Effects of sea horse (*Hippocampus abdominalis*)-derived protein hydrolysate on skeletal muscle development

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Abstract *Hippocampus abdominalis*, the big belly sea horse, is widely known for its medicinal value in Chinese folk medicine. In this study, extract obtained by proteolytic degradation of this species was investigated for its effects on skeletal muscle development, both *in vitro* and *in vivo*. Muscle cell lines (C₂C₁₂ and L₆) treated with the bioactive peptide did not have any detrimental effects on the cell viability, which was above 80%. Optical microscopy analysis on the morphology of the sea horse extract (SHE)-treated cells showed enhanced differentiating ability with myotube formation. Moreover, cells incubated with the hydrolysate displayed decreased proliferation rate, as recorded by the electric cell substrate impedance sensing system, thereby supporting enhanced differentiation. For a period of 12 weeks, mice models were fed with SHE and simultaneously subjected to treadmill exercise, which increased the expression of Myogenin, a key myogenic regulatory factor. In addition, there was an increase in the expression of AMPK- and Cytochrome C, both of which are important in mitochondrial biogenesis. Thus, the SHE from *Hippocampus abdominalis* can be a promising candidate as protein supplement aiding muscle development.

Keywords Bioactive peptides · Mitochondrial biogenesis · Protein hydrolysate · Protein supplement · Sea horse

Introduction

The marine ecosystem is the Earth's largest aquatic system and is the habitat for marine organisms that are highly rich in medicinal value. Protein hydrolysates, a mixture of amino acids that are the product of protein hydrolysis obtained from marine species are gaining notable attention as therapeutic resources, as these products have high nutritional and pharmaceutical significance (Bordbar et al. 2011). The complex peptide molecules obtained from these hydrolysates have gained much interest in the field of nutritional clinical medicine when compared to the administration of intact proteins and free amino acids, due to their enhanced gastrointestinal absorption ability, high digestibility and supply of di- and tri-peptides. Common preparation methods for these hydrolysates include enzymatic hydrolysis, solvent extraction, and microbial fermentation (Vijaykrishnaraj and Prabhasankar 2015; Hou et al. 2017). Other common sources for protein hydrolysates includes maize, canola, egg white and yolk albumin, casein, whey, soybean, meat and pork among others (McCarthy et al. 2013; Lene Meinert et al. 2016).

Marine protein hydrolysates from fish exhibit several bioactive roles, such as anti-hypertension, anti-oxidant, immunomodulating, anti-appetizing, antimicrobial, anti-cholesterol and opioid antagonist (Imen et al. 2015; Vincenzo et al. 2016). In addition, administration of these short range peptides increases the plasma amino acid concentration and skeletal muscle protein anabolism thereby improving muscle hypertrophy and accelerating recovery of muscle cells making them suitable for sports nutrition (Manninen 2009). This is attributed to the ability of human skeletal muscle to oxidize externally supplemented amino acids such as isoleucine, valine, glutamate, asparagine, aspartate, alanine and leucine.

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Moreover, there is repression of muscle protein degradation and an increase in the catabolic activity on carbohydrates and lipids, to supply the energy required for muscle anabolism (Eva et al. 2006; Philips 2011).

The sea horse is a marine teleost fish that is a well-known ingredient in traditional Chinese medicines, due to its pharmacological significance (Feixia et al. 2016). *Hippocampus abdominalis*, the big belly sea horse, is one of the largest sea horse species, and has great medicinal value (Perera et al. 2016; Nalae et al. 2017). The majority of studies in this species focus on biochemical analyses, immunomodulating ability, radical scavenging, steroidogenic capacity and anti-fatigue property. However, scarce data exists regarding the biological and clinical characteristics of this sea horse and its role in muscle hypertrophy.

There is a strong correlation between myogenesis and the key transcription factors known as myogenic regulatory factors (MRFs) (Pacagnelli et al. 2016). Physical exercise increases protein synthesis and energy efficiency in the body, therefore analysis of the primary MRFs such as Myf5 and MyoD involved in cell proliferation and secondary MRFs such as Myogenin (Myo G) and Mrf4, responsible for myotube formation can be used to evaluate the effect of exercise on muscle growth (Valente et al. 2013). In addition, analysis of AMPK- expression is one of the primary indicators of ATP production due to energy expenditure by a process termed mitochondrial biogenesis (Richter and Ruderman 2009).

The present work aims to characterize the effects of administering marine protein hydrolysate obtained from the sea horse *Hippocampus abdominalis* on myogenic regulatory factors during physical training, both *in vitro* and *in vivo* to be applied to the field of sports nutrition.

Materials and Methods

Cell line and culture medium

Mouse myoblast cells (C₂C₁₂ cells) and Rat myoblast cells (L₆ cells) were obtained from American Type Culture Collection (ATCC CRL-1772 and ATCC CRL-1458 respectively) and were cultured in Dulbecco's Modified Eagle Medium (Corning Cellgro, Manassas, VA, USA) supplemented with 10% Fetal Bovine Serum (heat inactivated FBS; Corning Cellgro, Manassas, VA, USA) in a 95% humidified chamber with 5% CO₂ at 37 °C. For cell differentiation studies, 2% Horse Serum Medium purchased (Sigma-Aldrich (Gyeonggi, Korea) was used.

Preparation of *H. abdominalis* protease enzymatic hydrolysates

Dried sea horse powder was provided by Haechunma Company Ltd., (Jeju, Korea). Distilled water (100 mL) was added to 5 g of powder. The solution was subjected to hydrolysis using alcalase enzyme with a substrate to enzyme ratio of 100:1 at 37 °C for 24 h. At the end of 24 h, the pH of the solution was neutralized

and the enzymes were inactivated by heating the solution to 100 °C for 10 min in a water bath. After centrifuging the solution for 20 min at 10,000 rpm, the soluble fraction was separated, freeze dried and stored at –20 °C until further use (Kim et al. 2016).

Cell viability assay

The effect of the sea horse hydrolysate on the C₂C₁₂ viabilities was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which determines viable cell count from active metabolism. Cells were treated with 0.05, 0.1, 0.2, 0.5 and 1 mg/mL hydrolysate for 24 h. At the end of treatment, 10 µL of MTT reagent (5 mg/mL in PBS) (Sigma Aldrich, Gyeonggi, Korea) -CAS: 298-93-1) was added to each well and left undisturbed until purple color precipitate was clearly observed under microscope. To this, 150 µL of dimethyl sulfoxide (DMSO) (Amresco, Solon, OH, USA) was added to each well and incubated for 30 min. Optical density was then measured at 570 nm.

Cell proliferation assay with the electric cell substrate impedance sensing (ECIS) system

Electric cell-substrate impedance sensing (ECIS) is a non-invasive biophysical approach for real time monitoring of cells in *in-vitro* conditions (Giaever and Keese 1984). This approach is based on the dielectric property of cells, which induces an increase in impedance as confluence of the cultured cells on the plate increases. C₂C₁₂ cells were seeded on electrode plates and after 24 h incubation, the cells were allowed to grown in 2% Horse serum (HS) medium containing hydrolysate concentrations of 0.05, 0.5 and 1 mg/mL. The entire electrode array was placed in a plate holder connected to the ECIS system, and cell proliferation analysis was performed using the ECIS system software (Applied Biophysics, Troy, NY, USA) (Sculcek et al. 2014). The growth curve was plotted between cell proliferation percentage and time.

Cell differentiation study

The effect of hydrolysate on cell differentiation was studied by allowing the myoblast cell line C₂C₁₂ to grow in DMEM in which 10%FBS was replaced with 2% HS with 1 mg/mL of the hydrolysate. The plate was then incubated at 37 °C and 5% CO₂ and observed under inverted microscope Olympus CKX41 (Olympus Corporation, Tokyo, Japan) once a day for the formation of myotubes. The medium in the culture plate was changed every 24 h.

In addition, cellular RNA was collected each day and analyzed by polymerase chain reaction (PCR) for the expression of transcription factors such as MyoD and MyoG, which aid in myotube formation. PCR instrument was purchased from Hangzhou Bioer Technology Co. Ltd., Zhejiang, China. Medium was removed from the cell culture dish at the end of each day followed by washing twice with 1× Phosphate Buffer Saline (PBS). Trizol (500 µL) was added to the washed cell culture dish

Table 1 RT-PCR conditions (A) Master Mix composition and (B) Reaction Condition

(A)	
Reagents	Volume 1X (μL)
Reaction buffer 5X	4
MgCl ₂ 2.5 mM	6
Reverse Transcriptase	1
dNTP	4
Test sample (RNA (2 μg) + DEPC + oligodT (1μL))	5
Total	20
(B)	
Experimental Step	Condition
Annealing	25 °C for 5 min
Extension	42 °C for 2 h
Reverse transcriptase inactivation	75 °C for 10 min
Storage	4 °C for 30 min

Table 2 PCR analysis of myogenic regulatory factors expressed in the C₂C₁₂ cells *in-vitro* after treatment with SHE. Primer sequence specifications

(A)		
Gene name		Primer Sequence (5'→3')
Myogenin	Forward	GAA GCG CAG GCT CAA GAA AG
	Reverse	GTC TGG GAA GGC AAC AGA CA
MyoD	Forward	CAT AGA CTT GAC AGG CCC CG
	Reverse	CAT TCA CTT TGC TCA GGC GG
GAPDH	Forward	TCAGATCCACGACGGACACA
	Reverse	CATCTTCCAGGAGCGAGACC

(B)	
Reagents	Volume 1X (μL)
10X buffer	3
20 mM dNTP	0.3
Forward primer (10 pmol)	0.5
Reverse primer (10 pmol)	0.5
Polymerase	0.1
DNA concentration (from RT-PCR)	1 μg
Distilled water	Make upto 30 μL
Total	30

(C)	
Experimental Steps	Experimental Conditions
	Myogenin MyoD GAPDH
Pre-Denaturation	95 °C for 5 min
Denaturation	95 °C for 45 s
Annealing	59 °C for 55 s 62 °C for 55 s 57.5 °C for 55 s
Extension	72 °C for 1 min
Final Elongation	72 °C for 2 min
PCR Cycles	35 45 35
Storage	4 °C for 30 min

and incubated at 4 °C for 20 min under shaking conditions, then collected from the dish with a cell scraper. RNA was collected by chloroform-isopropanol extraction, and purity was assessed by DU series 730 UV/Visible spectrophotometer (Beckman Coulter, Inc. Brea, CA, USA). Complementary DNA (cDNA) was synthesized with reverse transcriptase enzyme (Promega, Madison, WI, USA) from the RNA by reverse transcriptase PCR (RT-PCR) (Table 1). PCR was performed on the synthesized cDNA using FIREPol DNA polymerase (Solis Biodyne, Estonia, Northern Europe) to analyze the expression of myogenic regulatory factors as MyoG and MyoD and the loading control GAPDH. PCR conditions and primer sequences are provided in Table 2. The Table 2 provides a detail description on the PCR conditions and the sequence of the forward and reverse primers that are designed for MyoG, MyoD and GAPDH. The designed primer sequences were ordered and purchased from Genotech, Daejeon, Republic of Korea. And the PCR instrument was purchased from Hangzhou Bioer Technology Co. Ltd., Zhejiang, China.

In-vivo study

The effect of *Hippocampus* protein hydrolysate on muscle development was studied *in-vivo* using ICR mice (about 5 weeks old) purchased from Orient Bio (Orient Bio Inc., Seongnam, Gyeonggi, Korea). Animals were housed at room temperature (23±2 °C) with constant humidity (55±15%) on a 12 h light/12 h dark cycle, with free access to food and water for one week to adapt the laboratory environment. After 7 days, the mice were randomly divided into 2 groups: Exercise and Non-exercise. Further, each of these groups are divided into three categories: control group (administered PBS only), test group 1 (administered Sea Horse Extract (SHE) 50 mg/kg) and test group 2 (administered Sea Horse Extract (SHE) 200 mg/kg) for analysis of the effect of protein hydrolysate on muscle development. The mice were fed with PBS and SHE through oral route of administration. Initially the groups were fed with the hydrolysate or control for one week followed by three weeks of treadmill training. From weeks 5-12, the mice were subjected to treadmill exercise at 20 meters/min for 30 minutes once a week.

In order to study the effect of SHE together with exercise on aged group, two groups of mice (about 24 weeks old), each containing n=4 were used. With the same housing conditions, the aged group mice were subjected to treadmill at a speed of 15 m/min for 10 min once a week with SHE of 50 mg/Kg. At the end of 3 months, hind limb tissues were taken from each group, followed by evaluation of the protein expression responsible for metabolic adaptation of the muscle cells under exercise and protein supplementation by western blotting was done.

Western blot analysis

Protein was extracted from the isolated hind limb tissues with T-

per buffer (Thermo Scientific, Rockford, IL, USA). To 0.1 g tissue, 600 μ L of buffer was added and the tissue was homogenized thoroughly. The homogenate was centrifuged for 15 min at 14,000 rpm at 4 °C and the supernatant was carefully collected to avoid contamination with fat content. Protein concentration was measured by BCA assay (Pierce Biotechnology, Rockford, IL, USA). The total protein concentration in the lysates was normalized to 20 μ g per lane and proteins were separated on a 10-12% sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis (SDS-PAGE) by loading equi-volume (18 μ L) of total protein in the gel. SDS was purchased from Biosesang, Gyeonggi, Korea. Proteins separated by SDS-PAGE were transferred to nitrocellulose blotting membrane (GE Healthcare, Life Science, German, UK) for 90 minutes at 250 mA followed by blocking with 5% nonfat dry milk dissolved in of tris-buffered saline (TBS) and Polysorbate 20 (Tween 20) (TTBS) at 4 for overnight. Membranes were incubated with the following primary antibodies: MyoD (1:200), MyoG (1:500), AMPK- α (1:1000), UCP-3 (1:1000), Cyt C (1:1000), AKT (1:1000), mTOR (1:1000) and β -actin (1:1000) as the loading control. All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, Santa Cruz, USA). After incubation with the primary antibody, membranes were washed with 1X TTBS followed by 1 h incubation with secondary antibody raised against corresponding primary antibody. The concentration of the secondary antibodies is as follows: Rabbit (1:20,000), Mouse (1:20,000) and Goat (1:40,000). Blots were washed and then developed using an enhanced chemiluminescence ECL kit (LPS solution, Daejeon, Korea). Bands were quantified using ImageJ software (<https://imagej.nih.gov/ij/>).

Statistical Analysis

All data herein are expressed as mean $\pm$ SD. The quantified data of the bands obtained through PCR and western blotting

techniques using ImageJ software were entered into a Microsoft Excel spreadsheet. The significant difference between the expression of the proteins from all the mice groups (Control, SHE50 mg/Kg and SHE200 mg/Kg of each exercise and non-exercise batch) were evaluated using independent *t*-test analysis with cut-off significance less than 0.05.

Results/Discussion

The effect of protein hydrolysate on myoblast viability was evaluated using the MTT assay with two different cell lines- C_2C_{12} (mouse myoblast cells) and L_6 (rat myoblast cells). In all cases, the cell viability was above 80%. 1mg/mL of the protein hydrolysate had the most significant effect on the C_2C_{12} cell viability followed by 0.5 mg/mL as seen from the Fig. 1. Concentrations between 0.05-0.2 mg/mL hydrolysate had a slightly lower cell viability compared to the untreated C_2C_{12} cell lines; whereas L_6 cells treated with 0.05-1 mg/mL of the protein hydrolysate has lower viability than the untreated L_6 control group thus making them less compatible with the SHE in terms of cell growth. The decrease in the viability might be attributed to slower cellular proliferation in the presence of these hydrolysates.

The effect of SHE on the differentiation of muscle cells was also evaluated and is shown in Fig. 2. Cells were cultured with 2% HS in DMEM and the medium was changed every 24 h. The impedance-based analysis on the proliferation of anchorage dependent myoblast cells under the influence of different concentrations of SHE hydrolysate and differentiation medium indicated that 1 mg/mL of SHE hydrolysate significantly slowed proliferation and differentiation of the muscle cells. Thus, further experiments were conducted to analyze the morphology of the differentiating muscle cells *in-vitro* under the influence of SHE hydrolysate at a concentration of 1 mg/mL.

Differentiation of myoblasts begins after 5 days, when the

Fig. 1 Cell Viability analysis using MTT assay on the effect of the sea horse extract on two different cell lines. (A) C_2C_{12} and (B) L_6 . Cells were treated with various concentrations of the protein hydrolysate for 24 h followed by incubation with MTT reagent. By solubilizing the formazan with DMSO, OD_{570 nm} was measured (where n, the number of replicates =6) and the results were plotted

Fig. 2 Dose dependent effect of sea horse derived protein hydrolysate on proliferation resistance of C_2C_{12} cells. Cells grown on an electrode array in the presence of 2% horse serum medium were subjected to electric cell substrate impedance sensing (ECIS) system, to study the proliferative ability of the cells under the influence of various concentrations of SHE hydrolysate. The results were plotted with proliferation % versus time

Fig. 3 Effect of SHE on differentiation of C_2C_{12} cell lines on consecutive days of SHE treatment. (A) Morphology analysis using optical microscope after culturing the cells in 2% horse serum medium containing 1 mg/mL SHE (Day 1&3: 100X magnification; Day 5,7&9: 200X magnification). (B) RNA expression of myogenic regulatory factors (MRFs) using PCR. Cells were collected every day and RNA was extracted using Trizol. (C) Graph indicating the relative expression of MRFs with respect to the loading control GAPDH. The bands from the gel electrophoresis of the PCR products were analyzed using ImageJ software and the graph was plotted. The results are presented as mean $\pm$ SD where the significance of the result is $p < 0.05$ compared to the control

singly nucleated myoblast cells elongate and fuses with each other to form multinucleated myotubes in a process termed myogenesis (Teng et al. 2015). From the Fig. 3A, it is observed that a well differentiated muscle cell milieu was observed on day 9. Fig. 3B and 3C shows the analysis of the mRNA expression of myogenic

regulatory factors from the control and SHE administered test group on the corresponding days indicating an increase in the level of MyoD, a primary regulatory factor responsible for directing proliferating myogenic satellite cells towards a myogenic lineage, and Myogenin, a secondary regulatory factor controlling

Fig. 4 Effect of 50mg/Kg SHE on ICR mice *in-vivo* after exercise. (A) The treadmill running exercise machine (B). Area of skeletal muscle tissue excised from the animal for further analysis

differentiation leading to the formation of myofibers. The increase in the transcription of both MyoD and Myogenin plays a role in promoting regeneration of muscle fibers (Borselli et al. 2010). Thus the ECIS result was consistent with the morphology analysis of the cultured cells under optical microscopy along with evaluation of mRNA expression of the transcriptional markers of proliferation and differentiation.

The effect of SHE hydrolysate was also evaluated *in-vivo* in mice. Fig. 4A shows the treadmill machine used in the study. After 10 weeks of treadmill exercise, muscle tissue from the hind limb (given in Fig. 4B) of both the groups was collected and their protein extracts were analyzed for the effect of hydrolysate and exercise on muscle development. Western blot analysis for the proteins extracted from muscle tissue given in Figs. 5A, B, C corresponds to the young mice non-exercise group, young mice-exercise group and old mice-exercise group respectively.

Dietary supplementation of protein along with exercise has been expected to impose positive impact on muscle remodeling by promoting muscle hypertrophy (Schoenfeld et al. 2013). Proteins extracted from the hind limb muscle tissue of the exercise and non-exercise mice groups were subjected to western blot experiment and analyzed for the protein factors involved in adaptive response towards the treadmill aerobic exercise.

The efficiency of the administered SHE together with the treadmilling in inducing myofibre maturation and muscle development is analysed by studying the expression of myogenic regulatory factors, MyoD and MyoG. MyoD, a muscle cell

proliferation marker is responsible for inducing the expression of MyoG, which in turn results in well differentiated and matured muscle myofibers. An increased expression is observed with both the MyoD and MyoG proteins in the SHE administered exercise group than their corresponding non-exercise and control group.

Increase in expression of the two protein kinases -AKT and mTOR- which contributes to the maintenance of body muscle, is observed in the SHE treated-exercise mice group when comparing the SHE treated non-exercise mice group and their corresponding control to which SHE is not administered, in both the groups. It is seen from the literatures (Fernandes et al. 2012) that, the activation and stimulation of mTOR results as a consequence of its upstream signalling pathway PI3K/AKT/mTOR. Muscle contraction and cellular stress experienced during the treadmill process stimulates the AKT signalling pathway. Upon stimulation, AKT, a regulator of glucose homeostasis, phosphorylates mTOR thereby increasing muscle protein synthesis and muscle hypertrophy, as a result of which increase in muscle mass is expected. Further, Phosphorylated AKT is said to induce lysosomal degradation of SIRT1 by translocating it to the cytosolic region from the nucleus, by which antioxidant property of the cell is enhanced (Raja et al. 2017). Previous reports states that, AKT, in addition to mTOR, phosphorylates wide range of proteins that includes FOXO. The phosphorylated FOXO exits the nucleus and fails to activate a major E3 ligase, atrogin-1, thus protecting the muscle protein from degradation (Paul et al. 2016). Both the increase in the synthesis and decrease in degradation of the muscle protein is said to enhance the myofibrillar protein content and muscle mass.

Undergoing physical activities/exercises tends to develop oxidative stress in the muscle regions. In addition, there creates a ATP deprivation in the cells with increase in AMP:ATP ratio. All these events lead to activation of AMPK, a energy sensor of the body, which upon phosphorylation restores the ATP level by phosphorylating peroxisome proliferator activated receptor γ -co-activator 1 α (PGC-1 α), a crucial regulator of mitochondrial biogenesis (Wengong et al. 2003; Collu-Marchese et al. 2015; Guadalupe-Grau et al. 2017). Activated PGC-1 allows mitochondria to get adapted to the stress condition imposed by increased energy expenditure, as observed in exercise, thereby maintaining them in functional state. Further, activated AMPK increases catabolic fatty acid oxidation and glucose uptake for poising the astrayed energy balance, besides downregulating the anabolic processes such as fatty acid synthesis and gluconeogenesis, a process in which glucose is generated, thereby increasing ATP generation and decreasing ATP consumption respectively.

Another mitochondrial biomarker, Cytochrome C (Cyt C) which quantifies the mitochondrial content and the mitochondrial DNA (mtDNA) is said to increase 2 to 3 fold during exercise (Hollooszy 1967; Terjung et al. 1973). Herein, Cyt C expression in the SHE administered exercise group is higher than the corresponding control in the same group, indicating that protein supplementation together with the aerobic treadmilling has a

Fig. 5 Western blots and quantification graph for the proteins extracted from hind limb muscle of the mice in (A) Young mice non-exercise group (B) Young mice-exercise group (C) Old mice-exercise group. Proteins were extracted from the excised muscle tissues and are separated by performing SDS-PAGE. Primary and secondary antibody treatments were done against the factors associated with mitochondrial biogenesis and the proteins were detected using ECL solution. The bands were analyzed with ImageJ software and the data are plotted as mean $\pm$ Standard error. p -value <0.05 (vs. Control) are considered statistically significant and are marked using * in the graph

synergistic effect on mitochondrial biogenesis. This increased expression level of the mitochondrial marker supports the fact that PGC-1 α , a downstream signalling molecule in the AMPK- α activation pathway has induced the mitochondrial biogenesis.

Nutrient depletion is said to increase the expression level of SIRT-1, a NAD⁺ dependant deacetylase protein, thereby reducing muscle atrophy under energy expenditure/starvation (Gao et al. 2014). In the present study, both the SHE administered mice groups -exercise and non-exercise- showed lower SIRT-1 expression level than their respective control groups, indicating that the protein supplement provides enough nutrients to the muscle tissue, thereby blocking them towards entering nutrition deprivation state (Data not provided in the figure).

Increased expression of UCP3, a mitochondrial anion carrier protein, is observed in the exercise group than the non-exercise group and their corresponding control groups, which accounts for lowering the mitochondrial membrane potential together with reducing the stress imposed by overload of fatty acid in the muscle cells, thereby overcoming the dreadful effects of ROS accumulation (Patrick et al. 2006; Busiello et al. 2015). The primary role of UCP3 in skeletal muscle is to protect the mitochondria by making them metabolically adapt to the stress induced during exercise, thereby supporting ATP synthesis and energy partitioning (Miranda and Joris 2008).

Aging results in muscle atrophy and decrease in muscle strength as a result of reduction in the number and size of the muscle fibers and loss of muscle motors (Keller and Engelhardt 2013). Thus the effect of SHE (50 mg/Kg per mice) to 24 week old mice along with treadmill exercise has been subjected to study. An increase in expression of AKT and mTOR is observed in the SHE administered exercise group when comparing the control, where SHE is not provided. It is earlier reported that increase in expression of AKT and mTOR after exercise results in enhanced myofibrillar protein synthesis along with muscle hypertrophy leading to increased muscle mass. Also the myogenic regulatory factors, MyoD and MyoG is found to be enhanced in the test group, where SHE administration along with aerobic treadmill, than the control. Though there is no increase in the mitochondrial biogenesis, as there is no increase in the expression of mitochondrial biomarkers, AMPK- and Cytochrome C, yet the increase in expression of UCP3 accounts for the mitochondrial protection against the stress created during treadmill onto the muscle fibres. Thus, SHE administration along with mild exercise in the old mice group results in better outcome, wherein muscle atrophy can be overcome thereby making it suitable as a protein supplement to old age people.

Thus, upon comparing the protein expression from the hind limb muscle tissue of the exercise and non-exercise with their corresponding subgroup of SHE administered test group and SHE non-administered control group, it is revealed that together with exercising, SHE protein supplementation is a promising agent for enhanced muscle protein synthesis thereby finding its way to be

dietary food supplement for endurance trainers in muscle hypertrophy.

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