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Impact of Lihocin on Immuno Haematological and Antioxidant Enzyme Indices of Carp Fish

D.Vineela and S. Janardana Reddy*
Department of Fishery Science and Aquaculture, Sri Venkateswara University, Tirupati, (AP) - India

Abstract

Acute toxicity of an organochlorine insecticide is characterized by their persistence and ability to accumulate in aquatic organisms. Our present study is aimed to elucidate the effect of Lihocin (Chlorocholine chloride) on Haematological indices and antioxidant system of carp fish *Catla catla*. 1/5th sub lethal concentration of Lihocin is 1.56mg/L (7.63mg/L for 96hrs) was used for the toxicity experiment. Blood samples are used to analyze for the haematological total erythrocyte count (TEC), total leucocyte count (TLC), total haemoglobin count (Hb) and Packed cell volume (PCV or Hct),and tissue samples are used for lipid peroxidation and antioxidant enzymes (CAT,SOD,GPx) of Lihocin on *Catla catla* for over of 45 days exposure period. The experimental fish treated with lihocin over an exposure period of 3, 7, 14, 30 and 45 days. Lihocin caused a significant decrease in total erythrocyte count (-70.3%), total haemoglobin content (-67.2%) and haematocrit (-63.2%) and induced a significant elevation in the total leucocyte count (95.6%) compared to control group. Lihocin also induced a gradual and significant ($P<0.05$) increase in Lipid Peroxidation and antioxidant enzymes like SOD and GPx, where as CAT activity is gradually decreased throughout the study period. The present study explained that even at sub lethal concentration level of lihocin is harmful to *Catla catla* like aquatic culture organisms and applications of Lihocin close to bodies of water lead to havoc to aquatic life, then to the humans.

Key-Words: Lihocin, *Catla catla*, Behavioural changes, Haematological indices, Antioxidant Enzymes, Lipid Peroxidation

Introduction

It is well known that the organochlorine insecticides (OCIs) are widely used worldwide resulted in a high contamination risk to aquatic environment[1]. OCIs are highly soluble in water so they can easily contaminate aquatic ecosystems, thereby increasing the exposure risk of aquatic flora and fauna[2]. The basic mechanism of action of all insecticides is to alter the transfer of signals along nerve fiber and across the synapse from one nerve to another. In fish, due to biomagnification the concentration of insecticide is higher in fish tissues than in the aquatic medium where the fish is living. The organochlorine compounds reduce the metabolic activity and oxygen consumption in various tissues of fish[3].

Lihocin, an Organochlorine insecticide, is highly toxic to fish which absorbs it directly from water or by ingesting contaminated food and bioaccumulate in their fatty acids due to its Lipophilic nature[4].

*** Corresponding Author**
E.Mail: sjreddy_7@yahoo.co.in,
sjanardanareddy@gmail.com

Lihocin entered in to the aquatic environment cause serious threatening to various aquatic organisms and also cause severe metabolic abnormalities in non target species like fish and freshwater mussels[5]. The present study is aimed to evaluate the effect of sub lethal concentration of lihocin on haematological indices, Lipid peroxidation, and detoxification enzymes in various tissues of carp fish, *Catla catla* over an exposure period of 45 days. These parameters have been used to assess the health of fish, monitoring stress responses and forebode systematic relationship and physiological adaptations of animals. They quickly reflect the weak condition of fish. Haematological parameters are considered as index of the total body and therefore they are important for diagnosing structural and functional strategy of fish[6]. The hydroxyl radicals can initiate lipid peroxidation (LPO) in tissues. Lipid peroxidation indicates the oxidative deterioration of lipids containing any number of carbon-carbon double bonds. Lipid Peroxidation refers to the oxidative degradation of lipids. Exposure to xenobiotics triggers the generation of reactive oxygen species (ROS). Aerobic

organisms can generate superoxide anions, (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (.OH^-) because of oxidative metabolism of xenobiotics. Lipids, proteins and nucleic acid are sensitive targets of ROS. Excessive ROS production leads to damage of cellular components and guides to oxidative stress. The cells have a complex defense system to protect themselves from ROS, including main antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and peroxidase and non-enzymatic scavengers[7]. The primary defense is offered by enzymatic antioxidants, which have been shown to neutralize ROS. Fish tissues, specifically the liver and kidney are endowed with antioxidant defense system consisting of catalase (CAT), superoxide dismutase (SOD), peroxidase etc. to protect them from oxidative stress[8]. Antioxidants contributes to the maintenance of relatively low level of the reactive and harmful hydroxide radical, the superoxide radical and hydrogen peroxide in the presence of Cu^{2+} and/or Fe^{3+}**.** Such markers measured at the molecular or cellular level in fish have been identified as sensitive "early warning" tool in environmental quality assessment tests[9].

Material and Methods

Technical grade Lihocin [2-Chloro-N, N, N-trimethylethanaminium chloride] is a chlorocholine chloride which is used as a planned growth regulator (PGR) is obtained from. They are highly insoluble in water, but are attracted to fats. Lihocin is very stable in both fresh and salt-water environments[5].

Live specimens of *Catla catla* of (30.0 ± 1.5g) were collected from AP Govt. Fish Breeding and Hatchery Centre, Kalyani dam, near Tirupati, Chitoor district and immediately transferred to transparent polypropylene tank of 500L capacity filled with filtered, well aerated and dechlorinated bore well water. The fish were fed with a commercial pelletized formulated fish feed twice a day. The water quality is maintained constantly throughout the experimental period in control medium and also in pesticide treated aquatic medium (Table-1).

LC_{50} of Lihocin for 96h is calculated by the static bioassay method[10]. Fish were starved 24h prior to the experimental period to nullify the metabolic changes. Five replicates of each containing twenty fish were subjected to Lihocin at various concentrations for 96 hours. The lethal concentration of Lihocin (LC_{50}/96hrs) and identified as 7.63mg/L and 1.56mg/L was selected as 1/5th sub lethal concentration for the further analysis.

Collection of Blood

Blood samples were obtained from live fish specimen through by puncture of the caudal vein using a heparin coated needle attached to a 1 ml syringe.

Collection of tissues

After the experimental period the fish were killed by pithing (by damaging the brain and severing the spinal cord between the head and trunk region using a sharp needle) and the tissues viz gill, liver and muscle were removed from its body. They were washed in ice-cold 0.33M sucrose and blotted dry and the desired amounts of tissue were weighed and used. The tissues are homogenized in 6 volumes of homogenizing buffer (50mM Tris-HCl mixed with 1.15% KCl and pH adjusted to 7.4) using Teflon homogenizer. The resulting homogenate was centrifuged at 16,000 g for 15min in a centrifuge at− 4 °C. The supernatant was decanted and stored at –20 °C in a deep freezer for until enzymatic analysis.

Table 1: Physico-Chemical Characteristics of Water

Parameters	Values
Temperature (°C)	28.5 ±0.3
Light and dark period	12h: 12h
P^H	7.5±0.3
DO (mg/L)	5.9±1.3
Hardness (as $Caco_3$) (mg/L)	523.5±6.4
Alkalinity	422.6±2.7
Total ammonia (mg/L)	1007±3.5

Determination of Haematological Parameters

Total erythrocyte counts (TEC) ($10^6 mm^{-3}$) were estimated using diluted with Dacie's fluid in the ratio 1:4 as per Blaxhall and Daisley[11] with haemocytometer under a microscope[12].

Total leucocyte (TLC) (in mm^3x10^3) cells are counted using an improved Neubaur Haemocytometer [13]. Blood was diluted to 1:20 with Turks diluting fluid and placed in haemocytometer at 640x.

The haemoglobin content (mg/100) of blood was analysed following the cyanmethaemoglobin method using Drabkins fluid. The absorbance was prescribed using a spectrophotometer at 546nm.

The hematocrit (PCV) is used to deliberate the volume percentage (%) of red blood cells in blood, and it can be determined by the Microhematocrit tube method.

Determination of Enzymatic Assays

Lipid peroxidation is determined by the Thiobarbituric Acid Reactive Substances (TBARS) assay used to deliberate lipid peroxidation by

measuring the malondialdehyde (MDA) concentration in each tissue lysate[14].

CAT activity is determined by the method of Xu et al[15] which is based on the first-order reaction of CAT with H_2O_2? The CAT activity was dictated by spectrophotometry at 240 nm.

SOD activity is determined by the method of Zou et al[16] based on inhibition of SOD by auto-oxidation of 1, 2, 3-benzenetriol.The SOD activity was dictated by spectrophotometry at 325 nm.

Glutathione Peroxidase (GPx) activity was deliberated by the method of Tappel[17].Glutathione Peroxidase was recorded by the spectrophotometer at 340nm.

Statistical analysis

The Probit mortality was found out using SPSS software 16.0 and biochemical data processed using SPSS 13 statistical program. All data were expressed as arithmetic mean ± SD, for the analysis of the experimental parameters Student's -t test was used.

Results and Discussion

In the present study the probit analysis confirms the LC_{50} value of Lihocin for 96hrs was estimated experimentally as 1.56mg/L and the upper and lower confidence limits were calculated as 1.37mg/L and 1.78mg/L respectively, indicating that Lihocin is toxic to fish at a low concentration.

Vittozzi and Angelis[18] reported 0.78 mg/l and 0.79 mg/l as 96 h LC_{50} values of Azodrin for bluegill and trouts respectively. Attempts were also made in the present study to observe carefully the demeanour conditions of the fish during the 96 h exposure of Lihocin. Behavioural changes such as curling of spine gradual increase in colour fading, a thick mucous film was formed on whole body and gills, in all test fish and vertical movement of the fish was observed during the experimental period. This may be due to loss of labyrinthine sense at high inebriation which makes the fish to turn upside down and finally died. The swimming performance is considered as one measure which could serve as possible sensitive indicator of sub-lethal toxic exposure. According to Little et al, [19] Ololade and Oginni[20] behavioural measurements may be indicators of sub lethal contamination due to concentrations even being lowers than those that effect growth. Behavioural functions are generally quite susceptible to contaminant exposures, and fish often exhibited these reactions first when exposed to pollutants[21].

In the present investigation haematological indices are ascertained due to the exposure of *Catla catla* exposed to $1/5^{th}$ sub lethal concentration of Lihocin for 45 days exposure ascertained that the sub lethal concentration of lihocin caused significant variations. The total number of red blood cell (RBC) count, the haemoglobin (Hb) content, and haematocrit (PCV) values have shown a gradual and significant decrease, and significant increase in WBC count of lihocin treated fish. It is perceived from our results that there is a significant decrease in RBC count, might be due to haemolysis and shrinkage of blood cells by the toxic shock of Lihocin (Table-1).

The decrease in haematological indices such as RBC, Hb and PCV of the fish exposed to sub lethal concentration of Lihocin may be due to haemolysis of red blood cells by Lihocin leading to significant decrease in haematocrit value which results anaemia. Similar observations were also reported in juvenile *C. gariepinus* treated with various pesticides[22]. It may also be attributed to haemodilution resulting from impaired osmoregulation across the gill epithelium.[23]Reduction in haematological indices may also be due to a considerable decline in the haematopoiesis. Similar decline in RBC was also reported in *Labeo rhoita* treated with Cypermethrin[24], in carp fish, *Cyprinus carpio* treated with diazinone[25] and in African cat fish, *C. gariepinus* treated with diazinone[26]. Similar reduction effects on RBC of fishes have reported by Deoi et al. (2004)[27], Neeraj Kumar et al. (2011)[28], Christopher Didigwu Nwani et al. (2010)[29] by various other toxicants.

Decline in Hb content of treated *Catla catla* , indicating the decline in haemoglobin synthesis as well as reduction in oxygen carrying capacity which may perhaps be as a result of interference of endosulfan with haem or globin synthesis pathway. Ramaswamy et al. (1996)[30] reported a significant reduction in Hb content and erthyrocyte count in the blood of a fresh water fish, *Sarotherodon mossambicus*, on exposure to dimecron and carbamate. A significant decrease in erythrocyte count, haematocrit and haemoglobin contents had been reported for catfish on acute exposure to diazinon[26]. A 4-week reatment to sub lethal concentration of endosulfan was caused to induce blood dyscrasia in fish, *Barbus conchonius* and clinical findings included erythropenia, anaemia, lymphocytosis, thrombocytosis, monocytosis and neutropenia[31]. The reduction in values obtained for haematological parameters of lihocin treated fish in this study showed that the physiological activities of the treated fish were affected. The decreased erythrocyte count and haemoglobin content observed in this study may be due to the disruptive

action on the erythropoietic tissue, which in turn affected the cell viability. The reduction in RBC count and Hb are often accompanied by a decrease in Hct and demonstrates the physiological dysfunction of the hemopoietic system.

Changes in the erythrocyte visibility suggest a compensation of oxygen inadequacy in the body due to gill damage and the nature of the changes shows a release of erythrocytes from the blood depots[32]. It is perceived from our results that a gradual significant decrement in hemoglobin level was recorded after 3days exposure of Lihocin to *Catla catla* (Table- 1) due to the release of immature cells from haemopoietic tissue into the blood strength as well as commotion of iron metabolism that lead to a defective hemoglobin synthesis. Similar results are also reported by Khattak and Hafeez [33].

The increase in WBC count can be correlated with an increase in antibody production, which helps in survival and recovery of the fishes exposed to the toxicant [34]. A significant increase in WBC count in the present study indicate a hypersensitivity of leucocytes to lihocin and these changes may be due to immunological reactions to produce antibodies to cope up with stress induced by lihocin.

It is perceived from our result that the significant decrement in PCV indicates the fish suffers from anemia. The reduction in the PCV values suggests that the fish endures from anemia or hemodilution. In addition, an alteration in the fish metabolism would also lead to decrease values of haematocrit in *Catla catla.* It is evinced from our result that, there is a significant increase in WBC count after chemical stress recorded in the present study is in conformity with the earlier reports[35, 36, 37]**.** Increase in WBC count constitutes leucocytosis, considered a modification of fish to prolong against Lihocin. Leucocytosis helps in the removal of cellular debris of necrosed tissue at a faster rate, with an increase in antibody production which helps in survival and recovery of the treated fish to lindane and malathion[38].

In the present study the studies on antioxidant enzymes such as superoxide dismutase, catalase and Glutathione peroxidase activities in different tissues of fish after lihocin exposure, there was an increased SOD, and Glutathione peroxidase activity and decreased catalase activity on lihocin 45 days exposure period. Glutathione peroxidase activity levels along with catalase and SOD are considered as the key enzymes within the antioxidative defense mechanism, which directly determines the concentration of O_2 and H_2O[39]. The increased activity of SOD and peroxidase under toxic condition may be for counteracting lipid peroxidation and removing toxic H2O2 or the organic hydroperoxide formed during lindane exposure. The results obtained in this study demonstrate that the sub lethal concentration of lindane can cause changes in biochemical responses and antioxidant activity in fish .This alteration might be potentially destructive on the survivability and normal functioning of *Catla catla*. The stress created by sub lethal concentration of lihocin leads to increased activity of antioxidant enzymes such as glutathione peroxidase and SOD. Glutathione peroxidase activity significantly increased ($p < 0.001$) in the present study, Similar results were also reported by Shalini Verma and Dubey (2003)[40]. Glutathione peroxidase activity might be expected to reduce the level of ROS by metabolizing H_2O_2. Thus the increase in antioxidant enzyme activities are to minimize the potential effect of ROS at cellular level.

It is discernible from the our present outcome that the antioxidant enzymes of carp fish treated with sub lethal concentration of Lihocin caused significant elevation in antioxidant enzymes such as LPO, SOD, GP_x activity. LPO along with CAT, GPx and SOD are considered as the key enzymes within the antioxidative defense mechanism, which directly determines the concentration of O_2 and H_2O_2[39]. It is discernible from our present outcome that the significant increase in LPO activity may indicate the susceptibility of lipid molecules to reactive oxygen species and extend of oxidative damage imposed on these molecules this increase in antioxidant activity is to minimize the potential effect of ROS in cellular level. It is evident from our results that the SOD activity in *Catla catla* showed an increase in various tissues of fish *Catla catla* under the treatment of lihocin may be an adaptive response to decrease the severity of ROS effect. The increased activity of antioxidant enzymes is considered as a critical phenomenon in the protection against post free radical damage known as "*preparation for oxidative stress*"[41]. It is discernible from our present outcome that the increased activity of GPx under toxic condition may due to counteracting Lipid peroxidation and removing toxic H_2O_2 or the organic hydroperoxide formed during the exposure of sublethal concentration of lihocin to exposed fishes. The present outcome obtained from our study may exhibit the sub lethal concentration of lihocin to *Catla catla* undergo stress and leads to increased activity of antioxidant enzymes and cause changes in biochemical responses and antioxidant activity in fish.

Catalase enzyme along with glutathione peroxidase and glutathione-S-transferase enzyme removes the H_2O_2 produced by dismutation of O_2^- (Superoxide radical) by superoxide dismutases (SODs) and the hydroperoxides produced by lipid peroxidation[42]. In our study catalase activity was gradually and significantly decreased in *Catla catla.* Decreased catalase activity may be due to reduction in NADPH concentration pertaining to the higher energy need or immense generation of free radicals on chronic exposure to lihocin. This indicates the potential effect of lihocin on inhibition of antioxidant enzymes as a result of superoxide accumulation. Similar observation was found in cat fish, *Clarius batracus* exposed to pesticide, phosphomidon[43] and in the renal and hepatic tissues of another cat fish, *H. fossillis* exposed to Endrin and Sevin respectively[43,44]. Pesticide-induced inhibition of catalase activity has been reported on the exposure of endosulfan[45]. Sayeed et al. (2003)[46] pointed out that a drop in CAT activity could be explained by the flux of superoxide radicals due to the oxidative stress caused by the exposure of Deltamethrin in liver, kidney and gill tissues of murrel fish, *C. punctatus*. Similar observation was found in exposure to metal intoxication in the killifish, *F. heteroclitus* exposed to cadmium[47]; in the liver of *Catla catla* exposed to many metals [48],and in different tissues of *C. batrcus* exposed to mercury [49].

In our present study there is a gradual decrement in Catalase activity of *Catla catla* exposed to lihocin. Decreased Catalase activity may be due to reduction in NADPH concentration pertaining to the higher energy need or immense generation of free radicals on chronic exposure to lihocin. This indicates the potential effect of lihocin on inhibition of antioxidant enzymes as a result of superoxide accumulation. Similar observation was found in Pesticide-induced inhibition, drop in CAT activity could be explained by the flux of superoxide radicals due to the oxidative stress caused by the exposure of Deltamethrin in liver, kidney and gill tissues of *Channa punctatus* [46].

Conclusion

The results of the present study indicate that lihocin exposure during sub lethal treatment induces significant changes in the behavioural, haematological indices and antioxidant parameters of *Catla catla*. The alterations of these parameters may provide early warning signals for the determination of sub lethal toxic level of pesticides and their effects in aquatic medium. The present study also provide a better understanding of the toxicological endpoint of aquatic pollutants and to ascertain a safer level of these chemicals in the aquatic environment and protection of aquatic habitants.

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Table 2: Variations in Haematological indices of *Catla catla* exposed to sub lethal concentration of Lihocin

Parameter	Control	Exposure periods to Lihocin				
		3d	7d	14d	30d	45d
RBC (10^6/mm3) % Change	1.89±0.005 ----	1.46±0.004 (-22.71)	1.31±0.003 (-30.68)	1.19±0.004 (-37.03)	0.87±0.002 (-53.96)	0.56±0.003 (-70.37)
Hb (g/100mL) %change	84.36±1.37 ----	73.26±1.32 (-13.15)	68.59±1.26 (-18.69)	51.67±1.16 (-38.75)	43.52±1.02 (-48.4)	27.63±0.96 (-67.24)
PCV (%) % change	46.93±0.67 ----	41.93±0.62 (-10.65)	39.64±0.58 (-15.53)	30.15±0.43 (-35.75)	22.37±0.26 (-52.33)	17.69±0.13 (-63.20)
WBC (10^4/mm3) % change	2.73±0.022 ----	2.86±0.004 (4.76)	3.16±0.008 (15.75)	3.92±0.017 (43.58)	4.68±0.025 (71.42)	5.34±0.037 (95.60)

Values are Mean±SD of six individual observations. Values are significant at $P < 0.05$.

Table 3: Variations in Lipid Peroxidation in gill, liver, kidney and muscle tissues of *Catla catla* exposed to sub lethal concentration of Lihocin

Parameters	Control	Exposure periods of Lihocin				
		3d	7d	14d	30d	45d
Gill % change	4.56±0.33 ----	4.87±0.31 (6.79)	4.99±0.36 (9.42)	5.08±0.39 (11.40)	5.63±0.42 (23.46)	6.29±0.48 (37.93)
Liver % change	7.83±0.54 ----	7.98±0.52 (1.91)	8.13±0.49 (3.83)	8.87±0.31 (13.28)	8.99±0.28 (14.81)	9.04±0.17 (15.45)
Kidney % change	5.68±0.79 ----	5.37±0.65 (5.45)	5.02±0.54 (11.61)	4.83±0.47 (14.96)	3.61±0.35 (36.44)	3.22±0.21 (43.30)
Muscle % change	6.56±0.37 ----	6.79±0.38 (3.50)	6.95±0.43 (5.94)	7.36±0.54 (12.19)	7.73±0.51 (17.83)	8.13±0.45 (23.95)

Values are Mean±SD of ten individual observations. Values are significant at $P < 0.05$.

Table 4: Variations in SOD activity in gill, liver, kidney and muscle tissues of *Catla catla* exposed to sub lethal concentration of Lihocin

Parameters	Control	Exposure periods of Lihocin				
		3d	7d	14d	30d	45d
Gill % change	6.68±0.12 -----	6.98±0.14 (1.74)	7.32±0.16 (6.70)	8.53±0.12 (24.3)	8.99±0.18 (31.04)	9.46±0.22 (37.90)
Liver % change	1.74±0.45 ----	1.83±0.48 (5.17)	2.19±0.39 (25.86)	2.37±0.49 (36.20)	2.58±0.52 (48.27)	2.92±0.54 (67.81)
Kidney % change	5.58±0.57 ----	6.14±0.58 (10.03)	6.78±0.61 (21.50)	7.32±0.64 (31.18)	7.56±0.69 (35.48)	8.33±0.71 (49.28)
Muscle % change	8.72±1.03 ----	8.93±1.06 (2.40)	9.04±1.03 (3.66)	9.77±1.10 (12.04)	10.43±1.19 (19.61)	10.67±1.23 (22.36)

Values are Mean±SD of ten individual observations. Values are significant at $P< 0.05$.

Table 5: Variations in Glutathione Peroxidase in gill, liver, kidney and muscle tissues of *Catla catla* exposed to sub lethal concentration of Lihocin

Parameter	Control	Exposure periods of Lihocin				
		3d	7d	14d	30d	45d
Gill % change	2.97±0.01 ----	3.24±0.03 (9.09)	3.69±0.05 (24.2)	4.57±0.07 (53.87)	4.81±0.09 (61.95)	5.29±0.11 (78.11)
Liver % change	5.84±0.19 ----	5.96±0.14 (2.05)	6.07±0.14 (3.93)	7.83±0.18 (34.07)	8.49±0.21 (45.37)	9.32±0.27 (59.58)
Kidney % change	0.59±0.04 ----	0.65±0.04 (10.1)	0.93±0.07 (57.62)	1.02±0.10 (72.8)	1.11±0.15 (88.1)	1.14±0.16 (93.2)
Muscle % change	2.45±0.32 ----	2.69±0.36 (9.79)	2.97±0.39 (21.2)	3.64±0.41 (48.57)	3.85±0.44 (57.14)	4.21±0.44 (71.8)

Values are Mean±SD of ten individual observations. Values are significant at P < 0.05.

Table 6: Variations in CAT activity in gill, liver, kidney and muscle tissues of *Catla catla* exposed to sub lethal concentration of Lihocin

Parameters	Control	Exposure periods of Lihocin				
		3d	7d	14d	30d	45d
Gill % change	2.96±0.54 ----	2.98±0.54 (-0.67)	2.39±0.42 (-19.25)	1.86±0.30 (-37.16)	1.41±0.19 (-52.36)	0.98±0.05 (-66.89)
Liver % change	11.52±0.44 ----	10.36±0.41 (-10.06)	9.63±0.37 (-16.40)	8.49±0.21 (-26.30)	8.13±0.15 (-29.42)	7.84±0.03 (-31.94)
Kidney % change	7.76±0.36 ----	7.54±0.32 (-2.83)	6.89±0.29 (-11.21)	6.03±0.24 (-22.29)	5.73±0.21 (-26.15)	4.29±0.11 (-44.71)
Muscle % change	9.36±0.54 ----	8.92±0.49 (-4.70)	8.35±0.38 (-10.79)	7.77±0.31 (-16.98)	6.98±0.25 (-25.42)	6.03±0.19 (-35.57)

Values are Mean±SD of ten individual observations. Values are significant at P < 0.05.

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