athletic performance enhancement, biomarkers, overtraining prevention, spirulina supplement.

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1. INTRODUCTION

Achieving success in sports results from a meticulously crafted training regimen (Maglisco, 2003). Training in athletes refers to a systematic and planned program designed to improve physical fitness, performance, and skill levels specific to their sport. It typically involves a combination of various exercise modalities, including cardiovascular workouts, strength training, flexibility exercises, and sport-specific drills (Granacher *et al.*, 2016). Athletes experience overtraining when the equilibrium between the intensity of training and the opportunity for recovery is disturbed, resulting in decreased performance despite ongoing training efforts. It includes persistent tiredness, mood fluctuations, physical alterations, and a higher susceptibility to injuries (Kreher & Schwartz, 2012). The objective of the training regimen is to induce metabolic, physiological, and biochemical adaptations that enhance athletes' competitive performance (Chatard *et al.*, 1990; Manna *et al.*, 2009; Wang, 1996). The adaptations occurring in response to training enable the body's systems to operate with greater effectiveness and efficiency during competitive events (Hayman, 2016). Biochemical assessments are extensively utilized to evaluate the health and fitness levels of athletes engaged in intense training. These



tests play a crucial role in monitoring training intensity, managing training programs, and enhancing athletic performance (Butova & Masalov, 2009).

The biochemical parameters of the current study include creatine phosphokinase, lactate dehydrogenase, testosterone, cortisol, and the iron profile hemoglobin, ferritin, iron, and total iron binding capacity. Serum Creatine Phosphokinase levels serve as an indirect marker of muscle cell damage, often rising after intense physical exertion (Chernozub et al., 2020; Nikolaidis et al., 2003). Lactate dehydrogenase (LDH) plays a significant role in monitoring training and diagnosing overtraining in athletes. LDH is an enzyme involved in the conversion of lactate to pyruvate, a critical step in anaerobic metabolism. Elevated LDH levels can indicate muscle damage, which is often associated with intense or excessive training. Monitoring LDH levels helps in assessing the physiological stress athletes experience during training and can serve as a marker for overtraining (Zhukova et al., 2023). Lactate dehydrogenase (LDH) is an enzyme involved in carbohydrate metabolism, and its activity serves as a crucial biochemical marker for evaluating muscle tissue performance (Butova & Masalov, 2009). Hormone levels, especially testosterone and cortisol, are significantly influenced by physical exercise. These hormones are essential in regulating physiological responses and adaptations during exercise, as well as in the recovery phase, by balancing anabolic and catabolic processes (Ambroży et al., 2021). Endogenous hormones play a crucial role in physiological responses and adaptations during physical activity. They also impact the recovery phase post-exercise by regulating anabolic and catabolic processes (Fry et al., 1992). In athletes, satisfying the requirement of nutrients is essential for energy metabolism to attain the best performance. The most imperative micronutrient is iron, which is required for the growth and maintenance of tissues as well as for the regulation of body processes. Iron plays a critical role in the physical performance of athletes. Athletes often face unique challenges in maintaining optimal iron levels due to increased demands from their training and the potential for iron loss through sweating, gastrointestinal bleeding, and intravascular hemolysis. Regular monitoring of iron status, including hemoglobin, serum ferritin, and transferrin saturation, is essential for diagnosing and preventing iron deficiency, which can impair performance and overall health (Alaunyte et al., 2015).

The current study helps to find the effect of *Spirulina platensis* intake on the prevention of overtraining and anemia and thereby improve performance. The study also aims to find out the protective effect of skeletal muscle damage anemia in moderately trained and overtrained rats.

2. METHOD

2.1. Experimental Animals

Thirty male Wistar rats, each weighing between 150 and 210 grams, were utilized in this study. These rats were kept in animal cages within a well-ventilated laboratory setting. A 14-day acclimatization period was provided prior to initiating the treatments. All animal handling procedures adhered to institutional and ethical guidelines sanctioned for scientific research.

A total of 30 rats were allocated into five groups:

1. Control
2. Moderate Training–1 hour of swimming daily, 5 days per week for 4 weeks
3. Overtraining–1 hour of swimming daily, 5 days per week for the first 2 weeks, followed by an abrupt increase to 4 hours per day for the remaining 2 weeks
4. Spirulina Supplementation for Moderately Trained rats–1 g/kg body weight of Spirulina given daily to moderately trained rats (Fig. 1)
5. Spirulina Supplementation for Overtrained rats–1 g/kg body weight of Spirulina given daily to overtrained rats (Fig. 1)

All rats were maintained at room temperature with a 12-hour light/12-hour dark cycle and provided with a standard diet and distilled water ad libitum. Prior to the experimental procedures, the rats were acclimated by placing them in a container with 1.5 cm of water at 23°C to 25°C for 2 minutes. This acclimation process aimed to minimize stress at the onset of the exercise regimen (Harri & Kuusela, 1986). After completing their exercise sessions, the rats were dried with a towel and returned to their respective cages.

1 g of spirulina per kg of body weight of rats was the dosage given to rats. 1 g of spirulina was dissolved in 5 ml of water. From this, 1 ml was given to rats by oral route.

2.2. Swimming Tank Specifications (Kegel, 2006)

The swimming tank was round, which helped the rats to swim continuously. Water temperature is maintained between 33°C and 36°C. The tank had dimensions of 65 cm in width, 75 cm in length, and 85 cm in height, with the water level reaching 50 cm.



Fig. 1. Spirulina supplementation via oral administration in rats.

2.3. Biochemical Assessment

The biochemical parameters used in the study were hemoglobin, ferritin, urea, CPK, LDH, testosterone and cortisol. Blood samples were taken at the conclusion of the 28-day training period (post-test).

2.4. Procedure for Blood Collection ([Parasuraman et al., 2010](#))

Necessary items included gloves for handling animals and rodents, a towel, cotton, and tubes for sample collection. Rats were anesthetized using diethyl ether at a concentration of 1.9%, and this concentration was produced with 0.08 ml (80 microliters) per liter of volume of a container. Ether was administered to rats via a cotton ball placed within a small chamber, with induction lasting between 5 to 10 minutes.

The anesthetized rat was positioned laterally on a table with its head facing downward. A micro-hematocrit blood tube was carefully inserted into the corner of the eye socket beneath the eyeball, angled at approximately 45 degrees toward the center of the eye socket. The tube was rotated gently between the fingers as it advanced. Gentle downward pressure was applied and then released once the vein was punctured, allowing blood to flow into the test tube. Bleeding was promptly and entirely halted upon removal of the tube. Then, the rats were sacrificed, and the soleus muscle was dissected for histopathology.

2.5. Histopathology

After completing the training protocol, rats were euthanized. The hind limbs were then dissected to extract the soleus muscles, which were immediately immersed in a formaldehyde solution. The isolated soleus muscles were subsequently cut into 5 mm segments and fixed in a 10% neutral formalin solution for 3 days. Following fixation, the muscle pieces underwent a thorough 12-hour wash under running water, followed by dehydration using progressively stronger alcohol solutions (70%, 80%, and 90%) over 12-hour intervals. Final dehydration was achieved using absolute alcohol, with three changes at 12-hour intervals. Cleansing was performed using xylene, with changes every 15 to 20 minutes. Once cleansed, the muscle pieces underwent paraffin infiltration in an automated tissue processing unit, preceded by a thorough wash under running water to remove residual formalin.

2.5.1. Embedding in Paraffin

Molten hard paraffin was poured into L-shaped molds. The soleus muscle pieces were swiftly immersed in the liquid paraffin and left to cool.

2.5.2. Sectioning

The molded blocks were sliced using a microtome to obtain 5-micron-thick sections. These sections were affixed to glass slides with egg albumin and left to air-dry.

2.5.3. Staining

Eosin, an acid stain, and hematoxylin, a basic stain, were employed to stain the sections of the soleus muscle.

2.6. Experimental Procedure

- The tissue sections were cleared of paraffin using xylene washes for approximately 15 minutes.

TABLE I: SPIRULINA INGESTION IN RATS

Genera organic spirulina powder		
	10 g contains	1 g contains
Protein	6 g	0.6 g
Carbohydrate	2.5 g	0.25 g
Fat	0.7 g	0.07 g
Calcium	17 mg	1.7 mg
Iron	12 mg	1.2 mg
Magnesium	50 mg	5.0 mg
Manganese	133 mcg	13.3 mcg
Potassium	136 mg	13.6 mg
Sodium	100 mg	10.0 mg
Chlorophyll	70 mg	7.0 mg

- Following deparaffinization, the sections were dehydrated by sequential immersion in alcohol solutions of decreasing concentrations (100%, 90%, 80%, and 70%).
- Subsequently, the sections were stained with hematoxylin for a duration of 15 minutes and rinsed in tap water.
- Microscopic examination revealed distinct nuclei and a predominantly light or colorless background.
- The slides were briefly rinsed in tap water.
- The sections were then immersed in ammonia water until achieved a bright blue color after 3 to 5 dips.
- Afterwards, the slides underwent running tap water washes lasting 10 to 20 minutes.
- The slides were then subjected to eosin staining for 15 seconds to 2 minutes, adjusted based on eosin concentration and desired counterstain intensity. To ensure even staining, slides were dipped multiple times in ammonium water before eosin application.
- Following staining, dehydration was carried out using 95% alcohol and absolute alcohol until excess eosin was removed, with intervals of 2 minutes between successive treatments of 90% alcohol, 80% alcohol, and xylene.
- Finally, the sections were mounted in a DPX (Diphenyl xylene) mounting medium. Staining results exhibited blue-colored nuclei and cytoplasm in varying shades of pink, reflecting different tissue components.

2.7. Data Collection and Analysis

The data is collected and processed using SPSS (Statistical Package for Social Sciences) MS Windows version 9.0.

3. RESULTS AND DISCUSSION

Physical activity alters the morphology of skeletal muscle and the biochemical profiles in rats. The responses to long-term exercise are influenced by exercise variables such as intensity, duration, and frequency (MacInnis & Gibala, 2017). Table II shows that the hemoglobin and ferritin levels are significantly different among the groups. The OT group has the lowest hemoglobin and ferritin level, which shows that overtraining induces iron loss and ends up in an anemic state. MTS and OTS groups, which are supplemented with spirulina, have improved iron profiles. The supplement contains 1.2 mg of iron, which helps the rats prevent anemia and overtraining.

Hemoglobin and ferritin are the markers of anemia/iron status, and the spirulina supplement helps to prevent anemia in rats.

The impact of physical activity on changes in various enzyme systems has been thoroughly examined in existing literature. (Powers et al., 2022; Thirupathi et al., 2021). Exercise in humans has been associated with heightened enzyme activities (Thirupathi et al., 2021). Similarly, research involving experimental animals has consistently yielded comparable results (Highman & Altland, 1963; Novosadova, 1969; Papadopoulos et al., 1968; Sangster & Beaton, 1966). Physical exercise or activity alters the enzyme synthesis and degradation in both humans and animals (De Angelis et al., 2017; Maughan, 2000; Schmidt & Lee, 2013). Several researchers have endeavored to describe the alterations in plasma or serum enzyme levels observed after physical activity. Among the enzymes found to increase in plasma or serum due to exercise are CPK and LDH. Several researchers have endeavored to

TABLE II: COMPARISON OF BIOCHEMICAL AND HORMONAL PARAMETERS ACROSS DIFFERENT TRAINING AND SUPPLEMENTATION GROUPS

Parameter	Group-I C	Group-II MT	Group-III MTS	Group-IV OT	Group-V OTS
Hemoglobin (g%)	15.83 $\pm$ 0.54	15.43 $\pm$ 0.51	16.00 $\pm$ 0.51	15.28 $\pm$ 0.50	15.83 $\pm$ 0.29
Ferritin (ng/ml)	66.60 $\pm$ 3.36	61.60 $\pm$ 2.98	89.73 $\pm$ 6.47	48.40 $\pm$ 3.44	71.00 $\pm$ 4.41
Urea (mg%)	50.46 $\pm$ 1.82	55.65 $\pm$ 3.80	50.62 $\pm$ 2.45	56.98 $\pm$ 6.64	53.33 $\pm$ 3.54
CPK (IU/L)	90.30 $\pm$ 5.58	127.12 $\pm$ 10.26	104.77 $\pm$ 6.94	203.20 $\pm$ 56.14	152.47 $\pm$ 30.14
LDH (IU/L)	338.03 $\pm$ 21.64	394.40 $\pm$ 7.24	381.77 $\pm$ 10.63	483.85 $\pm$ 17.33	404.10 $\pm$ 10.65
Testosterone(ng/ml)	3.05 $\pm$ 0.72	4.87 $\pm$ 0.73	6.42 $\pm$ 0.56	4.30 $\pm$ 0.49	6.15 $\pm$ 0.63
Cortisol (ng/ml)	104.93 $\pm$ 4.26	122.50 $\pm$ 3.11	115.70 $\pm$ 4.83	141.83 $\pm$ 5.01	130.25 $\pm$ 3.84
T/C ratio	0.0292 $\pm$ 0.007	0.04 $\pm$ 0.06	0.0553 $\pm$ 0.004	0.0303 $\pm$ 0.004	0.0473 $\pm$ 0.006

Note: C-Control, MT-Moderately trained, MTS-Moderately trained with spirulina, OT-Overtrained, OTS-Overtrained with spirulina.

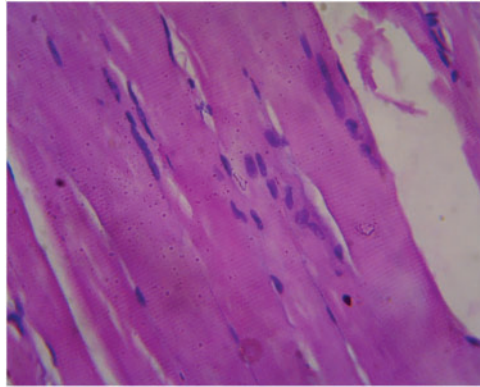


Fig. 2. Control.

describe the alterations in plasma or serum enzyme levels observed after physical activity. Among the enzymes found to increase in plasma or serum due to exercise are CPK and LDH (MacInnis & Gibala, 2017). In the present study, the CPK level of the OT group is a twofold increase of the C group, which shows the muscle cell damage and leakage of CPK enzymes to blood increases. The MT group has a higher CPK level than the C group, which shows the adaptation to exercise. MTS and the OTS groups that have been given the spirulina supplement of 1 g/Kg of body weight show improvement in their CPK values, and this result shows that the spirulina supplement helps mitigate the effects of overtraining (Table II). As spirulina possesses anti-inflammatory properties due to the presence of phycocyanin in it, it helps to reduce inflammation caused by training. Spirulina supplementation includes antioxidants like beta-carotene and vitamin E, which aid in the neutralization of free radicals and the reduction of oxidative stress (Deng & Chow, 2010; Karkos et al., 2011).

LDH level of different groups of rats shows significant differences (Table III). The OT group has the highest LDH level, which implies muscle enzyme leakage into the blood. Supplemented groups MTS and OTS have reduced the LDH values more than the MT and OT groups, which shows a protective effect on muscle cells when they are stressed (Table II).

Protein catabolism is a protein degradation process, which is cleaved into amino acids and ultimately ends with the biomolecule urea. When the rats are overtrained, the protein catabolism is higher, and so the urea levels are higher in the OT group than in the other groups. But, when the rats are supplemented with spirulina, which contains 0.6 g of protein, it can help to replenish protein content in rats (Table I). The urea level of the MTS and OTS groups is lower than that of the MT and OT groups. Spirulina contains the essential amino acids that help reduce muscle breakdown and muscle recovery. Hence, with protein supplementation, the rats can be stressed more without muscle damage and protein breakdown.

Positive athlete's training adaptation depends on the balance of the anabolic and catabolic processes. The disparity between catabolic and anabolic processes leads to overreaching or overtraining syndrome. This condition is marked by reduced sport-specific physical performance, quicker onset of fatigue, and subjective signs of stress (Cadejani, 2020). Athletes often dread overtraining, yet there remains a shortage of clear, measurable criteria for its identification and prevention (Cadejani, 2020). Serum urea level, enzymes CPK, and LDH level are used to monitor training load. In addition to that, testosterone and cortisol are also used. Hormones play a crucial role in the biochemical and physiological responses and adjustments in our body during exercise. The changes that occur affect post-exercise recovery through both anabolic and catabolic mechanisms (Kraemer & Ratamess, 2005). Steroid hormones like Testosterone and cortisol have a substantial impact on protein and carbohydrate

TABLE III: ANOVA COMPARISON OF BIOCHEMICAL AND HORMONAL PARAMETERS ACROSS TRAINING AND SUPPLEMENTATION GROUPS

Parameter		Sum of squares	df	Mean square	F	Significant	Post-hoc test (LSD)	Significant
Hemoglobin (g%)	Between groups	63.70	4	15.93	18.27	0.003	I vs II	0.038
	Within groups	21.79	25	0.87			I vs IV	<0.001
	Total	85.49	29				II vs III	0.007
							II vs IV	<0.001
							III vs IV	<0.001
Ferritin (ng/ml)	Between groups	3783.47	4	945.87	44.20	<0.001	IV vs V	<0.001
	Within groups	534.96	25	21.40			I vs III	<0.001
	Total	4318.44	29				I vs IV	0.002
							II vs III	<0.001
							II vs V	0.002
Urea (mg%)	Between groups	205.60	4	51.40	3.202	0.030	III vs IV	<0.001
	Within groups	401.29	25	16.05			III vs V	<0.001
	Total	606.90	29				IV vs V	<0.001
							I vs II	0.034
							I vs IV	0.009
CPK (IU/L)	Between groups	47573.95	4	11893.49	14.01	<0.001	II vs III	0.039
	Within groups	21222.64	25	848.91			III vs IV	0.011
	Total	68796.58	29				I vs V	0.001
							II vs IV	<0.001
							III vs IV	<0.001
LDH (IU/L)	Between groups	67502.34	4	16875.59	80.54	<0.001	III vs V	0.009
	Within groups	5238.20	25	209.53			IV vs V	0.006
	Total	72740.54	29				I vs II, III, IV, V	
							II vs IV	<0.001
							III vs IV	<0.001
Testosterone (ng/ml)	Between groups	45.82	4	11.46	28.69	<0.001	III vs V	<0.001
	Within groups	9.98	25	0.40			IV vs V	0.013
	Total	55.80	29					<0.001
							I vs II, III, V	<0.001
							I vs IV	0.002
Cortisol (ng/ml)	Between groups	4723.15	4	1180.79	64.91	<0.001	II vs III	<0.001
	Within groups	454.78	25	18.19			II vs V	0.002
	Total	5177.93	29				III vs IV	<0.001
							IV vs V	<0.001
							III vs IV, V	0.004
T/C ratio	Between groups	0.003	4	0.001	24.26	<0.001	IV vs V	<0.001
	Within groups	0.001	25	0.006			I vs II	0.002
	Total	0.004	29				I vs III, V	<0.001
							II vs III	<0.001
							II vs IV	0.006
							II vs V	0.031
							III vs IV	<0.001
							III vs V	0.020
							IV vs V	<0.001

metabolism. Hormones act competitively as agonists at the receptor level within muscle cells. The ratio of testosterone to cortisol serves as a marker for evaluating the balance between anabolic and catabolic processes in the human body (Adlercreutz *et al.*, 1986; Majumdar & Srividhya, 2010; Mangine *et al.*, 2018). The testosterone/cortisol ratio is greatly influenced by the intensity and volume of training. The ratio may decrease due to overtraining and improper recovery. The ratio of testosterone to cortisol

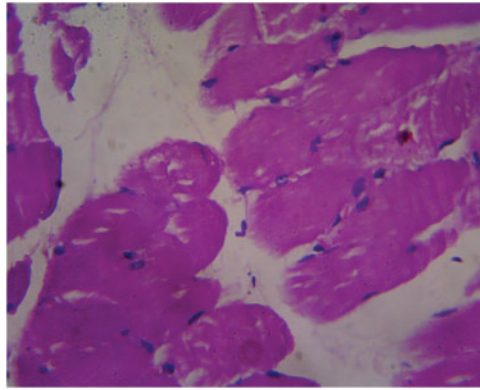


Fig. 3. Moderately trained. Section from skeletal muscle shows edematous change.

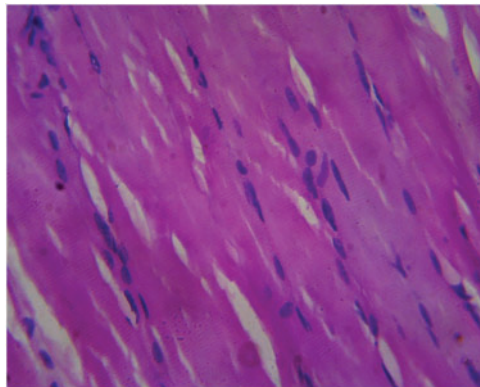


Fig. 4. Moderately trained with spirulina supplement.

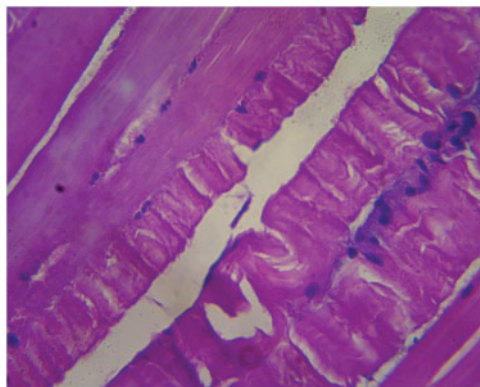


Fig. 5. Overtrained. Section from skeletal muscle shows inflammation.

reflects the real physiological stress during training rather than indicating overtraining syndrome specifically (Urhausen *et al.*, 1995). Testosterone is a hormone known for its role in promoting protein synthesis, contributing to anabolic processes in the body. In the OT group, the level of testosterone is less compared with the MT, MTS and OTS groups. Even though exercise increases the testosterone level, overtraining will decrease the level, leading to low testosterone levels, which eventually disturbs the protein metabolism. Supplements help to increase the level of MTS and OTS in the groups. Cortisol is a catabolic hormone, and the level is higher in the OT group. MTS and OTS groups have low levels of cortisol hormone, which shows that the rats fed with spirulina supplements can tolerate more stress with less muscle damage (Table II).

Urea, CPK, LDH, Testosterone, and cortisol are overtrained markers, and the spirulina supplement helps prevent and protect the cell against this stress. Chronic training adaptation leads to changes in skeletal muscle morphology, physiology, and biochemical properties.

In the control section of skeletal muscle (Fig. 2), no significant abnormalities are observed. In the overtrained section (Fig. 5), there is noticeable inflammation. In the moderately trained section (Fig. 3), edematous changes are evident. When supplements are introduced, the overtrained muscle section (Fig. 6) shows reduced inflammation, and the moderately trained muscle section

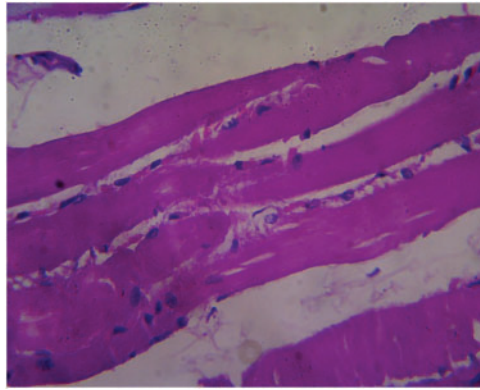


Fig. 6. Overtrained with spirulina supplement.

(Fig. 4) demonstrates diminished edematous changes compared to their respective non-supplemented counterparts.

4. CONCLUSION

The study concludes that Spirulina supplementation helps to avoid overtraining and anemia in rats. Optimum exercise loading with adequate protein and iron intake can help to improve performance. Optimum exercise helps to prevent muscle cell damage. Spirulina supplements with multi-nutrients helps from cell damage and protects/prevents overtraining and anemia.

CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

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