The Effect of Ginseng on Muscle Injury and Inflammation

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Abstract : The effect of *Panax ginseng* administration in muscle inflammatory process induced after eccentric exercise, that causes myofibrillar disruption, was studied. Changes in lipid peroxidation, inflammation, glycogen levels in muscle and release of myocellular proteins to blood were measured. The analyses were performed immediately after eccentric exercise and over week since this period are necessary for the muscle damage-repair cycle. The ginseng extract (100 mg kg^{-1}) was orally administered to rats for three months, before the eccentric exercise performance. The results showed the protective role of ginseng against skeletal muscle damage. This effect could be associated with their membrane stabilising capacity since creatine kinase (CK) activity was significantly decreased 96 h post-exercise from 523±70 to 381±53 and 120 h post-exercise from 443±85 to 327±75 in treated animals. β-glucuronidase activity, as indicator of inflammation, showed a significant reduction of about 15-25% in soleus, vastus and triceps in these post-exercise times. The lipid peroxidation, measured by malondyaldehyde levels, was significantly decreased in the 24 h post-exercise period in soleus and vastus intermedius muscles and on the recovery period. Finally ginseng administration reduced significantly the decrease of the glycogen levels immediately after exercise and when the regenerative process took place (72-168 h post exercise). Collectively, the results have showed that ginseng did not inhibit the vital inflammatory response process associated with the muscle damage-repair cycle but presumably ameliorate the injury.

Key words : ginseng, inflammation, eccentric exercise, muscle damage, glycogen, lipid peroxidation, Creatine kinase

INTRODUCTION

Panax ginseng roots have been used in traditional Chinese medicine for their important therapeutic qualities. The pharmacological effects of ginseng have been demonstrated in the cardiovascular, immune, endocrine and nervous systems.[1] Until recently, the use of ginseng has been primarily empirical. However, systematic research has provided evidence for the antineoplastic, antistress and antioxidant activity actions of *Panax ginseng*.[2,3] Most pharmacological actions of ginseng are attributed to ginsenosides, which can act in a wide range of tissues. Among all ginsenosides, Rb_{1-2} and Rg_1 are the most abundant.[4] Some of the individual effects of ginsenosides have been studied; thus Rb_1 increases thermogenesis and cold tolerance;[5] restores the action potentials of free radical-damaged cells to normal levels indicating its anti-oxidative action, and shows calcium channel blockade activity, like Rb_2 or Rb_3.[6] In addition, the non-ginsenoside constituents of ginseng also exert pharmacological effects.[7] Ginsenosides are amphiphilic in nature and have the ability to become intercalated in the membrane environment.[1,8]

Evidence points out that the medicinal efficacy of ginseng has been closely linked to its protective properties against free radical attack.[9,10,11] *Panax ginseng* administration in the rat prevented myocardial ischemia-reperfusion damage induced by hyperbaric oxygen.[10] We have reported the hepatoprotective effects of Panax ginseng on oxidative stress induced by exhaustive exercise.[12] Ginseng extract was also reported to scavenge superoxide radicals[13] and inhibit lipid peroxidation through transition metal chelation[8] to diminish oxidative DNA damage caused by Fenton reagent.[14] The active role of Ginseng extract scavenging hydroxyl radical and protecting unsaturated fatty acids may contribute to stabilize the structure of lipid membrane perturbed by free radical attack.[8] Although the mechanism of ginseng actions remains unclear because of the complex composition of ginseng extract which precludes a structure-function activity.[15]

Free radicals play an important role as mediators of skeletal muscle damage and inflammation after strenuous exercise and muscle disease.[16,17]

Eccentric exercise (EC) produces high muscular tension when the muscles are stretched and is associated to delayed onset muscle soreness (DOMS) provoking ultra-

structural and metabolic changes in the muscle cells. The injuries are subcellular and generally in small areas of the muscular fibre, therefore they suffer a transient reduction in strength, subsequently muscular pain and finally an inflammatory process, that causes further tissue deterioration. Direct evidence of inflammatory cells within skeletal muscle has been reported in both animals and humans. Cellular infiltration is associated with proteolytic degradation of muscle tissues and is only associated with the eccentric exercise. The early changes in exercised-induced muscle injury consist of a disruption in the myofibrillar banding pattern and disruption of the sarcolemma. From 2 to 6 h later, secondary changes such as fibre autophagy and heterophagy by macrophages are shown in the muscle fibres. This stage continues for the next 2-4 days. Regenerative processes usually start within 4-6 days.[18] Eccentric exercise is also responsible for the low levels of glycogen.[19] Muscle glycogen levels not only reflects the muscle activation and exercise metabolism but the phases of inflammation-status post-exercise.

The aim of the present work was to study the effects of long-term ginseng administration in muscle inflammatory process induced after eccentric exercise in opposite to animals without ginseng treatment. Specifically eccentric exercise leads to severe inflammation and edema. This condition provides an informative experimental model for evaluation of compounds believed to exhibit therapeutical properties such as antioxidant and antinflammatory agents. The amount of muscle injury was measured biochemically by analysing the activity of the following: creatine kinase (CK) in plasma as indicator of membrane integrity, muscle β-glucuronidase activity as lysosomal enzyme indicator of inflammation, malondialdehyde (MDA) levels as index of lipidic peroxidation and muscle glycogen concentration.

MATERIALS AND METHODS

Drugs and Chemicals

The standardized ginseng extract used was obtained. This ginseng extract contains about 4% of ginsenosides, as determined by HPLC.[20] The roots of *Panax ginseng* C.A. Meyer contain other compounds such as polysaccharides, peptides, polyacetylenic alcohols, fatty acids and minerals. Standard enzymes or products were obtained from Sigma Chemical Company (Spain).

Animals and exercise protocol

Male Wistar rats with an approximate initial weight of 200±30 g, supplied by Iffa Credo (Madrid, Spain), were used. The animals were kept 2 or 4 to a cage under controlled conditions of temperature, light and humidity. The feed consisted of a standard diet marketed by Panlab S.A. (Barcelona, Spain). Drinking water and food were supplied ad libitum throughout the study period. All animal handling practices complied with the principles of Council Directive 86/609/ of the European Community concerning the "Protection of Animal used for Experimental an other Scientific Purposes". The ginseng treated animals (Ginseng) received 1 ml of ginseng extract at a dose of 100 mg kg^{-1} day^{-1} along 3 months of the experimental period by gastric intubation and performed the exercise protocol described below, a subgroup (n=6) of these rats did not perform exercise (Rest-Ginseng). This dose was chosen based in our previous works.[2] The control group (Control) received saline instead ginseng extract for 3 months and at the end performed eccentric exercise. The control group included a rest group (n=6) which did not perform exercise (Rest-Control).

The eccentric exercise performed was on a rodent treadmill with the following protocol described by Armstrong.[21] The rats ran an intermittent protocol downhill ($-16°$ incline) at 16 m min^{-1} for a total of 90 min; 5-min bouts (18 bouts) separated by 2-min rests.

Taking into account that the development of damage associated with eccentric contraction does not occur immediately after exercise and that it shows temporal variability, the study was performed immediately after eccentric exercise and over one week after this. So, the exercised rats (both Ginseng and Control) were divided in eight subgroups (n = 6 for each subgroup) and killed at 0 (immediately following exercise), 24, 48, 72, 120, and 168 h after the realization of the exercise. At the time of slaughtering the animals were anaesthetized with pentobarbital sodium (5 mg 100 g body wt^{-1} i.p.). Blood was sampled from the tail vein and used to determine plasma creatine kinase levels. Muscles were rapidly excised, trimmed of extraneous fat and connective tissue, immersed in isopentane, dropped into liquid nitrogen and stored at $-70°$ until further analysis. Tissues were homogenised at a proportion of 1:10 w v^{-1} in a phosphate buffer 0.1 M (pH 7.4) at 0°C and centrifuged at 750 g in order to separate the cell residues.

The muscles studied were *extensor tonic muscles*-soleus, vastus intermedius, red part of triceps brachii, and red part of gastrocnemius (the percentage of fibres type I and IIa is higher than 90%); *extensor phasic muscles*-rectus femoris, extensor digitorum longus (EDL),

white part of triceps brachii and white part of gastrocnemius (the percentage of fibres IIb is higher than 75%) and *flexor phasic muscles*-plantaris, tibialis anterior and biceps braquii (the percentage of fibres type IIa and IIb is higher than 90% in plantaris and tibialis, but biceps braquii shows a low IIb type proportion of 8%).

Biochemical analyses

Plasma CK levels were analysed using a commercially available kit (Sigma Chemical), immediately after (0 h) or in different times (24, 48, 72, 96, 120, 144 and 168 hours) after eccentric exercise.

β-glucuronidase

β-glucuronidase was assayed according to measuring the hydrolysis of *p*-nitrophenyl-β-D-glucuronide.[22] The homogenate (200 µl) was incubated for 60 min at 37°C at a final volume of 1 ml containing 0.2 M sodium acetate buffer, pH 4.5, 0.1% bovine serum albumin, and 10 mM *p*-nitrophenyl- β-D-glucuronide. The reaction was stopped by adding 2.0 ml of 0.1 M NaOH and 3 ml of water, and absorbance was measured at 400 nm. One unit is the activity that catalyses the release of 1 µmol of *p*-nitrophenol per minute from the substrate.

Malondialdehyde (MDA) measurements

Malondialdehyde (MDA) was assayed by quantifying the release of malonyldialdehyde (MDA) in muscle homogenates using the 2-thiobarbituric acid (TBA)-trichloroacetic acid (TCA)-HCl reagent, following the method of method of Buege and Aust.[23] An aliquot of the homogenate and 1 ml of reagent were added to the centrifuge tubes. The mixture was kept at 100°C. After a short centrifugation at 3000 *g* for 10 min, the absorbance of the supernatant was measured by spectrophotometry at 535 nm. Parallel standards containing tetramethoxy-propane 5 µM (TMP) were processed.

Glycogen

Glycogen concentrations of the muscle samples were measured according to Varnier.[24] Samples of muscle (160 mg) were homogenized in a 1 ml of water at 0°C. The homogenate was hydrolyzed in 100 µl of HCl 6M, incubating at 100°C. Subsequently the samples were cooled, neutralized with 285 µl of KOH 2M and centrifuged at 1000g for 10 min. The glycogen concentrations were analysed using 15 µl of centrifuged sample and a commercially available kit (Test Glucose Sigma Diagnostics).

Statistical Analysis

The results are presented as means±SD. Statistical evaluation of the data of the groups was performed by ANOVA followed by a *post-hoc* Newman-Keuls test. The level of significance was set at the level of p<0.05.
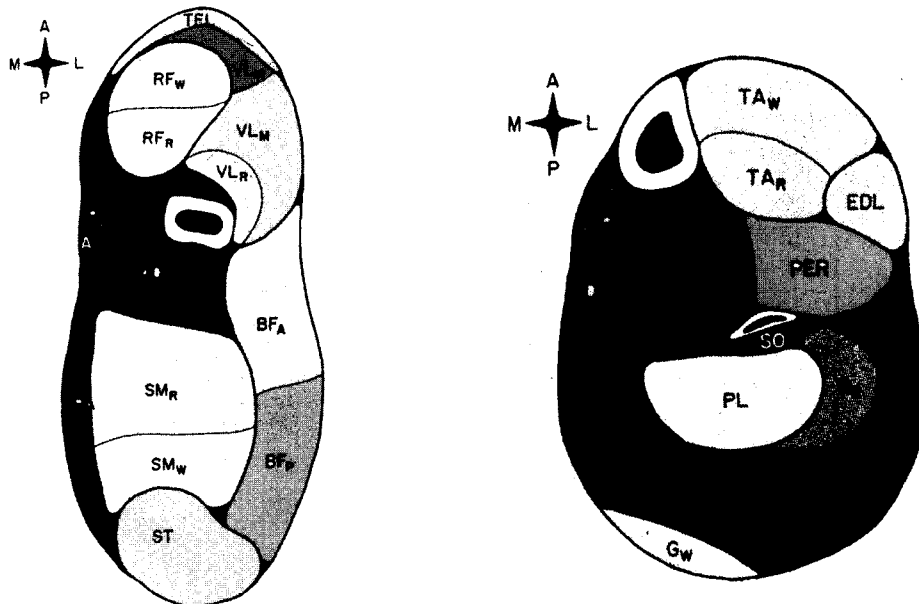
Schema 1. Cross sectional view of the rat hindlimb. Directions indicated are anterior (A), posterior (P), medial (M) and lateral (L). *Thigh muscles*: Rectus femoris (RF), Vastus intermedius (VI). *Leg muscles*: Tibialis anterior (TA), Extensor digitorum longus (EDL), Soleus (SO), Plantaris (PL), Gastrocnemius (G).
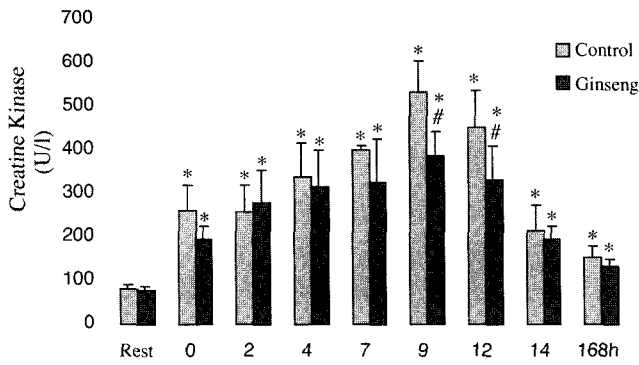
Fig. 1. Plasma Creatine Kinase (CK) activity determined from Control (C) and Ginseng (G) administered animals in rest and after eccentric exercise. Values are expressed as mean±SD. *Significant differences from rest groups. #Significant different from control groups. $P<0.05$.

RESULTS

Fig. 1 shows a time course of plasma CK in sedentary and exercised animals. Immediately following exercise (0 h) a significant increase in plasma CK activity was observed. Plasma CK levels were analysed over a very long period of time: up to 168 h after the performance of eccentric exercise, when they almost returned to basal values. The highest levels of CK activity were noted 96 h after exercise (523 ± 69 U l^{-1}), and the ginseng-treated group showed significantly ($P<0.05$) lower values when compared with their control (381 ± 53 U l^{-1}) CK levels were also significantly ($P<0.05$) lower in ginseng-treated groups after 120 h of exercise.

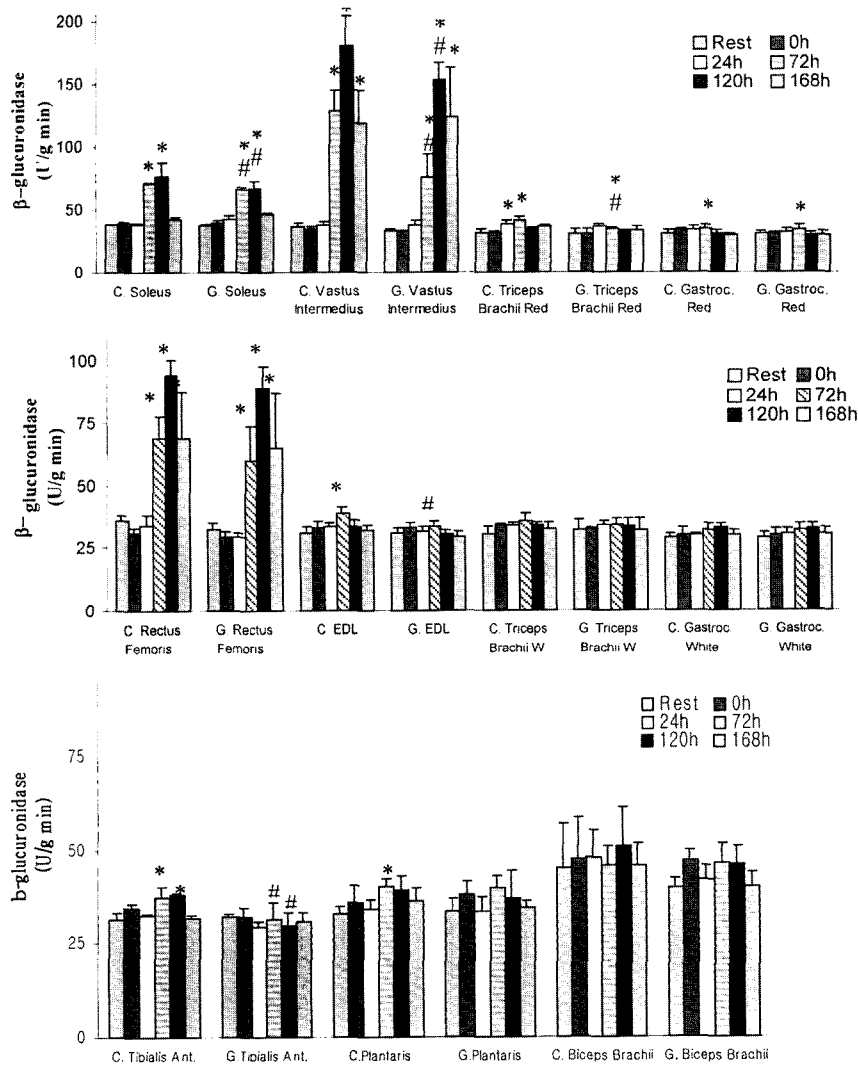β-Glucuronidase activity from 11 different muscle is

Fig. 2. β-glucuronidase activity determined from muscles of Control (C) and Ginseng (G) administered animals. Values are expressed as mean±SD. *Significant differences from Rest groups. #Significant differences from Control groups. $P<0.05$.

Table 1. MDA (nmol g muscle^{-1}) in Control (saline administered) and Ginseng treated animals in rest and after eccentric exercise

Control	Soleus	Vastus	Triceps	Rectus	EDL	Tibialis
Rest.	40.22±3.82	23.39±2.71	25.05±2.13	22.61±1.39	24.02±2.57	22.90±4.31
0 h	42.71±3.90	25.44±3.34	26.12±1.81	24.51±5.36	22.36±1.17	21.39±4.27
24 h	59.88±1.08*	31.68±1.13*	26.12±0.16	25.63±5.88	26.66±3.72	22.71±2.32
72 h	44.91±4.45*	23.88±2.63	31.59±1.19*	27.29±2.45	34.07±1.70*	29.98±2.53*
120 h	37.68±2.35	23.49±2.28	25.63±1.54	32.51±1.76	27.19±2.82	28.95±2.86*
168 h	37.49±3.59	26.90±1.53*	23.73±0.90	26.56±1.67	26.56±1.98	24.07±4.55
Ginseng						
Rest.	38.95±2.65	20.17 ±1.45	23.63 ±1.61	20.17 ±1.45	24.22 ±1.67	22.31 ±2.40
0 h	40.08±1.03	23.19 ±1.92	23.09 ±1.10	23.00 ±1.12	26.32 ±2.72	21.44 ±1.87
24 h	50.37±1.13*#	27.73 ± 1.89*#	24.71 ±2.71	23.88 ±1.92	25.83 ±1.33	22.17 ±2.16
72 h	39.83±2.71#	22.12 ±0.58	29.73 ±0.92*	25.97 ± 2.18*	29.99 ±0.82*#	25.68 ±2.20#
120 h	36.66±1.64	23.34 ±2.66	23.63 ±2.47	28.85 ±0.18*#	26.95 ±2.54	25.63 ±1.87#
168 h	37.88±1.13	22.41 ±1.16*#	22.85 ±2.72	26.32 ±0.93	26.75 ±2.16	22.07 ±2.60

Values are means ± SD, n=6 animals in each time. *$P<0.05$ vs Rest groups. #$P<0.05$ vs Control groups.
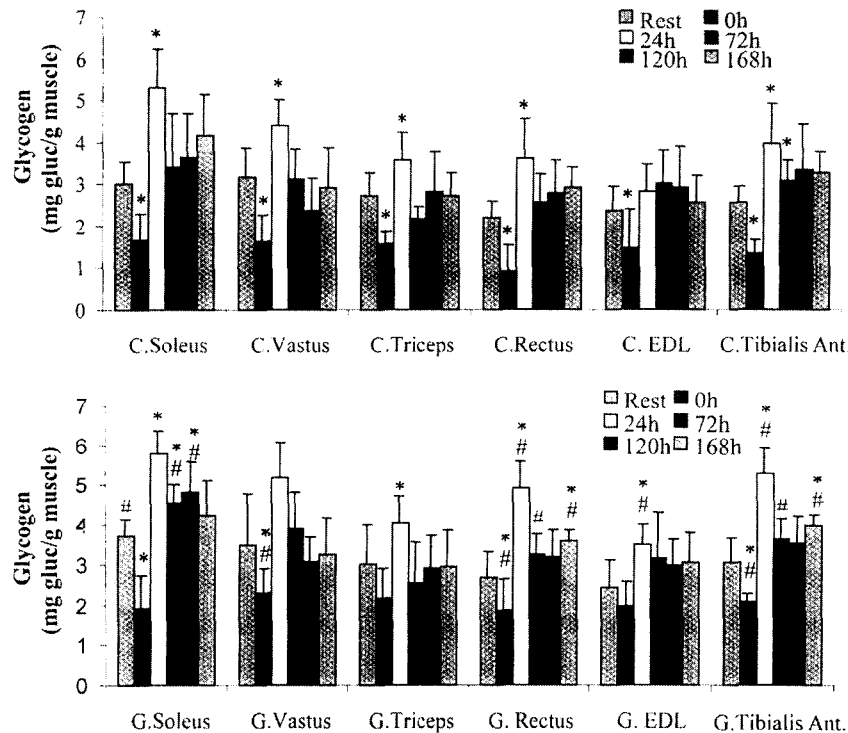

Fig. 3. Glycogen determined from muscles of Control (C) and Ginseng (G) administered animals. Values are expressed as mean±SD. *Significant differences from Rest groups. #Significant differences from Control groups. $P<0.05$.

shown in Fig. 2. There is an initial increase of the enzyme activity followed by a reduction lasting for about 7 days, with a peak on 120 h post-exercice. Our results showed that the extensor muscles as soleus, and vastus intermedius are the most affected in an eccentric activity. The soleus presented an increase of about 100% compared with Rest-control group 120 h after exercise; the vastus 402% after 120 h, the triceps 29% after 72 h; the rectus 168% after 120 h; EDL 25% after 72 h; and the tibialis

anterior 19% after 120 h.

In the ginseng-treated group a significant ($P<0.05$) reduction of about 15-25% in enzyme activity was found in soleus, vastus and triceps in these post-exercise times. The EDL and tibial and muscles showed the lowest increases in these enzyme activities, and in a similar manner the activities were significantly ($P<0.05$) reduced by ginseng extract administration.

The modest increase in β-glucuronidase activity of the

gastrocnemius red (16%), indicates low damage. The non-injured muscles are basically composed by fibres type IIb (plantaris, biceps brachii, white gastrocnemius and white triceps) and were excluded in the further analyses.

Table 1 shows the levels of MDA after eccentric exercise. Significantly higher MDA levels, as an indicator of membrane disruption, were noted a few hours (24 h) after the exercise in soleus and vastus. The highest differences between the rest and control exercised rats were shown in the muscles with the highest percentage of glycolytic fibres (less oxidative potential): i.e., rectus, EDL and tibial muscle after the inflammatory process developed (72-120 h). However, with ginseng treatments MDA concentrations were significantly ($P<0.05$) reduced in all the muscles studied over the same period of time stated for the muscle enzymes above. The reduction percentages associated to ginseng administration varied between 15-20% in the chosen muscles except for triceps muscle with a significant decrease of only 8%.

Fig. 3 shows the results of muscle glycogen concentration. Ginseng administration increased significantly the muscle glycogen stores in soleus from 3±0.5 in Rest-Control group to 3.73±0.4 in Rest-Ginseng rats. The overall results clearly show an increase of glycogen concentration in ginseng treated animals that can be divided into 3 phases. Immediately after exercise (0 h) there is a significant reduction in glycogen contents. The glycogen concentration decreased about a 50% in all studied muscles but in vastus, rectus and tibialis the ginseng administration diminished the decrease about 20%. After 24 h there is a significant increase and the glycogen contents were higher than control values with significant differences respect to control groups in the rectus, EDL and tibialis of ginseng treated rats. In the subsequent days, when the regenerative process takes place, the glycogen levels tend to decline and reach basal levels. Ginseng treated animals showed significant increased levels from 72 to 168 h in soleus, rectus and tibialis muscles.

DISCUSSION

The events of EC injury at cellular, biochemical and biomecanical levels are unclear. EC damage seems to be fiber type specific. In human experimental models this kind of injury appears to be more frequently associated to type II fibres,[25] while in animals the extensor muscle (deep vastus intermedius), basically composed of fibres type I was preferentially damaged.[26] It has been also pos-

tulated that muscle damage from EC is as a function of oxidative capacity. Selective damage in fast glycolytic fibers in rabbit tibialis anterior muscle after EC contraction was demonstrated[27] to be due to the low oxidative capacity that predisposed them to injury, but the increased oxidative capacity did not protect the muscle against electrical stimulation-induced injury,[28] neither the supplementation with soluble antioxidants and vitamins.[29] There is a large variability depending on the fibre type pattern and exercise model demonstrating that is necessary to clarify this kind of injury. The results obtained here showed clearly a differential muscle response in the inflammatory process after eccentric contraction. The first parameter studied related to this process is the β-glucuronidase activity as a sensitive and quantitative marker of muscle injury and its total activity correlates with the overall histological changes in injured muscle.[30] Several works have demonstrated an increase of this activity associated with exhaustive exercise both in humans and animals.[31,32] β-glucuronidase have shown that the tonic muscles (vastus, soleus and red triceps) and phasic extensors (rectus, and EDL) were the most injured because they facilitate the forward movement and acts as a break against gravity. The soleus have as main function to sustain the body against gravity. Running or walking, they are responsible for the increase of force necessary for the elevation of the body and its forward movement. Successively, as the velocity increases, the gastrocnemius is incorporated. The vastus and the rectus are directly responsible of the knee extension, the first one responsible for the antigravity maintenance of the body in the resting state. Downhill they are overloaded because they have the main responsibility of breaking. The EDL and the tibialis are responsible for the extension of the toes and dorsiflexion of the foot respectively, during walking or running, provoking a forward movement. The red triceps is an antigravity muscle acting as a break to maintain the velocity when moving.

The time course of CK efflux is coincident with the high values of β-glucuronidase and Ginseng had a protective role decreasing elevated CK levels significantly, although it did not prevent leakage of CK from the muscle to the blood after exercise. Increased levels of CK in the blood stream show myofibrillar disintegration and is taken as evidence of the disruption or increased permeability of the muscle cell membranes.[33] β-glucuronidase activity showed decreased activity in soleus, vastus, red triceps, EDL and tibial after ginseng administration. The highest protection was found in the vastus in the early

stage of the inflammatory process (72 h). β-glucuronidase activity reflects the overall damage associated to injured fibres, inflammatory cells and affected but surviving muscle fibres.[30] The highest protection by ginseng extract was observed in the most damaged muscles (vastus intermedius and soleus) composed mainly of type I fibres.

Regarding to oxidative stress and lipid peroxidation, there are several mechanism involving free radicals that could contribute to the damage process after eccentric exercise and lipid peroxidation so free radicals alter the normal permeability barrier provided by the sarcolemma, or mitochondrial and lysosomal membranes, permitting abnormal diffusion of molecules such as Ca^{2+}.[34,35] The altered Ca^{2+} homeostasis actives various Ca^{2+} dependent degradative pathways in the cell such as endogenous proteases (e.g. calpain).[36] In addition free radicals may also initiate and or amplify via the upregulation of genes involved in the inflammatory response such as nuclear factor κB and contribute to cellular damage during phagocytic stage of muscle injury.[34] However, the role of antioxidants in the damage prevention after eccentric exercise are controversial since supplementation with vitamins C and E and N-acetyl-cysteine not attenuate the muscle injury but increases oxidative stress in humans after eccentric exercise.[29,37] The protective effects of ginseng related to increased MDA levels were mainly shown in the 24 h period in soleus and vastus intermedius muscles. Mechanical experiments reveal that excessive sarcomere strain is the primary cause of injury and allows extracellular or intracellular membrane disruption, and the hydrolysis of structural proteins.[36] The significant lipid peroxidation measured by MDA in triceps, rectus, EDL and tibialis was over 72-120 h and could be associated to the inflammation that occurs after injury which degrades the tissue. Concerning membrane integrity and damage induced by free radicals, ginseng may stabilize the lipid structure of the membrane perturbed by the attack of free radicals, the stabilising effect was reflected in the percentage of MDA decrease.[1] reported the ability of ginsenosides to target the cell membrane modifies its physical properties, interacts directly with membranes proteins, and even ginsenosides become incorporated in membranes. Moreover estrogen have a membrane stabilising effect and a protective role against skeletal muscle damage.[38]

Other possible mechanism that could explain the suppression of lipid peroxidation is through the inhibition of enzymes such as lipoxygenase involved in the metabolism of unsaturated fatty acids, arachidonic acid, to prostaglandins. In addition compounds that block the effect of pros-

tanglandin synthesis might reduce or abolish the stimulatory action of bradykinin, factor involved in the inflammatory response.[33] Ginseng has well known anti-inflammatory actions[39] and such effects could be caused by a decrease in phospholipase A_2 activity[40] and by the reinforcement of muscle fibre membranes in a similar manner to another anti-inflammatory agents; i.e., prednisone.[41] Several authors have found that decreased phospholipase A_2 activity can lead to a decrease in the hydrolysis of membrane phospholipids, decreased membrane fluidity, and a decreased Ca^{2+} influx and muscle damage.[41,42]

Wang and Lee[43] showed that after exhaustive exercise skeletal muscle glycogen levels of ginseng saponin treated rats were slightly higher than those of saline treated.

As reported in previous studies,[21,32] we also observed an alteration of glycogen levels in the period immediately after the eccentric exercise, commonly named first phase. Others authors, found a decrease from day 1 to day 10 after exercise.[19] The glycogen pool is dependent of glucose transport across the membrane from the blood by the GLUT-4 transporter. Eccentric exercise can reduce GLUT-4 expression and in this way reduce glycogen storage in muscular cells.[44,45] observed a reduction of RNAm for GLUT-4 in skeletal muscles of rats submitted to eccentric exercise.

After eccentric exercise there were significant differences in glycogen concentration between saline and ginseng treated groups in vastus, rectus and tibialis.[46] demonstrated that depletion of muscle glycogen also modifies Ca^{2+} release reducing contractile performance and leading to muscle fatigue. The concomitant reduction in the maintenance of energy used may be an important step in maintaining the structural and functional integrity of the cell.

Regarding to the second phase of glycogen levels after eccentric exercise,[47] found a significant increase in muscular glycogen in the first 24 h of exercise in a treadmill that also resulted in several muscle injury. In spite of the reduction in GLUT-4, eccentric exercise can increase GLUT-1, and this transporter is involved in glycogen reposition in resting conditions.[48] Over 72-168 h the presence of inflammatory cells increase glucose utilization and reduce glycogen resynthesis.

In animal models, ginseng have been shown to alter the mechanism of fuel homeostasis incresing the capacity of skeletal muscle to oxidize free fatty acids in preference to glucosa for celular energy production; ginseng increases the antioxidant capacity reducing the effects of the oxidative stress induced by exhaustive exercise. This work was based on eccentric exercise whose energy cost is unusu-

ally low and the magnitude of the force produced unusually high. Both properties are physiologically fundamental since muscles exposed to chronic eccentric training respond with significant increases in strengh, size and spring properties. These responses have clinical and physical performance consequences. Our results have showed that ginseng did not inhibits the vital inflammatory response process associated with the muscle damage repair cycle but presumably ameliorate the injury as well as the pain. Further studies with humans will be required to confirm this findings.

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