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Use of combinations of gum arabic, maltodextrin and soybean protein to microencapsulate ginkgo leaf extracts and its inhibitory effect on skeletal muscle injury

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ABSTRACT

In this study, ethanol extracts of ginkgo leaf were microencapsulated with maltodextrin, gum arabic or a soluble soybean protein by spray-drying. The results indicated that, for the microcapsules, the encapsulation efficiency of 81.3% was achieved when air inlet temperature was 181 °C. The oxidation of ginkgo leaf polyphenol under the conditions was retarded by its microencapsulation with gum arabic, maltodextrin or the soybean protein. Thus, microencapsulation of ethanol extracts of ginkgo leaf significantly improved its oxidative stability. Pharmacological experiment showed that ethanol extracts of ginkgo leaf could enhance ALP activities and collagen I in mouse osteoblast MC3T3-E1 cells. Rabbits pretreated with microcapsules of ethanol extracts of ginkgo leaf significantly inhibited ischemia/reperfusion-induced oxidative injury in rabbits' skeletal muscle.

1. Introduction

The concept of microencapsulation was first demonstrated in the 1960s in Chang's report on encapsulating proteins into stable microcapsules with semipermeable polymer membranes (Chang, 1964). Thereafter, microencapsulation technologies for immobilization of a variety of biologically active species such as enzymes and living cells, have been developed and applied in biotechnologies for developing bioreactors, biosensors, and hybrid bioartificial organs (Chang, 1995; Parthasarathy & Martin, 1994; Perols, Piffaut, Scher, Ramet, & Poncelet, 1997; Sun, Cai, Shi, Ma, & O'Shea, 1987). The simplest microcapsule consists of a core surrounded by a wall or barrier. The core is the component requiring protection that may be composed of one or more ingredients. The wall may be single or multi-layered (Pothakamury & Barbosa-Canovas, 1995). This technology is used in foods and beverages to control the release of active ingredients, protect ingredients from the environment, lower flavor loss during the product shelf-life, extend the flavor perception and mouthfeel over a longer period of time, and enhance the ingredient bioavailability and efficacy (Berry, 2004; Shefer & Shefer, 2003).

In a controlled release system of encapsulation, degradation of matrix material occurs as a determining factor for release of the encapsulant (Imam et al., 1998; Pothakamury & Barbosa-Canovas, 1995). Extract from leaves of ginkgo (Ginkgo biloba) (EGb) includes flavonoid glycosides, diterpenes (ginkgolides A, B, C and M) and a sesquiterpene as active ingredients (DeFeudis, 1991), and isolated constituents have been found to be active in a variety of assays. For example, ginkgolide B has a potent platelet activating factor antagonist (Lamant, Mauco, Braquet, Chap, & Douste-Blazy, 1987; Zablocka, Lukasiuk, Lazarewicz, & Domanska-Janik, 1995), bilobalide protects cultured rat hippocampal neurons against damage caused by glutamate (Krieglstein et al., 1995), and the flavonoid fraction contains free radical scavengers (Bastianetto, Zheng, & Quirion, 2000). Other biological effects of EGb have also been reported: it attenuates ischemia/reperfusion damage of brain tissue (Karcher, Zagermann, & Krieglstein, 1984; Krieglstein, Beck, & Seibert, 1986) and enhances brain functions including learning and memory (Hadjiivanova & Petkov, 2002). Also, it may help people with Alzheimer or dementia to become more alert, sociable, feel better, and think more clearly (Le Bars et al., 1997; Kanowski, Herrmann, Stephan, Wierich, & Horr, 1996).

Various mechanisms have been implicated to explain the development of ischemia/reperfusion (I/R) injury in skeletal muscle (Nanobashvili et al., 2002). Stimulated generation of superoxide (O_2^-) and reduction of nitric oxide (NO) production are believed to

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play a key role in this process (Freischlag & Hanna, 1991; Huk et al., 2000). The molecular interactions that occur in reperfusion injury are known to involve the formation of reactive oxygen species (ROS), lipid peroxidation, eicosanoid generation, neutrophil activation, infiltration, complement activation and cytokine generation (Appell, Glöser, Soares, & Duarte, 1999). ROS including hydroxyl radical (UOH), superoxide anion radical ($O_2{}^-$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and nitric oxide (NO) can cause cellular injury when they are generated excessively and hazardous to lipids, proteins, carbohydrates and nucleic acids (Clanton, Zuo, & Klawitter, 1999; Cheeseman, 1993; Marx & Chevion, 1986). Oxidative stress means an alteration in the delicate balance between free radicals and the scavenging capacity of antioxidant enzymes in favor of free radicals in the body systems (Frei, 1994).

The purpose of this work is to determine the effect of gum arabic, maltodextrin, soybean protein and their blends, as wall materials, on the preparation of ginkgo leaf extract microcapsules. Then, we tested if pre-treatment of ginkgo leaf ethanol extracts microcapsules might positively influence skeletal muscle ischemia and reperfusion (SKIR) injury in skeletal muscle.

2. Materials and methods

2.1. Preparation of emulsions

Coating materials or wall (gum arabic, maltodextrin, soybean protein) and ginkgo leaf extract were dissolved in purified water, in a 3 L plastic jar, using Ultra-Turrax (T50, IKA Labortechnik, Staufen, Germany) at 10,000 rpm. The emulsions were prepared at 50% (w/w) total solids with an extract load of 25% (dry basis). The coarse emulsions were then further homogenized using a laboratory homogenizer at 270/40 bar and using Gann homogenizer at 200 bar.

2.2. Microencapsulation efficiency

Total extract content of the powder was determined by a modification of the Röse-Gottlieb method (Richardson, 1985). Extractable core material was determined by gently shaking 2.5 g of powder with 100 mL petroleum ether in a sealed 250-mL glass bottle at 25 °C for 15 min. The solvent was filtered (Whatman 41), and result solvent was dried to obtain core material. Microencapsulation efficiency (ME) was calculated as follows: ME = [(total core material − extractable core material)/total core material] × 100.

2.3. Emulsion viscosity

Emulsion viscosity was measured through the determination of steady-shear flow curves (shear stress × shear rate), using a controlled stress Physica MCR301 rheometer (Anton Paar, Graz, Austria) with stainless steel plate–plate geometry with a diameter of 75 mm and a gap of 0.2 mm. Three flow ramps (up, down and up-cycles) were obtained in a range of shear stress corresponding to shear rates from 0 to 300 s^{-1}, in order to eliminate any possible thixotropy effect. Trials were performed in triplicate.

2.4. Spray drying

Emulsions were spray-dried in a Niro Minor dryer (A/S Niro Atomizer) equipped with a rotating disc for the atomization of the emulsion into small droplets at the top of the chamber. The dryer was operated at air temperatures of 120 and 220 °C for inlet and 100–130 °C for outlet. Emulsions were fed by means of a peristaltic pump with flow rates between 22 and 68 mL min^{-1}.

2.5. Cell culture

Mouse osteoblast MC3T3-E1 cells were cultured in α-MEM medium supplemented with 10% fetal calf serum, 100 units/mL of penicillin, and 100 μg/mL streptomycin. All cells were maintained at 37 °C in a humidified incubator with an atmosphere of 5% CO_2. In all experiments, the cells were incubated with different concentrations of ethanol extract for 10 days.

Whole cultures were washed with phosphate buffered saline (PBS) and 500 μL 0.25 M sucrose/35 mm dish was added. The cells and the matrix were scrubbed off the dish and transferred into a cryotube and frozen at −80 °C. Immediately before the assays, the cells were thawed and sonicated (3× 20 s) at 70 W and 20 kHz on ice. The samples were centrifuged at 600 × g for 7 min to remove cell debris and the volume of the supernatant was determined.

2.6. Alkaline phosphatase activity (ALP) and collagen I

We used Sigma Kit No. 245 (Sigma, Buchs, Switzerland) for the determination of alkaline phosphatase activity. Collagen type I ELISA kit was used for the detection of collagen type I in cells. Total protein: the Bio-Rad protein assay kit II (BioRad, Glattbrugg, Switzerland) was used with bovine serum albumin as standards.

2.7. Animal experiment

Forty-eight rabbits were housed at a room temperature of 25 ± 2 °C, relative humidity of 75 ± 5%, and 12-h darklight cycle. The rabbits were provided with basal diet in the form of pellets and water ad libitum. Approval of the China Animal Ethics Committee was obtained for the study.

Animals were divided into four groups: normal control, skeletal muscle ischemia and reperfusion (SKIR) model groups and three medicine-treatment groups. In the medicine treatment groups (n = 8), ginkgo leaf ethanol extracts microcapsule, 130 mg/kg (as equal as ginkgo leaf ethanol extracts 30 mg/kg) or 260 mg/kg b.w. (as equal as ginkgo leaf ethanol extracts 60 mg/kg), ginkgo leaf ethanol extracts 60 mg/kg, were orally administered 10 days prior to induction of ischemia, respectively. Normal control and SKIR model groups (n = 8) were orally administered an equal volume of vehicle.

One hind limb of the anesthetized animals was subjected to periods of ischemia and reperfusion by clamping of the femoral artery and vein. At the end of the experiment samples of the tibialis anterior muscle were was frozen rapidly in liquid nitrogen and stored at −70 °C for biochemical analysis.

2.8. Histology Examination

Skeletal muscles were dissected and embedded in paraffin. Transverse cross-sections of the muscle were stained with hematoxylin and eosin (H&E) and examined for histologic signs of injury.

2.9. Statistical analysis

Results were expressed as the mean ± S.E.M. The difference between the groups was compared using one-way analysis of variance (ANOVA) followed by the Dunnett's post hoc test. A value of $P < 0.05$ was considered statistically significant.

3. Results and discussion

Numerous wall materials or encapsulating agents were available for food application. Gums arabic, hydrolyzed starches, and emulsifying starches were most commonly used as wall materials (Kenyon, 1995; Shahidi & Han, 1993). Mixtures of maltodextrins or corn syrup solids with whey proteins were reported as effective wall materials for microencapsulation of ethyl caprylate (Sheu

Table 1
Viscosity, stability and encapsulation percentage of the emulsion solution.

Mass fraction (%) in 100 mL emulsion				Viscosity (mPa s)	Stability	Encapsulation percentage (%)
Core material	Gum arabic	Maltodextrin	Soybean protein			
4.6	4.1	13.8	2.5	13.2	Stable	59.5
5.2	3.64	12.15	4.01	11.1	Stable	73.2
6.1	2.87	11.75	4.28	9.6	Stable	82.4

& Rosenberge, 1995). Barbosa, Borsarelli, and Mercadante (2005) reported that maltodextrin with emulsifier Tween 80 had the ability to encapsulate a higher amount of bixin than maltodextrin alone. A blend of gum arabic:maltodextrins:modified starch at a 4/6:1/6:1/6 was reported to provide a better protection of cardamom oleoresin than gum arabic (Krishnan, Bhosale, & Singhal, 2005).

Table 1 showed the dependence of the viscosity, stability and encapsulation percentage on the volume of core material, gum arabic, maltodextrin and the soybean protein. It was concluded that the microencapsulation efficiency (%) of the resultant ginkgo leaf extract microcapsules was about 82.4% when core material, gum arabic, maltodextrin and the soybean protein in the ratio of 6.1:2.87:11.75:4.28, and the loading amount of extract in the microcapsules was about 58 wt.%. The results further showed that the emulsion stability of the microcapsules was influenced by the type of encapsulant materials used.

Microcapsules were prepared using three different viscosity grades of maltodextrin by coacervation-non solvent addition technique with no change in other experimental parameters. The effect of homogenization number and homogenization pressure on viscosity and stability of the emulsion solution was shown in Table 2. It was observed that higher the homogenization number and homogenization pressure, higher was the viscosity and stability of the emulsion solution. It showed that the viscosity and stability of the emulsion solution were ideal when the homogenization number and homogenization pressure were 3 and 40 MPa.

The encapsulation yield of extract with air inlet temperature was listed in Table 3. When air inlet temperature was 80 °C, the encapsulation yield of extract was the highest. During membrane emulsification, emulsions were produced with low mechanical stress compared with conventional emulsification techniques, such as homogenization and the rotating stirrer method (Schröder, Behrend, & Schubert, 1998). Therefore, a high encapsulation yield of the extract could be explained by the mild action of droplet formation during membrane emulsification. The difference in extract caused by different drying conditions may be attributed to both a higher inlet and outlet temperature at 210/90 °C. For both parameters an increase in extract content had been reported due to formation of vacuoles and pores (Sloth Hansen, 1980) or formation of cracks (Bhandari, Dumoulin, Richard, Noleau, & Lebert, 1992). Furthermore the difference in particle size accounted for the increase in extractable oil at different drying conditions.Osteoarthritis and osteoporosis are not only the most frequent degenerative diseases of the skeleton, but they are also the most frequent degenerative diseases in developed countries (Cooper & Melton, 1996). Alkaline phosphatase (ALP, ALKP) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. The process of removing the phosphate group is called dephosphorylation. As the name suggests, alkaline phosphatases are most effective in an alkaline environment. It is sometimes used synonymously as basic phosphatase (Tamás, Huttová, Mistrk, & Kogan, 2002). Fig. 1 shows the effect of the ethanol extract on ALP activity in mouse osteoblast MC3T32E1 cells. The ALP activity in mouse osteoblast MC3T32E1 cells increase with the increasing ethanol extract content.

Collagen is the major constituent of bone matrix. It is a crystalline fibroprotein fibril with characteristic X-ray diffraction and electron microscopic pattern, having a periodicity of about 6400 nm, although its length, diameter and density vary with age. Collagen is also the major extracellular protein of the body and comprises some 30% today body protein. Important advances have been made in the elucidation of the structure of the precursors, or procollagens, of tissue collagen, in the discovery of new types of collagen and in the mechanisms of collagen degradation. Type-I collagen is the most abundant collagen of the human body. It is present in scar tissue, the end product when tissue heals by repair. It is found in tendons, the endomysium of myofibrils and the organic part of bone (Choi, Lee, Christ, Atala, & Yoo, 2008; Gentry, Andries Ferreira, McCambridge, Brown, & Phillips, 2010). The effect of the ethanol extract on collagen I in mouse osteoblast MC3T3-E1 cells was evaluated (Fig. 2). Addition of the ethanol extract to the medium caused an increase in collagen I. The increase was displayed in a dose-dependent manner.

Table 2
Effect of homogenization number and homogenization pressure on viscosity and stability of the emulsion solution.

Homogenization number	Viscosity (mPa s)	Homogenization pressure (MPa)	Stability
1	9.65	20	Stable
2	10.5	35	Stable
3	11.73	40	Stable
4	11.8	40	Stable

Table 3
Inlet temperature and encapsulation percentage.

Air inlet temperature (°C)	Encapsulation percentage (%)
120	77.2
160	79.9
180	80.4
210	76.4

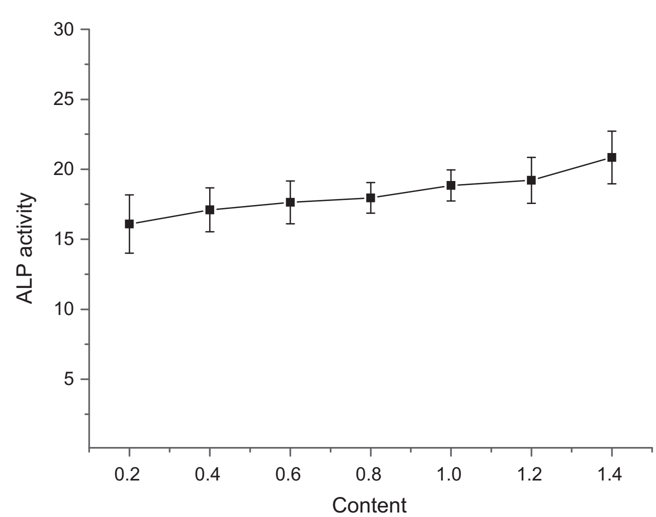

Fig. 1. Effect of ethanol extracts of ginkgo leaf on ALP activities.

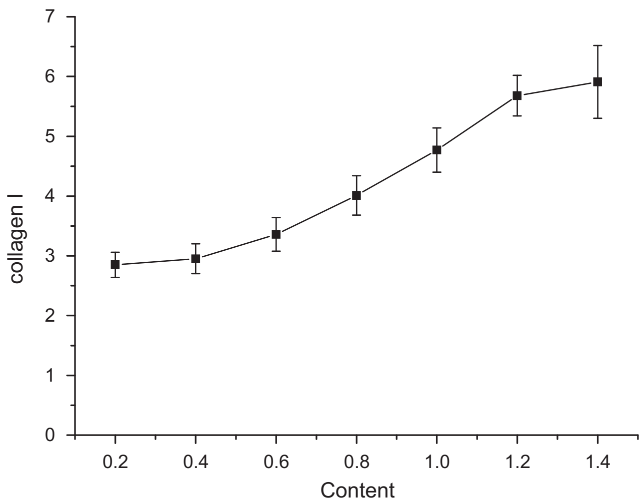

Fig. 2. Effect of ethanol extracts of ginkgo leaf on collagen I.

As shown in Fig. 3, level of serum CK and LDH was significantly increased in the SKIR model control group compared with the normal control group. Pretreatment of ginkgo leaf ethanol extracts microcapsule, 130 mg/kg (as equal as ginkgo leaf ethanol extracts 30 mg/kg) or 260 mg/kg b.w. (as equal as ginkgo leaf ethanol extracts 60 mg/kg) markedly decreased the level of serum CK and LDH in GLEE-treatment groups (I, II and III). In addition, pretreatment of ginkgo leaf ethanol extracts (60 mg/kg) also significantly decreased the level of serum CK and LDH in GLEE-treatment groups (I, II and III).

As shown in Fig. 4, level of skeletal muscle MPO was significantly increased in the SKIR model control group compared with the normal control group. Pretreatment of ginkgo leaf ethanol extracts microcapsule, 130 mg/kg (as equal as ginkgo leaf ethanol extracts 30 mg/kg) or 260 mg/kg b.w. (as equal as ginkgo leaf ethanol extracts 60 mg/kg) markedly decreased the level of skeletal muscle MPO in GLEE-treatment groups (I, II and III). In addition, pretreatment of ginkgo leaf ethanol extracts (60 mg/kg) also significantly decreased the level of skeletal muscle MPO in GLEE-treatment groups (I, II and III).

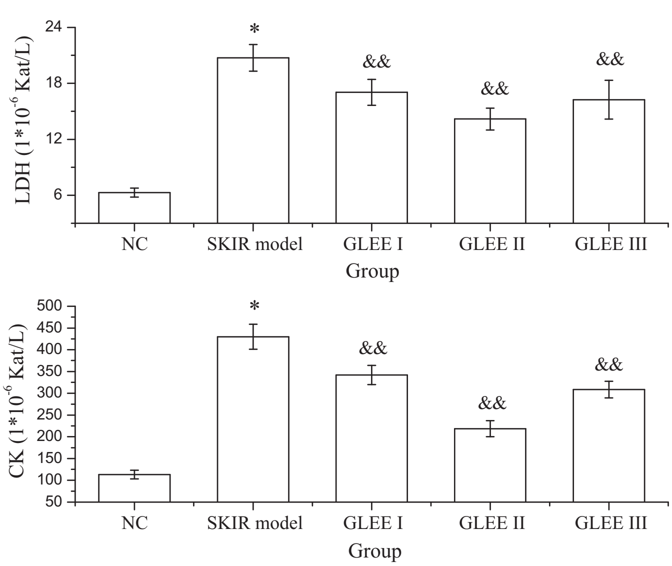

Fig. 3. Effect of ethanol extracts of ginkgo leaf on serum CK, LDH activities. Each value represents mean ± SD; $n = 8$. $^{**}P < 0.01$, compared with normal control; $^{\&\&}P < 0.01$, compared with diabetic control.

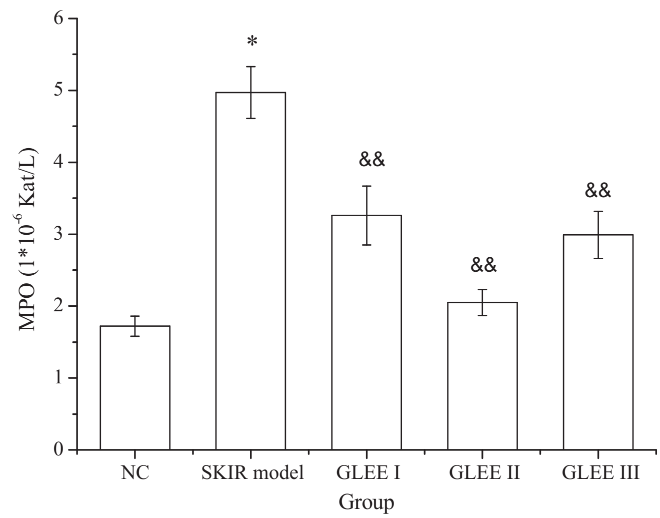

Fig. 4. Effect of ethanol extracts of ginkgo leaf on skeletal muscle MPO activities. Each value represents mean ± SD; $n = 8$. $^{**}P < 0.01$, compared with normal control; $^{\&\&}P < 0.01$, compared with diabetic control.

Muscle disuse is common in patients who are restricted to bed rest or limb immobilization for a period of time. Muscles exposed to disuse present with many cellular adaptations such as reduced muscle strength, decreased muscle mass, and reduced blood flow. Wang et al. find men who experienced 4 weeks of severe intermittent hypoxia have decreased anti-oxidant capacity and increased oxidative damage. These changes lead to vascular endothelial dysfunction and vascular hemodynamics impairment. Compared to other tissues, like cardiac and cerebral tissues, skeletal muscle has been described to be relatively resistant to ischemia because the maintenance of its metabolic capacity is assumed to cease only after 5–7 h (da Cruz, Massuda, Cherri, & Piccinato, 1997; Harris et al., 1986; Idström, Soussi, Elander, & Bylund-Fellenius, 1990; Rubin et al., 1992). However, an ultrastructural study on biopsies obtained during surgery, i.e. after various periods (15–90 min) of ischemia, but without reperfusion, clearly pointed out that already under such conditions skeletal muscle ultrastructure showed eventual pathological alterations, especially extending to metabolically important organelles (Appell, Glöser, Duarte, Zellner, & Soares,

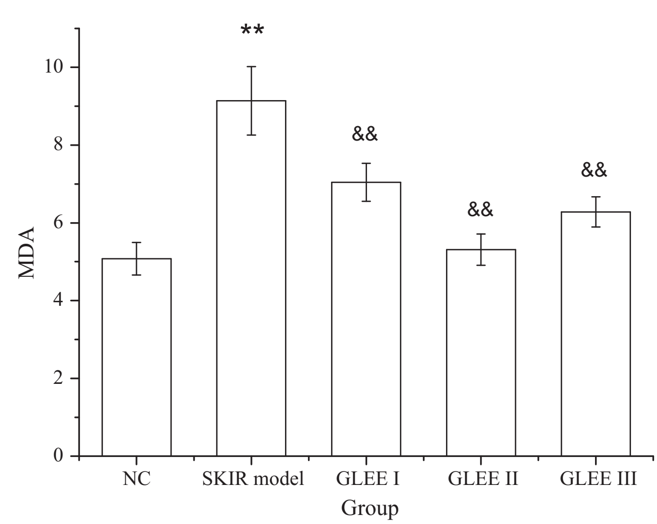

Fig. 5. Effect of ethanol extracts of ginkgo leaf on rabbits' skeletal muscle MDA. Each value represents mean ± SD; $n = 8$. $^{**}P < 0.01$, compared with normal control; $^{\&\&}P < 0.01$, compared with diabetic control.

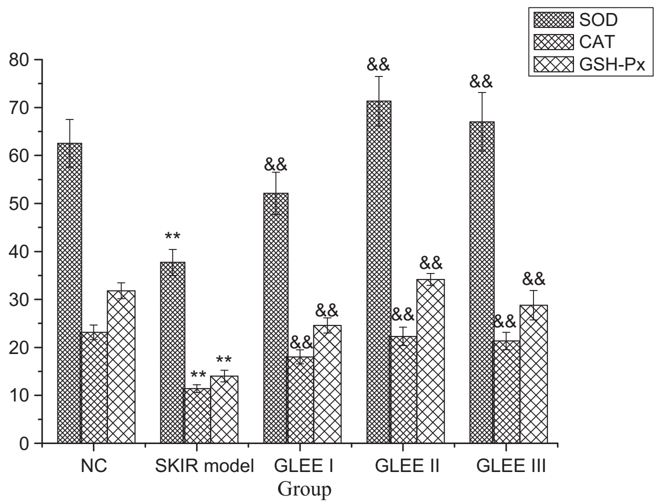

Fig. 6. Effect of ethanol extracts of ginkgo leaf on rabbits' skeletal muscle SOD, CAT and GSH-Px. Each value represents mean ± SD; $n = 8$. $^{**}P < 0.01$, compared with normal control; $^{\&\&}P < 0.01$, compared with diabetic control.

1993). It can be expected that these alterations will be aggravated during reperfusion. Figs. 5 and 6 exhibited that SKIR operation increased oxidative injury in rabbits' skeletal muscle, as indicated by rise in MDA level. Oral pre-treatment of rabbits with ethanol extract microcapsule (100 and 200 mg/kg b.w.) significantly ($P < 0.01$) decreased skeletal muscle MDA level. Similarly, there appears to be a parallel decrease in the activities of antioxidant enzymes in rabbits' skeletal muscle (Fig. 6). It was shown that Oral pre-treatment of rabbits with ethanol extract microcapsule (100 and 200 mg/kg b.w.) significantly ($P < 0.01$) increased skeletal muscle antioxidant enzymes activities (SOD, CAT and GSH-Px). In addition, pretreatment of ginkgo leaf ethanol extracts (60 mg/kg) also significantly ($P < 0.01$) increased skeletal muscle antioxidant enzymes activities (SOD, CAT and GSH-Px) in GLEE-treatment groups (I, II and III).

Compared with control rats (Fig. 7A), I/R injury of rats resulted in severe skeletal muscle damage, characterized by rhabdomyolysis and necrocytosis (Fig. 7B). Rats fed with microencapsulate ginkgo leaf extracts had less severe injury in skeletal muscle (Fig. 7C and D).

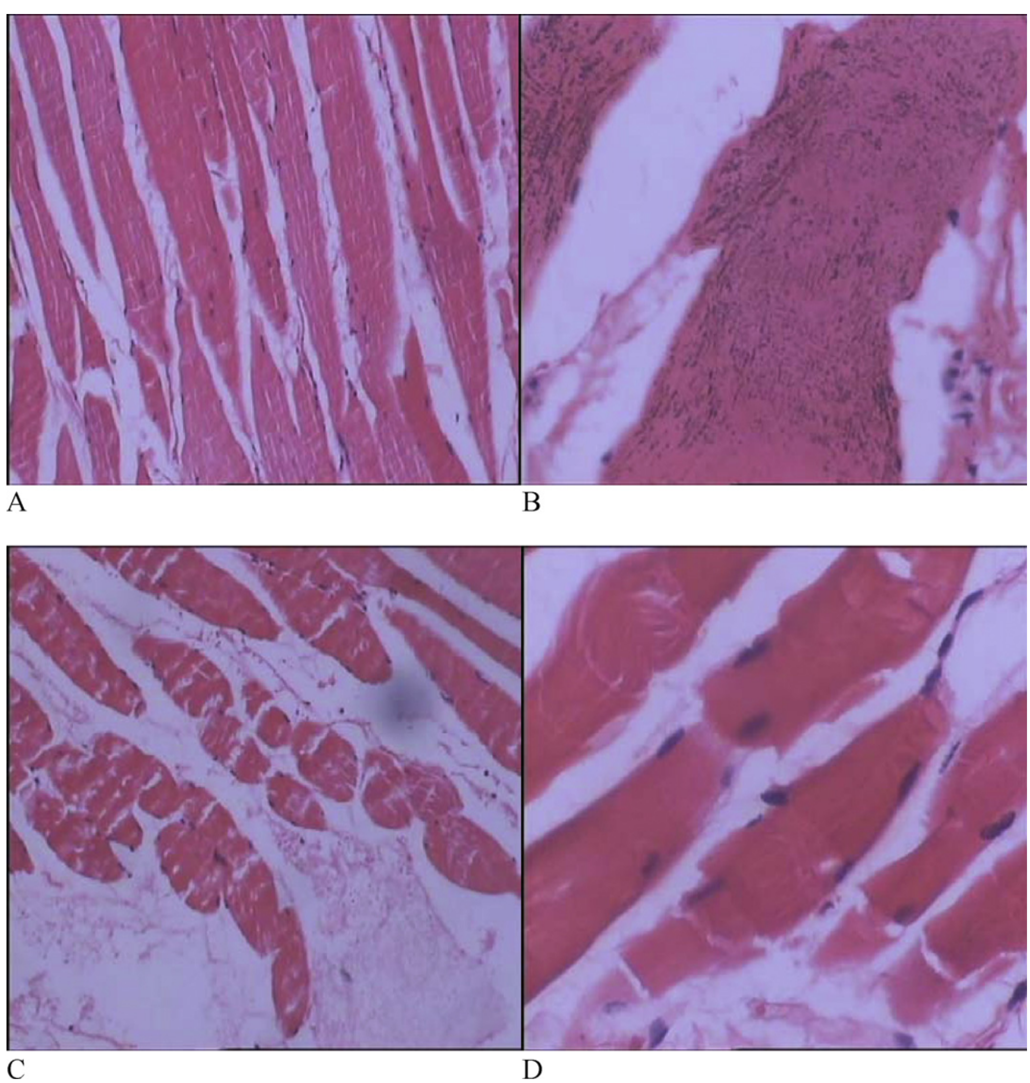

Fig. 7. Histology examination (H&E stainning).

4. Conclusion

Ginkgo leaf ethanol extracts microcapsules were produced using maltodextrin, gum arabic or a soluble soybean protein as a coating material by spray and freeze drying. It showed that the viscosity and stability of the emulsion solution were ideal when the homogenization number and homogenization pressure were 3 and 40 MPa. The results still indicated that, for the microcapsules, the encapsulation efficiency of 81.3% was achieved when air inlet temperature was 181 °C. At last, pharmacological test showed that ginkgo leaf ethanol extracts could enhance ALP activities and collagen I in mouse osteoblast MC3T3-E1 cells. In addition, pretreatment of ginkgo leaf ethanol extracts microcapsules could markedly skeletal muscle oxidative injury in IR rabbits. At the same dose, microencapsulation of ginkgo leaf ethanol extracts could exhibit better pharmacological function. A possible explanation is that microencapsulation may retard the release of active ingredients in ginkgo leaf ethanol extracts and maintain the longer pharmacological activities in body.

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