

# Assessing the Efficacy of Eufortyn - A Terclatrated CoQ10 and Creatine Combination Therapy on the Aging Rat Model

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With the progression of age, the organism enters a highly oxidized state resulting from impaired mitochondrial function. As the susceptibility to reactive oxygen species heightens, a decline of both size and number of skeletal muscle fibers manifests in chronic fatigue syndrome known as sarcopenia in the elderly. Consumption of antioxidant as part of a diet may help regulate oxidative damage by reducing the rampant activity of reactive oxygen species in the cell, yet the poor bioavailability of most commercial supplements limit the capacity of the antioxidant to quench free-radicals. Eufortyn is a terclatrated formulation of Coenzyme Q10, creatine, and ginseng extract that retains the integrity of the antioxidant moiety, while allowing maximal absorption into the intestinal mucosa. A Eufortyn pellet was fed orally to 19 and 27-month old Fischer 344 x BNF1 rats for six weeks. Grip strength improved (12%) in 19-mos, but not in 27-mos old rats. Water maze analysis exhibited sharpened cognitive performance in Eufortyn-treated old-age rats compared to their age-match peers. Significant advancement in mitochondrial calcium retention capacity (66% in 19-mos and 19% in 27-mos), diminished non-heme iron levels (54% in 27-mos), and suppressed nucleic acid oxidation (31% in 19-mos and 24% in 27-mos) were observed in skeletal muscle in Eufortyn-treated cohorts. These findings indicate that treatment is more effective in the middle-aged animals in comparison to the 27-month old rats suggesting that intervention with Eufortyn needs to be initiated at an earlier age than 27-month old to see optimal effects.

## Introduction

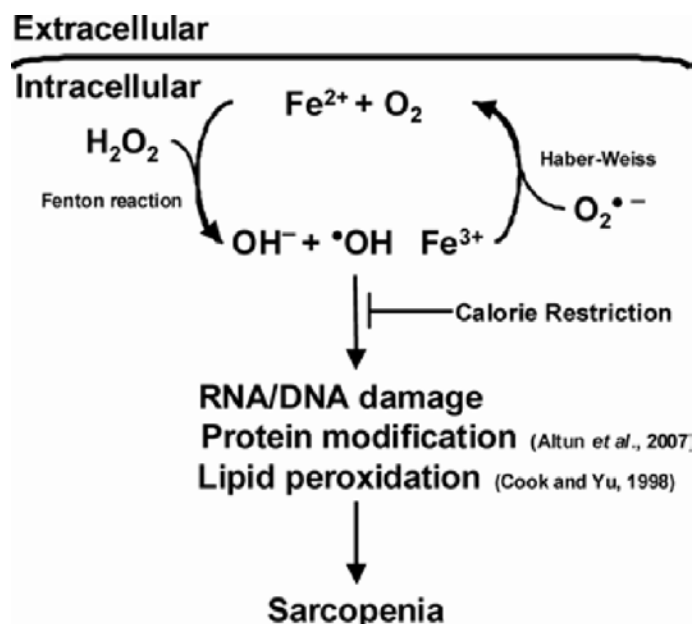
Aging is a natural, degenerative process occurring throughout the life cycle of all organisms. In the elderly, chronic fatigue syndrome is an expected, yet degenerative symptom of aging evidenced by the decline of muscle fibers, diminished cognitive function resulting in the departure from a self-sufficient lifestyle<sup>1</sup>. The impairment of mitochondrial bioenergetics is theorized to be the central mechanism behind tissue dysfunction, fatigue and aging<sup>2,3</sup>. In normal aging, cellular matter undergoes a highly regulated form of cell death characterized by morphological, biochemical, and molecular events referred to as apoptosis<sup>4-7</sup>. When apoptosis afflicts skeletal muscle, the decline in both the size and number of both kinds of muscle fibers, particularly the Type II (fast-twitch fiber) contributes to the condition known as sarcopenia. Sarcopenia is a debilitating condition commonly associated with diminished muscle mass, strength and increased disability and dependence<sup>8</sup>. Apoptosis, in healthy cells, allows for turnover and removal of defective cellular matter as a way to promote tissue homeostasis. Regrettably, once apoptosis strikes post-mitotic cells (skeletal muscle fibers), regeneration is not possible. Thus,

progression of apoptosis is linked to the increasing frailty experienced in aging. Being that apoptosis is strictly regulated by controlled signaling pathways<sup>1</sup>, it is apparent that any oxidative damage afflicting internal cellular homeostasis will inevitably lead the cell to programmed death. The central theory of aging states that mitochondrial free-radical formation destabilizes the internal cellular balance because endogenously occurring antioxidants fail to manage the rampant pro-oxidant activity<sup>9</sup>.

## Free-Radical Chemistry

Senescence is tied to the accrual of tissue damage by free-radicals that results from a marked shift in pro-oxidant activity<sup>10,11</sup>. Normally, there is a balance between pro-oxidant generation and anti-oxidant defense. However, in aging, the ratio of pro-oxidants increases relative to the endogenously occurring anti-oxidant activity<sup>12</sup>. The free-radical formation pathway begins in the mitochondria of the skeletal muscle where superoxide anion ( $O_2^{\cdot-}$ ) and hydrogen peroxide ( $H_2O_2$ ) diffuse in and out of skeletal muscle cells. When ferrous ( $Fe^{2+}$ ) iron, a pro-oxidant, reacts with diatomic oxygen, the produced  $H_2O_2$  is converted to the highly unstable hydroxyl radical ( $\cdot OH$ ) via Fenton chemistry (Fig. 1). Additionally, ferric ( $Fe^{3+}$ ) iron can in turn react with hydroxyl radical and hydroxide ion to further contribute to overall superoxide ion concentration via Haber-Weiss reactions<sup>13</sup>. The resulting oxidation is

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**Figure 1: Intracellular Free-Radical Generation Cycle.** The release of iron from heme-protein or ferritin protein via  $H_2O_2$  and superoxide ion results in free iron capable of reacting with diatomic oxygen to generate the highly-reactive hydroxyl radical via Fenton chemistry. This free-radical, in turn, has the capacity to alter the physiological structure of nucleic acids, proteins, and lipids, thus compromising the integrity of the cell. This ultimately leads to cellular apoptosis – a widely recognized contributor of sarcopenia.

then at liberty to modify the delicate structure of nucleic acids, lipids, and proteins<sup>14, 15</sup>. Cell death, the theorized culprit behind degenerative disease, is facilitated by the compromised integrity of the cell.

### Coenzyme Q10

Naturally present within the inner mitochondrial membranes is Coenzyme Q10 (CoQ10), a critical biomolecule crucial to ATP synthesis. Functioning as a ubiquinone and an important Complex II electron carrier in the mitochondrial electron transport chain (ETC), CoQ10 is a powerful antioxidant that scavenges free-radicals and thus, maintains tissue health, potentiates cell growth, and enhances vigor<sup>16</sup>. Comparative studies on various mammalian species have observed an inverse relationship between the generation of super oxide anion radical and sub mitochondrial CoQ10 content, implying the effective antioxidant properties of CoQ10<sup>16</sup>. Another study investigated age-related changes in lipid peroxidation and functionality of liver and skeletal-muscle mitochondria in rats fed a diet rich in polyunsaturated fatty acids that was either supplemented or not with CoQ10. Results for the

supplemented groups showed a decrease in peroxidizability index, an increase in catalase activity in skeletal muscle, and modulation of the age-related changes in the mitochondrial ETC components. The shifts in these biomarkers from CoQ10 supplementation suggest key underlying mechanisms associated with the age-delaying properties of CoQ10<sup>17</sup>.

### Creatine Monohydrate

Creatine monohydrate (C) is a high energy compound that anaerobically recycles ATP during intense muscular exertion and is therefore concentrated in fast-twitch (Type IIB fiber) muscle. Synthesized in the liver, pancreas, and kidneys, creatine is transported in the bloodstream to muscle cells where 95% of all creatine is found. Supplementation can improve stores of phosphocreatines and thus optimize muscular output during periods of high-intensity exercise as well as lessen recovery time thereafter<sup>18</sup>. Recent studies suggested that creatine aids patients suffering from muscular dystrophy as well as attenuate sarcopenia by rehabilitating disuse atrophy<sup>19</sup>. Resistance training for the elderly has proved beneficial, yet some muscular loss is still problematic in the elderly; a lack of a nutritional component may be the culprit. Creatine supplementation has the potential to override muscle atrophy during resistance training, although the mechanism for its ergogenic effect is unclear<sup>20</sup>. Additionally, creatine decreases cytoplasm  $Ca^{2+}$  levels and increases phosphocreatine stores intramuscularly which allows for possible musculoskeletal effects, including cellular hydration, increases in myogenic transcription factors, and up-regulation of myosin heavy chains possibly involved in muscle hypertrophy<sup>19</sup>.

### Ginseng Extract

Ginseng extract is regarded widely as an effective adaptogen - a natural herb product used to increase an organism's resistance to stress, trauma, anxiety, and fatigue<sup>21</sup>. Prolonged administration of standardized ginseng to rats reduced oxidative stress in certain tissues by modifying specific antioxidant enzyme activities that are required to eliminate free radicals, thus mitigating tissue peroxidation end-products<sup>22, 23</sup>. Some earlier studies reported greater oxygen uptake and transport in elderly subjects as well as enhanced energy levels in athletes.

### Recognizing the Need for Bioavailable Combination Therapy

With the progression of age, the susceptibility to reactive oxygen species expedites the deterioration of

skeletal muscle fibers<sup>24, 25</sup>. The multitude of degenerative processes that influence the aging process manifest in the total reduction of slow oxidative muscle fibers (Type I muscle) and fast glycolytic fibers (Type IIB muscle)<sup>8, 26</sup>. It is hypothesized that the specific combination of CoQ10 and creatine may attenuate the deterioration of both Type I/IIB fibers and provide a therapeutic effect<sup>16, 27</sup>. In aging studies, oxidative stress is a major factor in diminishing integrity of functional tissue by destroying nucleic acids coding for appropriate proteins needed to build those tissues. When considering the amount of antioxidants a normal individual would assimilate from diet alone, this amount appears to be deficient in producing changes in the progression of tissue oxidation. Antioxidant supplements help regulate oxidative damage by reducing the unbridled activity of reactive oxygen species in the cell. However, most over-the-counter products have poor bioavailability when taken in a standard-quality, generic form, thus their effects go unnoticed in the organism. Despite the benefits associated with CoQ10 supplementation, its slow and ineffective absorption into the body due to its lipophilic nature tends to be the major obstacle in getting access to the nutrient through ingestion<sup>28</sup>. This very problem has been addressed by biochemist in their development of the innovative Eufortyn compound (Scharper Company, Milan, Italy), which is mainly comprised of CoQ10, creatine, and ginseng extract. Eufortyn's unique terclatrated structural composition makes it a highly soluble, multicomposite entity while maintaining the integrity of the CoQ10 moiety, thereby greatly increasing absorption into the mucosa.

### **Unique Properties of Eufortyn®**

Clatration (from the latin word, *clatrum*, i.e. cage) uses mechanical energy to create a multicomposite substance; that is, a combination of two or more chemical entities that interact with each other without modifying their respective physio-chemical properties, but end up with physical characteristics that are unique to that multicomposite. Clatration can be used when certain physical properties, considered to be restrictive for pharmaceutical purposes, need to be improved. According to Giorgio Bianchi, Ph.D, a leading development expert of Qter®, a terclatrate (ie, three-in-a-cage) designates a multicomposite arising from delivering mechanical energy to three moieties:

1. A biologically active substance whose physical properties need to be modified.
2. A pharmacologically inactive polymer acting as a passive matrix that traps single moieties of the active ingredient inside each of its loops, thus preventing the trapped moieties from getting in contact and interacting with each other.

3. A small molecule that acts as a catalyst for the formation of the clatrate, enabling clatration to occur at room temperature thus, keeping chemical reactions from occurring between the components of the multicomposite.

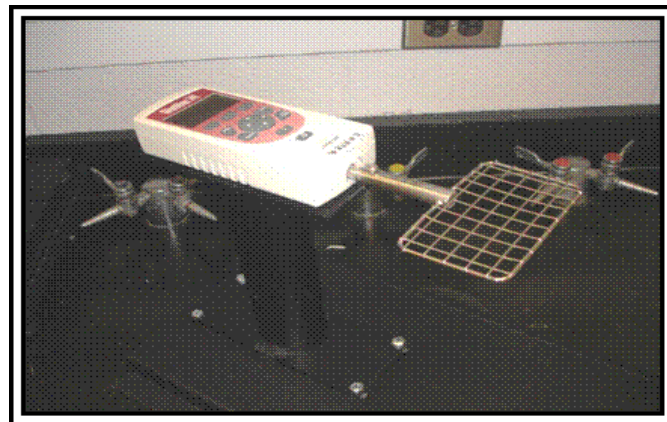
Qter® is a terclatrate multicomposite in which the active moiety is coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>), the inactive polymer is a commonly used pharmaceutical excipient, and the catalyst is a naturally occurring amino-acid. Native CoQ<sub>10</sub> has poor water solubility as a substance, forming a waxy and highly electrostatic powder: impossible to be administered by intravenous route, poorly absorbed by the GI mucosa when orally administered, and troublesome in the industrial pharmaceutical setting. Qter® is aimed at overcoming these limitations, by trapping CoQ<sub>10</sub> in the passive matrix; electrostatic interactions between moieties are prevented, and a finely dispersible, water soluble powder is obtained, showing a fairly improved bio-availability profile in humans, when compared to native CoQ<sub>10</sub>.

### **Materials and Methods**

The Eufortyn pilot was conducted in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. In an effort to examine the efficacy of Eufortyn on the physical, cognitive and biochemical aspects of an organism, the combined therapy (Eufortyn) was fed for 6 weeks to male Fischer 344 x Brown Norway rats obtained from the National Institute of Aging colony (Harlan Sprague Dawley, Indianapolis, IN) at 6, 19, and 27 months of age. The F344xBN is an excellent model often used for aging research. The stock and strain of these hybrid models have been characterized under well-defined environmental and genetic conditions. All animals were singly housed in a temperature (20±2.5°C) and light-controlled (12:12h light-dark cycle) environment. The animals were provided with water, food (*ad libitum* NIH31 pellets #7017 and a daily Eufortyn pellet dosed at either 938 mg/tab or 375 mg/tab depending on rodent weight at time of feeding), and were given two week acclimation time before the experiments began. Animals with documented pathology were not included in the final analyses. All three age groups were randomly selected into either control or experimental groups (C6, C19, E19, C27, or E27) followed by a further separation into one of four cohorts (Table 1), which were then staggered throughout the treatment process to allow for sufficient time to perform all necessary physical and cognitive analysis prior to sacrifice and tissue extraction. Eufortyn treatment began in line with pilot schedule (Fig. 2). No treatments were given to these animals so as to limit confounding variables in tissue biochemistry analysis. Animals were euthanized by decapitation using a dedicated

Groups	Control	Control	Eufortyn	Control	Eufortyn
Age (months)	6	19	19	27	27
Rats	7	8	8	7	7

**Table 1: Eufortyn Pilot Animal Distribution.** Randomized selection of animals into experimental and control groups followed by placement into cohorts C6, C19, E19, C27 or E27.



**Figure 3: Grip Strength Analysis.** The grip strength apparatus measured forelimb strength and is predictive of future physical disability. Grip strength results were expressed as total grip strength force (kg of force) and total force divided by body weight (kg of force/kg body weight).

	Week	Cohort 1	Cohort 2	Cohort 3	Cohort 4
Arrival/No Treatment	1				
No Treatment	2				
Treatment	3				
Treatment	4				
Treatment	5				
Treatment	6				
Physical Study/Treatment	7				
Treatment	8				
Sacrifice	9				
	10				
	11				
	12				
	13				
	14				
	15				

**Figure 2: Eufortyn Pilot Study Design Plan.** Cohorts were staggered so as to allow for sufficient assimilation, treatment, physical study, and sacrifice time.

guillotine to avoid interference of anesthesia on mitochondrial functions. All discomfort, distress, pain, or injury was accounted for in this pilot study. Select muscle (gastrocnemius, plantaris, soleus, EDL, and quadriceps) and organ tissue (heart, kidney, liver, and brain) were extracted fresh, weighed, and flash-frozen in liquid nitrogen followed by storage in -80°C until analysis. These methods were all consistent with the recommendation of the Panel of Euthanasia of the American Veterinary Medical Association ©.

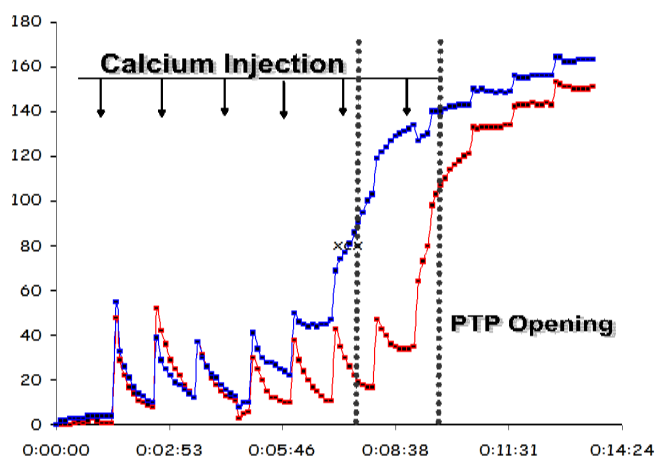
**Grip strength-behavioral/functional testing**

Five weeks into treatment, functionality analyses of the forelimb grip were assessed to determine treatment effect on retention of musculoskeletal performance of the three age groups. An automated grip strength meter was used as a standard measuring device for all animals (Fig. 3). The mean force in grams was determined with a computerized electronic pull strain gauge fitted directly to the grasping ring. To normalize, the resulting measurement would then

be divided by body mass. After 3 successful trials were conducted, an average was taken to determine the final outcome.

**Permeability Transition Pore**

In aging, progressive deregulation of the mitochondria organelle leads to decreased life-sustaining functions such as ATP production, intracellular Ca<sup>2+</sup> buffering, and regulation of cellular redox balance and apoptosis<sup>29, 30</sup>. Additionally, increases in non-heme iron positively correlate with age-related mRNA oxidative damage, diminishing mitochondrial capacity to handle influxes of Ca<sup>2+</sup> in skeletal muscle. This theory is central to the idea that the pro-oxidant effects of non-heme iron influences mitochondrial impermanence thus, triggering cellular apoptosis and the overall dilemma of neuromuscular degeneration<sup>31</sup>. In a highly oxidized state, such as in senescence, mitochondrial integrity is compromised by its inability to tolerate membrane impermeable calcium uptakes before opening and releasing cytotoxic factors. This mitochondrial suicide is catalyzed by the permeability transition pore (mPTP), a voltage-dependent, high-conductance, non-specific passive pore that infiltrates the mitochondrial matrix and the outer and inner mitochondrial membranes. The opening of mPTP compromises mitochondrial membrane potential and leads to a transition in membrane permeability<sup>32</sup>. The negative relationship between Ca<sup>2+</sup>-retention capacity and iron contents implies that higher iron content renders the mitochondria more susceptible to Ca<sup>2+</sup>-induced mPTP opening and thus, apoptosis<sup>33</sup>. The mPTP of both control and Eufortyn

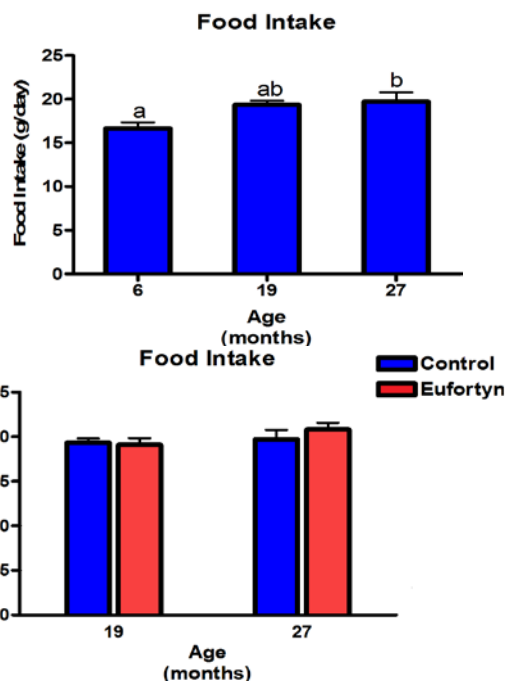


**Figure 4: Example of mitochondrial permeability transition pore.** Mitochondria were energized with succinate and a known amount of  $\text{Ca}^{2+}$  ( $10 \mu\text{M Ca}^{2+}$ ; Calcium Green 5N) was added stepwise after each minute, reflective of fluorescence pulses. The increase of fluorescence is directly linked to an increase of extra-mitochondrial  $\text{Ca}^{2+}$  and the reduction in fluorescence pulses shows the uptake of calcium by the mitochondria. The total content of calcium intake prior to PTP is used as data for PTP. The control data points (blue) reach maximum capacity prematurely as indicated by the expulsion of mitochondrial contents whereas the Eufortyn treated mitochondria (red) indicate imperviousness to additional  $\text{Ca}^{2+}$  at the same point, opening substantially later in the injection process.

samples were infused with Calcium Green – 5N and the uptakes were measured using micro plate reader with automatic injectors (Fig. 4).

### Non-heme iron assay

In homeostatic biological organisms, free-radical formation is a natural, continuous phenomenon and is typically kept in check by endogenously occurring antioxidants localized in specific areas of the skeletal muscle<sup>9</sup>. Despite the unremitting scavenging of free-radicals by antioxidants, oxidant production in aging may surpass the capability of antioxidants to fully shield oxidants from incurring extensive oxidative damage on the muscle cell<sup>12</sup>. Iron is widely recognized as a powerful pro-oxidant that catalyzes the formation of free radicals within cells. Despite a natural tendency to form free-radicals, iron may inflict further oxidation creating a summation effect of oxidation<sup>31</sup>. Conducting the non-heme assay provided insight into non-heme iron accumulation in muscle tissue with normal aging as well as non-heme iron status in Eufortyn-treated muscle tissue. Gastrocnemius muscle non-heme iron content was measured after performing an iron assay described by Xu et al.



**Figure 5: Food Intake Trends.** Aging effect on food intake of control diet age-cohort animals (left panel). Eufortyn-effect on food intake of age-match groups.

### RNA and DNA oxidation measurement using HPLC-ECD

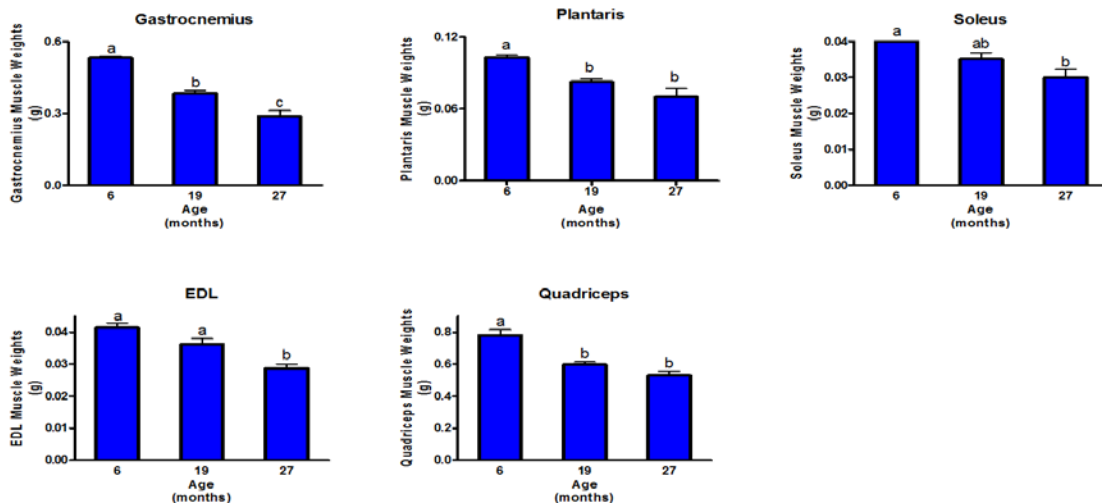
Previously, studies have observed a strong correlation between state of oxidation and mitochondrial levels of iron. It is hypothesized that the antioxidant properties of Eufortyn may have mediated some of the effects of non-heme mitochondrial iron on vulnerable nucleic acids resulting in greater longevity of the organelle in an aging organism. Investigation of the oxidation status of muscle tissue established the intracellular role of Eufortyn on pro-oxidant regulation. Total RNA and DNA oxidation levels and RNA/DNA ratios of plantaris muscles were analyzed using a novel HPLC-ECD method<sup>34</sup>.

### Results

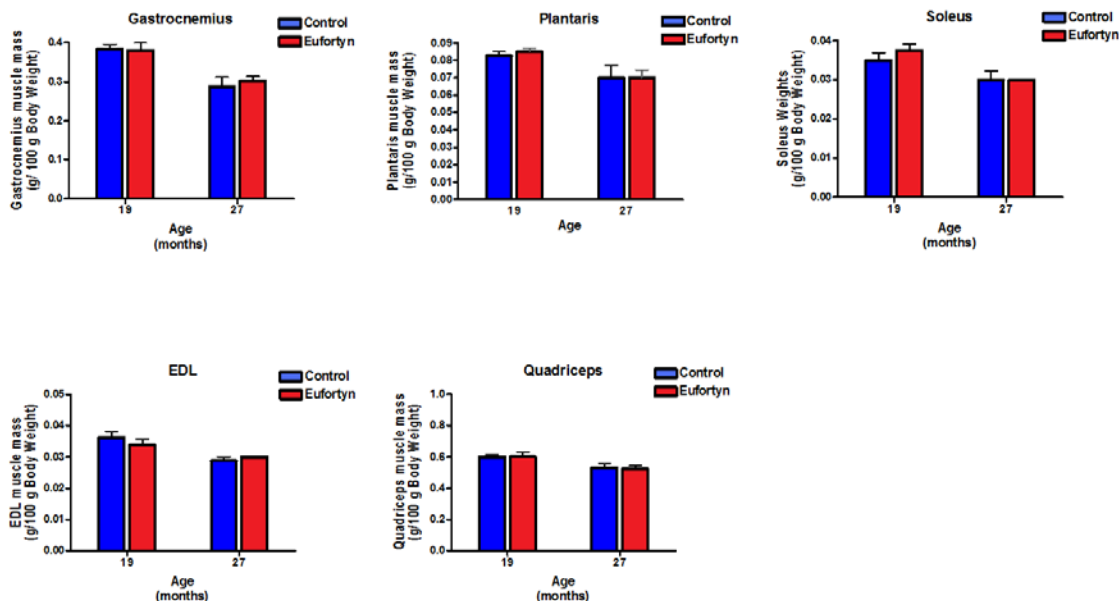
Eufortyn supplementation during the 6-weeks showed a significant increased in body weight between 6 and 19 months of age, but did not show a further increase at 27 months of age. No adverse changes in body weight were experienced by any of the groups. Food intake increased in relation to age-cohorts; both 19-month and 27-month old animals consumed greater amounts of food as compared to 6-month old controls. There was no significant difference in food intake between Eufortyn groups and age-matched control groups in the animals at 19 and 27 months of age (Fig. 5). Muscle weight in the gastrocnemius, plantaris,

soleus, extensor digitorum longus (EDL), and quadriceps muscle of the 19-month and 27-month old animals progressively decreased in contrast with the 6-month old rats (Fig. 6). No significant difference was seen in all muscles weights when comparing age-matched controls

with Eufortyn groups (Fig. 7). Heart weight, kidney weight and liver weight of the 19-month and 27-month old rats gradually increased with age as compared to the 6-month old animals. In contrast, brain weight did not change with age.



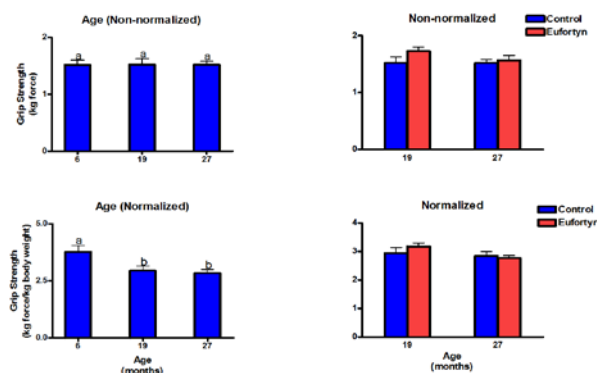
**Figure 6: Control Animal Muscle Tissue Weights.** Aging effect on various muscle groups of control diet age-cohorts.



**Figure 7: Treatment Effect On Muscle Tissue.** Eufortyn effect on various muscle groups consisting primarily from Type II skeletal muscle tissue. Supplementation with Eufortyn did not affect muscle tissue weights in either cohort.

### Grip Strength Analysis

Grip strength results were expressed as total grip strength force (kg of force) and total force divided by body weight (kg of force/kg body weight) (Fig. 8). Control cohorts expressed diminished grip strength capabilities with age when the kg force/kg body weight were normalized with body weight of the animal. The Eufortyn treated cohorts did not exhibit significantly

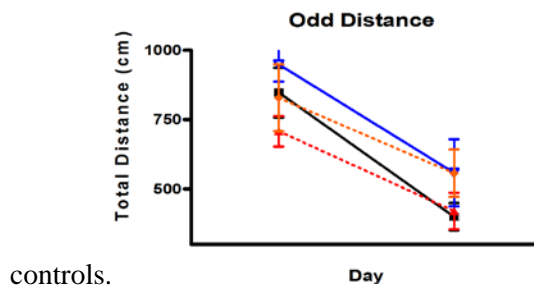


**Figure 8: Grip Strength Analysis.** Absolute grip strength force did not change with age (Fig 8A upper Panel). In contrast, grip strength expressed by body weight showed a decline in the 19-month and 27-month old animals as compared to the 6-month old controls (Fig 8A lower Panel). There was a marked increase (12%) in total grip strength force for the 19-month old Eufortyn group as compared to their age-matched controls after 4-weeks of treatment with Eufortyn (Fig 8B upper panel). In addition, forces normalized by body weight showed a similar (14%) increase in the 19-month old Eufortyn treated group (Fig 8B lower panel). The older 27-month old cohorts exhibited no comparable difference due to Eufortyn treatment.

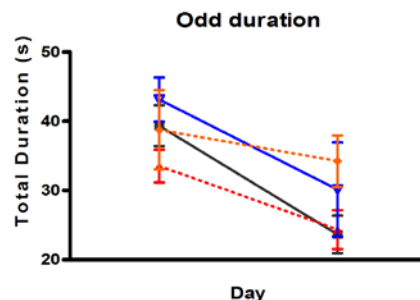
improved strength, however, there was a slight improvement (12%) in total grip strength for the 19-month old Eufortyn cohort compared to their control counterparts. Eufortyn produced no distinguishable effects for the 27-month old cohorts in terms of grip strength.

### Morris Water Maze Spatial Discrimination

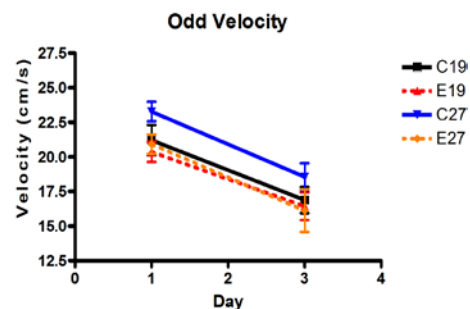
Initial (day 1) and terminal learning performance (day 3) using the distance measure were determined. A significant effect of both day ( $p < 0.001$ ) and age ( $p = 0.02$ ) was seen in that all groups improved between initial and terminal learning and this effect was larger in the younger versus older animals (Fig. 9). Though, there was no significance observed in either aging or treatment effect, there was a promising trend observed for the initial (day 1) trial of the



controls. **Figure 9:** Distance in maze measurement



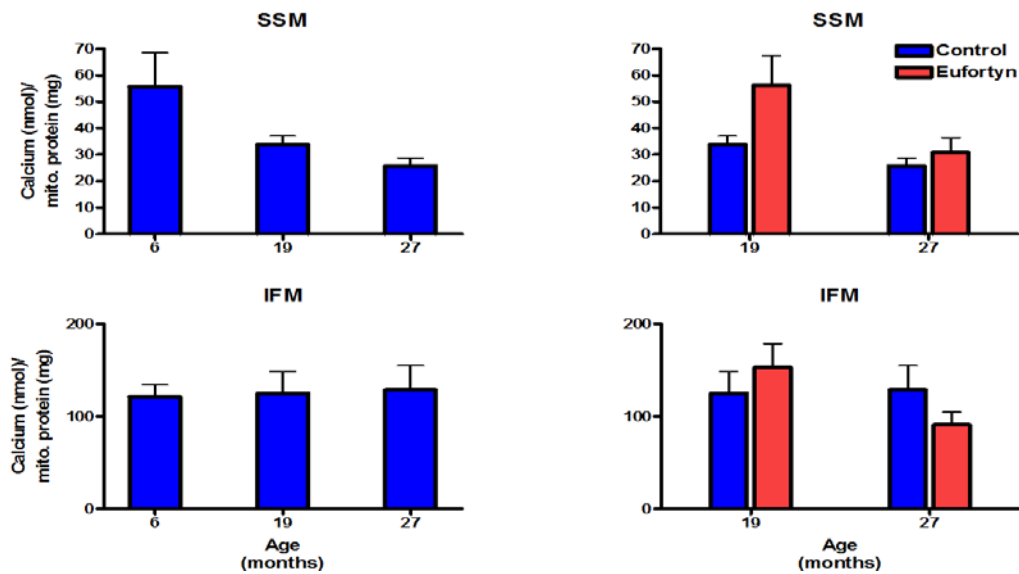
**Figure 10:** Duration in maze measurement



**Figure 11:** Velocity in maze measurement

experiment; the Eufortyn groups achieved a shorter distance to the platform. This indicated that the Eufortyn groups experienced improved cognitive performance relative to the

Over the course of the experiment, the learning curve of the Eufortyn and control groups began to coincide and finally no significant difference in duration was seen on the final day of water maze. The similar pattern was observed for the distance traveled by each cohort and group (Fig. 10). The Eufortyn groups started off with an advantage in that they had to travel the shortest distance to reach the hidden platform as compared to the control, however at the final stage, all groups tended to travel the same small distance resulting from the inevitable assimilation to the water maze in general. The velocity at which the rats traversed to the hidden platform was consistently greatly in the Eufortyn groups than the control groups (Fig. 11). The



**Figure 12: SSM and IFM Calcium Retention.** The calcium retention capacity of the subsarcolemmal mitochondria (SSM) decreased significantly with age in the 19- and 27-month old animals compared to the 6-month old control (Fig 12A lower panel). One-way ANOVA revealed a significant age effect for SSM (Fig 12A lower panel;  $p < 0.05$ ). Eufortyn treated mitochondria demonstrated a marked increase in the calcium retention capacity of the SSM isolated from the 19-month old rats (66%) and a modest increase in the 27-month old rats (19%) (Fig 12B upper panel). IFM mitochondria did not exhibit any significant trend.

19-month Eufortyn treated rats appeared to be fastest initially, but eventually leveled off in speed with the 27-month Eufortyn treated rats. Nevertheless, the control groups consistently lagged behind their age-matched Eufortyn groups throughout the course of the experiment.

### Permeability transition pore (PTP) opening

The maximal calcium loading capacity of isolated mitochondria was determined by using membrane impermeable fluorescent probe, Calcium Green-5N. The calcium uptakes of subsarcolemmal (SSM) and intermyofibrillar (IFM) mitochondria were measured by using the microplate reader with automatic injectors. In control 19 and 27-month old rats as compared to the 6-month olds, the SSM endured more calcium additions before pore opening and expulsion of cell-death markers (Fig. 12). In other words, with age the mitochondria uptake less calcium additions (in nmol of calcium/ mg of mitochondrial protein) for the opening of the permeability transition pore and release of cytotoxic factors such as the pro-apoptotic proteins cytochrome c and apoptosis-inducing factor.

### Eufortyn and non-heme iron

The total non-heme iron content of gastrocnemius

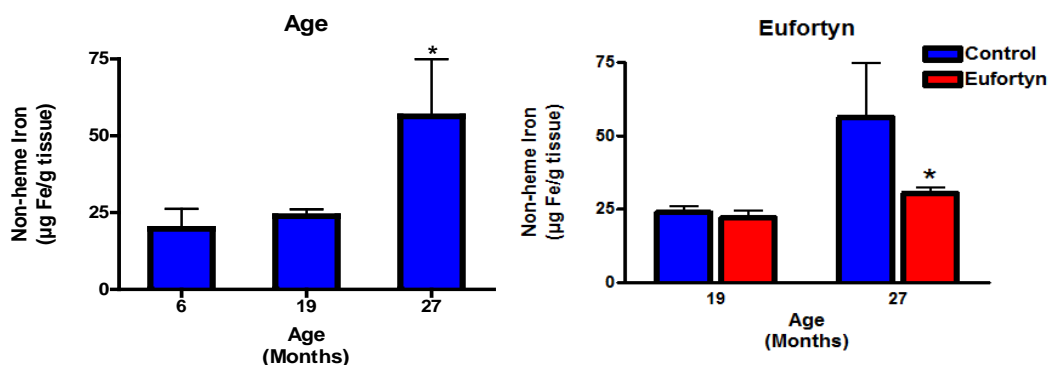
muscle in control cohorts showed significant iron accumulation as part of a normal aging effect (Fig. 13). Eufortyn treatment of age-matched cohorts showed no distinction at the 6 and 19-months, but a significant (54%,  $p < 0.05$ ) mitigation in iron content was observed in the 27-month old senescent rats.

### Eufortyn and oxidative stress

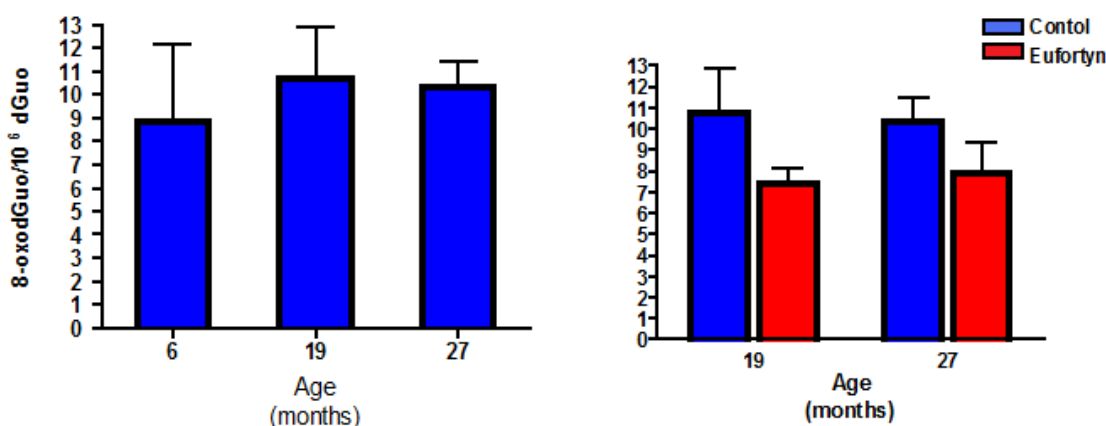
Oxidized DNA was assessed by the levels of oxidative products 8-oxo-7,8-2'-deoxyguanosine from control and Eufortyn rats at 6, 19, and 27 months of age. One-way ANOVA showed no significant age effect for the DNA oxidative damage (Fig. 14 left panel). However, DNA oxidative damage in both 19- and 27-month old rats was attenuated by 31% and 24% individually in the Eufortyn groups as compared to age-matched controls (Fig. 14 right panel). Two-way ANOVA indicated a significant treatment effect ( $p < 0.10$ ), suggesting that Eufortyn effectively mitigates the DNA oxidative damage in the gastrocnemius muscle.

### Conclusion

As a whole, these results have advanced our understanding of Eufortyn's effectiveness on intracellular energy regulation in skeletal muscle mitochondria by way



**Figure 13: Non-Heme Iron Aging and Eufortyn Effect.** A substantial aging effect depicted by total non-heme iron content measured in gastrocnemius muscle tissue of *ad-libitum* fed 6, 19, and 27-month old rats (left panel) normalized to micrograms of iron per gram of wet tissue weight ( $p < 0.05$ ) by Tukey's Multiple Comparison Test,  $n = 7-8$  per group. Total non-heme iron content measured in gastrocnemius muscle tissue of 19 and 27-month old *ad-libitum* and Eufortyn fed rats (right panel) normalized to micrograms of iron per gram of wet tissue weight. With respect to 19-month old control group, Eufortyn treatment showed no effect, but in the 27-month old age Eufortyn treated animals show a remarkable suppression of non-heme iron (54%) as compared to control. ( $p < 0.05$ ),  $n = 7-8$  per group.



**Figure 14: Effects of aging and eufortyn on DNA oxidation levels in control and eufortyn-treated gastrocnemius muscle.** Levels of oxidized DNA were assessed by examining levels of oxidative products 8-oxo-7,8-2'-deoxyguanosine using HPLC-ECD. One way ANOVA exhibited no significant difference in DNA oxidative damage between control cohorts (Fig 14 left panel), but oxidative damage in both 19 and 27 month old rats was diminished by 31% and 24% respectively in the Eufortyn groups as compared to the control cohort matches (Fig 14 right Panel). Two-way ANOVA suggested a significant treatment effect ( $p < 0.10$ ), indicating that Eufortyn successfully lowered DNA oxidative damage in the gastrocnemius muscle.

of physical and cognitive improvements of the aging organism. Our results demonstrated the treatment's role in altering biomarkers specific to mitochondrial bioenergetics and skeletal muscle aging. All tissue weights appeared unaffected by Eufortyn treatment when compared with age-matched controls indicating that the diet utilized has no negative effects on organ weight and is safe for consumption. Moreover, Eufortyn had no negative effects on body weight, food intake and muscle weights in this

study. These findings establish the safety of Eufortyn as a supplement. The distance measurements delineate that the Eufortyn groups had a higher initial performance capacity and were therefore able to exhibit outstanding performance compared to the control groups. Resultantly, Eufortyn may play a role in delaying cognitive deterioration with age by extending its protective properties beyond the blood brain barrier. The beginning trials of the water maze experiment in which the Eufortyn groups display a distinct lead over

their control counterparts support the notion that the Eufortyn treatment boosts mental acuity. The velocity (cm/s) measure confirms the two previous measures of distance and duration in corroborating the positive effect of the treatment on cognition and therefore, facilitation of learning, habituation, and application of problem solving strategy in the 19 and 27-month old animal models.

In the PTP analysis of the Eufortyn treated groups, the calcium retention capacity increased by 66% in the 19-month old rats and 19% in the 27-month old rats establishing sufficient evidence that Eufortyn prevented mitochondrial-mediated apoptosis and reduced inclinations toward cell death *in vivo*; this is a remarkable result pointing out the treatment's preventative features in delaying mitochondrial-mediated apoptosis and reducing the susceptibility to cell death *in vivo*.

The non-heme iron assay results provided a positively correlating trend between the aging rats and non-heme iron concentration found in gastrocnemius muscle tissue. The typical increase of iron seen in aging skeletal muscle has been substantially suppressed by supplementation of Eufortyn just over a six week period providing strong evidence that Eufortyn helped diminish non-heme iron levels in aging muscle.

Nucleic acid oxidation results propose that the treatment is clearly more promising in the middle-aged animals than in the 27-month old rats; 27-month olds failed to benefit as much as 19-month olds because of irreversible damage. The intervention with Eufortyn needs to be initiated at an earlier age than 27-month old to see optimal effects. Considering all the trends, it is imperative for future clinical trials to take place. Further, in-depth studies will work to validate Eufortyn as an effective therapy in delaying aging effects while improving the strength, fatigue resistance, and independence of the elderly population.

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