JPET/2002/46144

Low molecular weight fucoidan promotes therapeutic revascularization in a rat model of critical hind limb ischemia.

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This study was supported by grants from the Institut National de la Santé et de la Recherche Médicale. C.E. Luyt was supported by the Fédération Française de Cardiologie.

JPET Fast Forward. Published on January 21, 2003 as DOI: 10.1124/jpet.102.046144
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Running title : Therapeutic revascularization with low molecular weight fucoidan

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Number of text pages : 14

Number of tables : 0

Number of figures : 6

Number of words in the *Abstract*: 245

Number of words in the *Introduction*: 289

Number of words in the *Discussion*: 605

Abbreviations used in this paper :

SMC : smooth muscle cell

LMW: low molecular weight

HMW : high molecular weight

EDL : Extensorum Digitorum Longus

FGF-2 : Fibroblast Growth Factor 2

SDF-1: Stromal derived factor 1

Section option : Cardiovascular

ABSTRACT

The therapeutic potential of low molecular weight (LMW) fucoidan, a sulfated polysaccharide extracted from brown seaweed devoid of direct antithrombin effect, was investigated *in vitro* and in a model of critical hind limb ischemia in rat. *In vitro* results showed that LMW fucoidan enhanced FGF-2-induced ^{3}H-thymidine incorporation in cultured rat smooth muscle cells. Intravenous injection in rats of LMW fucoidan significantly increased Stromal-Derived-Factor-1 (SDF-1) level from 1.2 +/- 0.1 to 6.5 +/- 0.35 ng/ml in plasma. The therapeutic effect of LMW fucoidan (5mg/kg/day), FGF-2 (1µg/kg/day) and LMW fucoidan combined with FGF-2 was assessed 14 days after induction of ischemia by 1) clinical evaluation of claudication, 2) tissue blood flow analysis, 3) histo-enzymology of muscle metabolic activity, and 4) quantification of capillary density. Both LMW fucoidan and FGF-2 similarly improved residual muscle blood flow (62.5 +/- 6.5% and 64.5 +/- 4.5% respectively) compared to the control group (42 +/- 3.5%, $p<0.0001$). The combination of FGF-2 and LMW fucoidan showed further significant improvement in tissue blood flow (90.5+/-3%, $p< 0.0001$). These results were confirmed by phosphorylase activity, showing muscle regeneration in rats treated with the combination of FGF-2 and LMW fucoidan. Capillary density count increased from 9.6+/-0.7 capillaries/muscle section in untreated ischemic controls, to 14.3+/-0.9 with LMW fucoidan, 14.5+/-0.9 with FGF-2 and 19.1+/-0.9 with the combination ($p<0.001$).

Thus, LMW fucoidan potentiates FGF-2 activity, mobilizes SDF-1 and facilitates angiogenesis in a rat model. This natural compound could be of interest as an alternative for conventional treatment in critical ischemia.

INTRODUCTION

In recent years, therapeutic angiogenesis has been proposed in the treatment of chronic ischemia. In animals, it was shown that basic Fibroblast Growth Factor (FGF-2) (Gospodarowicz, 1974; Maciag et al., 1984), which is mitogenic for vascular endothelial cells, fibroblasts and smooth muscle cells, can induce angiogenesis *in vivo* (Yanagisawa-Miwa et al., 1992; Lefaucheur and Sebille, 1995; Sellke et al., 1996; Yang et al., 1996; Shou et al., 1997). FGF-2 binds to heparan sulfates that stabilize it by protecting it from proteolytic cleavage and enhance its bio-availability (Aviezer et al., 1994; Roghani et al., 1994; Pellegrini, 2001). Tissue heparan sulfates thereby serve as co-receptors for growth factors.

Fucoidans are vegetal sulfated polysaccharides extracted from brown algae. High molecular weight (HMW) fucoidans are known to bind growth factors, such as FGFs, and protect them from proteolysis (Belford et al., 1993). HMW fucoidans can release the glycosaminoglycan-bound Stromal Derived Factor-1 (SDF-1) from its tissue storage sites. SDF-1 mobilises medullary progenitors (Frenette and Weiss, 2000; Sweeney et al., 2000; Sweeney et al., 2002) which could participate in angiogenesis with VEGF and FGF (Salvucci et al., 2002). Therefore we supposed that fucoidans would have therapeutic potential in critical muscle ischemia.

A fraction of low molecular weight (LMW) fucoidan (7 +/- 2 kDa) was obtained by radical depolymerization of HMW extracts from brown seaweed (Nardella et al., 1996), and was devoid of any direct antithrombin effect (Haroun-Bouhedja et al., 2000). We have tested here the ability of LMW fucoidan to potentiate the effect of FGF-2 *in vitro* and to mobilize SDF-1 *in vivo* and have assessed the *in vivo* therapeutic effect using a rat model of critical hind limb ischemia previously developed in our laboratory (Luyt et al., 2000).

MATERIALS AND METHODS

LMW fucoidan extraction

Low-molecular-weight fucoidan (LMW fucoidan) was obtained by radical processing of HMW extracts from brown seaweed (Nardella et al., 1996). On the basis of previously reported analytical methods (Chevolot et al., 1999), the characteristics of LMW fucoidan were: weight-average molecular mass: 7 $\pm$ 2 kDa (polydispersity 1.7) ; fucose content: 35% (w/w) ; uronic acid content: 3% (w/w) and sulfate content: 34% (w/w). The anticoagulant activity of the LMW fucoidan was measured by activated partial thromboplastin (APTT) (Millet et al., 1999); the amount of LMW fucoidan required to obtain an APTT of 80 seconds (control, 40 seconds) was 25 µg/mL, a high concentration providing evidence of the low of direct ligation of thrombin.

Cellular pharmacology

Primary cultures of Wistar rat smooth muscle cells were used (Battle et al., 1994a). The effects of 10µg/ml of LMW fucoidan or LMW heparin (molecular weight 5 +/- 2 kD, Sigma, Saint-Louis, USA) on the FGF-2-induced DNA synthesis in SMCs were studied by ^{3}H-thymidine uptake (Battle et al., 1994b).

SDF-1 and MMP9 levels in plasma

HMW fucoidan (Sigma) (5mg/kg) or LMW fucoidan was injected at 5mg/kg into the jugular vein in 5 anesthetized rats. After one hour, blood was sampled on citrate, centrifuged and plasma collected. Plasma MMP-9 activities were determined by gelatin zymography as previously described (Jacob et al., 2002). Similarly, MMP-9 levels were measured *in vitro* after a one hour incubation with increasing concentrations (0 to 1 mg/ml) of LMW and HMW fucoidans with citrated rat blood. Plasma concentrations of SDF-1 were determined using an ELISA kit (Quantikine, UK).

Experimental model of critical hind limb ischemia

Surgical procedure

The surgical procedure has been described elsewhere (Luyt et al., 2000). Male Wistar rats (Iffa-Credo, L'Arbresle, France), weighing 280-320g and aged 10 weeks, were used for this study. The experimental design complied with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the care and use of the laboratory animals (NIH publication N.86-23 revised 1989. Authorization N° 00577, Paris, France). The animals were anesthetized with 50 mg/kg of sodium pentobarbital. Under a surgical microscope, the right external iliac and femoral arteries were dissected free from the origin of the external iliac artery (figure 1). Because external iliac artery ligation alone did not induce critical ischemia at rest in rats, ischemia was achieved by injection into the internal iliac artery via a retrograde catheter of 10,000 microspheres (Cytodex 2, Amersham Pharmacia Biotech AB) of 150 μm in diameter (figure 1). The catheter was removed, the external iliac artery ligated and excised, and the skin sutured. The contralateral hind limb was sham-operated by incision of the skin and dissection of the external iliac and femoral arteries.

Therapeutic design

To evaluate the effect of LMW fucoidan on critical ischemia, 4 groups of animals underwent the surgical procedure. In group A (control group, n = 10), animals received vehicle alone, i.e. Phosphate Buffer Saline (PBS) containing 1 % bovine serum albumin (BSA). In group B (n = 8), animals received 5 mg/kg/day of LMW fucoidan. In group C (n = 8), animals received 1μg/kg/day of recombinant FGF-2 purified as previously described (Patry et al., 1994). In group D (n = 8), animals received 1 μg/kg/day of FGF-2 plus 5 mg/kg/day of LMW fucoidan. The treatments or vehicle were administered immediately after surgery and then daily during the 14 days of the study by intra-muscular injection in the sham-operated hind limb. All 4 groups were evaluated 14 days after induction of ischemia by

clinical examination to assess claudication, measurement of tissue blood flow, detection of muscle phosphorylase activity and capillary count.

Tissue blood flow

A laser-doppler flowmeter (Perimed, Sweden) was used for evaluation of tissue blood flow (Nilsson et al., 1980; Luyt et al., 2000). Three muscles (tibialis anterior, biceps femoris and adductor) on each hind limb were studied. After removal of the skin, the probe was placed on the muscle and a signal was recorded. The Doppler signal was taken as an index of microvascular perfusion of the muscle area under the probe (6 mm) and at a depth of 1 mm. Three to five measurements on each muscle were recorded and averaged. Results represent "residual blood flow" in the ischemic hind limb and are expressed as a percentage of the muscular blood flow in the sham-operated hind limb. For each animal, 3 determinations were performed : before surgery, immediately after surgery and 14 days later.

Phosphorylase activity

Ischemic and non-ischemic Extensorum Digitorum Longus (EDL) muscles were excised in all groups at day 14, frozen in liquid nitrogen-cooled isopentane, and stored at –80°C (Luyt et al., 2000). The muscles were sectioned transversally at 8 µm using a cryostat and stained with hematoxylin and eosin for topographical examination. Additional sections were stained with 1% lugol substrate to study phosphorylase activity (Carlson and Gutmann, 1975). Phosphorylase activity indicates muscle glycogenolysis, and metabolically active skeletal muscle fibers stain brown, whereas a yellow stain indicates absence of metabolic activity (Carlson and Gutmann, 1975).

Capillary count

To detect angiogenesis, we performed a capillary count in ischemic and control muscles. Frozen sections of the EDL muscles (see above) were used. Sections were incubated with lectin from *Bandeiraea simplicifolia* (Sigma) as previously described (Alroy et al.,

1987). The reaction was amplified using Extravidin-Peroxidase (Sigma, Saint-Louis, USA) and revealed by diaminobenzidine (DAB)(Sigma, Saint-Louis, USA). Sections were counterstained with methyl-green, dehydrated and coverslipped before examination. The total number of capillaries on the area of the entire muscle cross section was counted.

Statistical analysis

All results are expressed as means +/- SEM. Comparisons of muscle perfusion in different groups were performed using a two-way ANOVA. Differences were analysed using a one-factor ANOVA with post hoc comparisons by the Fisher test. A Chi-2 test was performed to compare the positive phosphorylase activity between groups. A p value of <0.05 was considered statistically significant.

JPET Fast Forward. Published on January 21, 2003 as DOI: 10.1124/jpet.102.046144
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RESULTS

Thymidine incorporation by cultured SMCs

Because heparin-like molecules can modulate SMC proliferation by potentiation of FGF-2, we tested the effect of LMW fucoidan with FGF-2 on ^{3}H-thymidine uptake by rat aorta SMCs.

In agreement with previously published data (Bjornsson et al., 1991; Goncalves, 1998), FGF-2 stimulated the ^{3}H-thymidine uptake in SMCs (figure 2). The addition of LMW heparin had no effect on the growth of FGF2-stimulated SMCs, whereas the adjunction of LMW fucoidan potentiated FGF-2-induced ^{3}H-thymidine incorporation into SMCs (figure 2).

MMP-9 and SDF-1 levels in plasma

To test whether fucoidan can directly stimulate release of MMP-9 by leucocytes, we studied the effect *in vitro* of HMW and LMW fucoidans at different doses (0 to 1 mg/ml). HMW and LMW fucoidans were incubated with total citrated rat blood for one hour. Blood was then centrifuged, and MMP-9 release in plasma was measured by zymography. HMW and LMW fucoidans did not modify MMP-9 levels *in vitro* (data not shown). In contrast, *in vivo* intravenous injection in rats of HMW fucoidan increased both MMP-9 ($p<0.01$) and SDF-1 concentrations ($p<0.01$) (figure 3). Interestingly, LMW fucoidan did not modify MMP-9 levels *in vivo* but significantly increased SDF-1 concentration ($p<0.01$) (figure 3).

Critical hind limb ischemia

To evaluate the ability of LMW fucoidan alone or combined with FGF-2 to modulate rat hind limb ischemia, we assessed its ability to improve clinical status, muscle blood flow and activity.

Clinical evolution

All animals 14 days after surgery presented ischemia at rest and muscular atrophy. All the animals in the control group presented claudication without skin necrosis. In the group B

(FGF-2-treated group) and C (LMW fucoidan-treated group), rats had less critical ischemia at rest than those in the control group. In group D (combined treatment of FGF-2 and LMW fucoidan), 3 of the 8 animals showed a complete resolution of their claudication after 14 days of treatment ($p < 0.05$ in comparison with control group).

Muscle perfusion

Functional evaluation before the induction of ischemia showed that muscular blood flow was similar in the two hind limbs for all animals. Immediately after surgery, the residual blood flow in the ischemic hind limb (compared to the sham-operated hind limb) decreased from 100 % to approximately 15 % (figure 4).

Fourteen days after induction of ischemia, the mean residual blood flows of the control group had risen from 15 +/- 5.1 % to 42 +/- 3.5 %. In the FGF-2-treated group and the LMW fucoidan-treated group, mean residual blood flows at day 14 were 64.5 +/- 4.5 % and 62.5 +/- 6.5 % respectively ($p<0.0001$ in comparison with control group). In the combined-treatment group, this value reached 90.5 +/- 3 % ($p<0.0001$ in comparison with other groups)(figure 4).

Phosphorylase activity

Fourteen days after surgery, no phosphorylase activity was detectable in the ischemic muscles of the control group. A diffuse yellow staining of ischemic muscle sections was observed whereas the normal muscles of the sham-operated side all stained brown, indicating ATP-dependent phosphorylase activity. On the hematoxylin and eosin-stained cross-sections of the ischemic muscles, there was evidence of severe diffuse cellular ischemia, shown by a loss of architecture of the muscle, disappearance of the muscle nuclei and inflammatory cell infiltration, as compared with sections from normal muscle (figure 5).

In the LMW fucoidan-treated group, all animals had partial regeneration of the ischemic muscle. In the FGF-2-treated group, one rat showed complete regeneration of the muscle, and the 7 others had partial regeneration of the muscle. In the combined-treatment group (group D), 3 animals had complete regeneration of the EDL muscle, observed both with

hematoxylin and eosin-staining and with phosphorylase activity, and the 5 others showed partial regeneration of the EDL muscle (figure 5).

Capillary count

Figure 6 shows the results of quantification of capillary density. In the control group, there were significantly less capillaries than in the other groups ($p<0.001$ in comparison with all groups). FGF-2 or LMW fucoidan alone significantly increased the number of capillaries per section as compared to untreated ischemic muscles ($p<0.001$). Combined treatment with FGF+LMW fucoidan enhanced further the capillary density ($p<0.001$) (figure 6).

DISCUSSION

FGF-2 is mitogenic for vascular cells (Burgess and Maciag, 1989) and enhances the migration of vascular cells both *in vitro* and *in vivo* (Lindner and Reidy, 1991). Previous studies in animals (Lefaucheur and Sebille, 1995; Sellke et al., 1996; Shou et al., 1997) demonstrated the ability of FGF-2 to improve revascularization *in vivo*. HMW fucoidan in association with FGF-2 was previously reported to improve endothelial cell proliferation *in vitro* (Giraux et al., 1998). Despite the direct *in vitro* and *in vivo* inhibitory effects of HMW and LMW fucoidan on vascular smooth muscle cell growth (McCaffrey et al., 1992; Logeart et al., 1997a; Logeart et al., 1997b; Deux et al., 2002), our results indicate that LMW fucoidan potentiates the effect of FGF-2 on ^{3}H-thymidine uptake. We have extended these concepts to experimental therapeutics, showing that LMW fucoidan promotes FGF-2 effects *in vivo*, suggesting its potential interest for use in vascular tissue repair (Deux et al., 2002) and angiogenesis (Religa et al., 2000; Matou et al., 2002).

Effects of FGF-2 *in vivo* are multiple and complex including an arterial vasodilatory effect (Cuevas et al., 1991) and mitogenic properties on vascular cells (Gospodarowicz, 1974; Maciag et al., 1984). The half-life of FGF-2 is short but is prolonged when sulfated polysaccharides are co-infused (Whalen et al., 1989; Lazarous et al., 1997). However, unfractionated HMW and LMW heparins alone were reported to have no therapeutic effects in angiogenesis (Rosengart et al., 1997). Contrasting with these results, we demonstrate here, for the first time, a beneficial effect in revascularization with of LMW fucoidan *in vivo*.

At the site of the injury, tissue repair is in part mediated by growth factors such as FGFs which are released from their extracellular or cellular glycosaminoglycan storage sites. As already described for HMW fucoidans (Belford et al., 1993) and for other natural and synthetic heparan sulfates (Belford et al., 1993; Aviezer et al., 1994; Roghani et al., 1994; Meddahi et al., 1995; Meddahi et al., 1996; Rusnati and Presta, 1996), LMW fucoidan may act *in vivo* by trapping and protecting endogenously released FGFs from deactivation and

proteolytic cleavage and may also displace endogenous FGFs from their tissue heparan sulfate storage sites thus increasing their bio-availability.

Another effect of fucoidan is the ability to promote progenitor stem cell mobilization via the release of SDF-1 from heparan sulfate storage sites (Amara et al., 1999; Sadir et al., 2001). SDF-1 is a heparin binding cytokine (Lortat-Jacob et al., 2002) involved in angiogenesis (Mirshahi et al., 2000). It has been recently shown that SDF-1 regulates endothelial cell branching morphogenesis (Salvucci et al., 2002) and conversely that FGF-2 and VEGF upregulate the expression of SDF-1 receptors (CXCR4) on endothelial cells (Salcedo et al., 1999). Thus, SDF-1 mobilization could be one of the molecular effectors of therapeutic revascularization. Our results indeed show an increased SDF-1 concentration in plasma after a single bolus injection of LMW fucoidan. This effect was previously described by Sweeney *et al.* for HMW fucoidan and other glycosaminoglycans such as dextran sulfates and chondroitin sulfates (Sweeney et al., 2002). Sweeney *et al.* also indicated that plasma MMP-9 significantly increased in response to intravenous injection of HMW fucoidan. In contrast, LMW fucoidan did not induce an increase in MMP-9 level *in vivo*. These results suggest that sulfated polysaccharides from the same family may exhibit different properties depending on their electrical charges, their degree of sulfatation and their molecular weight.

In conclusion, this study demonstrates for the first time, the therapeutic potential of LMW fucoidan in experimental critical hind limb ischemia, providing a promising new tool for the promotion of revascularization. In addition, this polysaccharide of natural origin has no direct antithrombic effect, allowing clinical applications without hemorrhagic side-effects.

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LEGENDS TO FIGURES

Figure 1 : Experimental model of critical hind limb ischemia. A catheter is introduced into the femoral artery and microspheres are injected into the internal iliac artery. The femoral and external iliac arteries are then excised(Luyt et al., 2000). Animals were treated during 14 days by intra-muscular injections of 5 mg/kg/day LMW fucoidan or 1 µg/kg/day FGF-2 or a combination of both, or vehicle alone.

Figure 2: Proliferation of vascular smooth muscle cells. H^3-Thymidine incorporation in aortic rat smooth muscle cells after addition of various concentrations of FGF-2 alone or associated with 10µg/ml of LMW fucoidan or LMW heparin. * indicates significant differences between FGF-2 and FGF-2+LMW fucoidan treatment ($p<0.001$).

Figure 3: Metalloproteinases and SDF-1 in plasma after intravenous injection of fucoidans. 5 mg/kg of HMW fucoidan (●) or LMW fucoidan (Δ) were injected intravenously in 5 Wistar rats**.** After one hour, plasma samples were analysed for MMP9 activities (Figure 3A) by gelatin zymography (inset figure A) and pro-MMP9 activities were quantified using NIH software analysis. Concentration of SDF-1 in plasma (Figure 3B) was quantified using an ELISA test (Quantikine). T0 = HMW fucoidan or LMW fucoidan injection, T1: one hour after HMW fucoidan or LMW fucoidan injection. Results are expressed as means +/- sem.

Figure 4: Effects of LMW fucoidan treatment with or without FGF-2 on blood flow. Residual blood flow (% of contralateral values) observed immediately (Day 0) and 14 days (Day 14) after induction of ischemia. Results are expressed as means +/- sem. ANOVA was used to compare the LMW fucoidan or FGF-2 groups with the control group ($p<0.0001$=*) and the LMW fucoidan + FGF-2 groups with the other groups ($p<0.0001$=**).

Figure 5: Morphological studies of muscle sections after 14 days of treatment. Transverse sections of control (sham operated animal), ischemic and treated muscle (LMW fucoidan)

were stained with hematoxylin-eosin and assessed for phosphorylase activity. In treated animals partial regeneration was observed. Magnification : x10.

Figure 6: Effects of LMW fucoidan treatment with or without FGF-2 on capillary density. The capillary count per muscle section was performed after 14 days of treatment. Statistical analysis was performed using ANOVA test and compared the control group with the LMW fucoidan and with the FGF-2 treated group (*=$p<0.001$) or the control group with the LMW fucoidan + FGF-2 treated group (**= $p<0.0003$).

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Figure 1

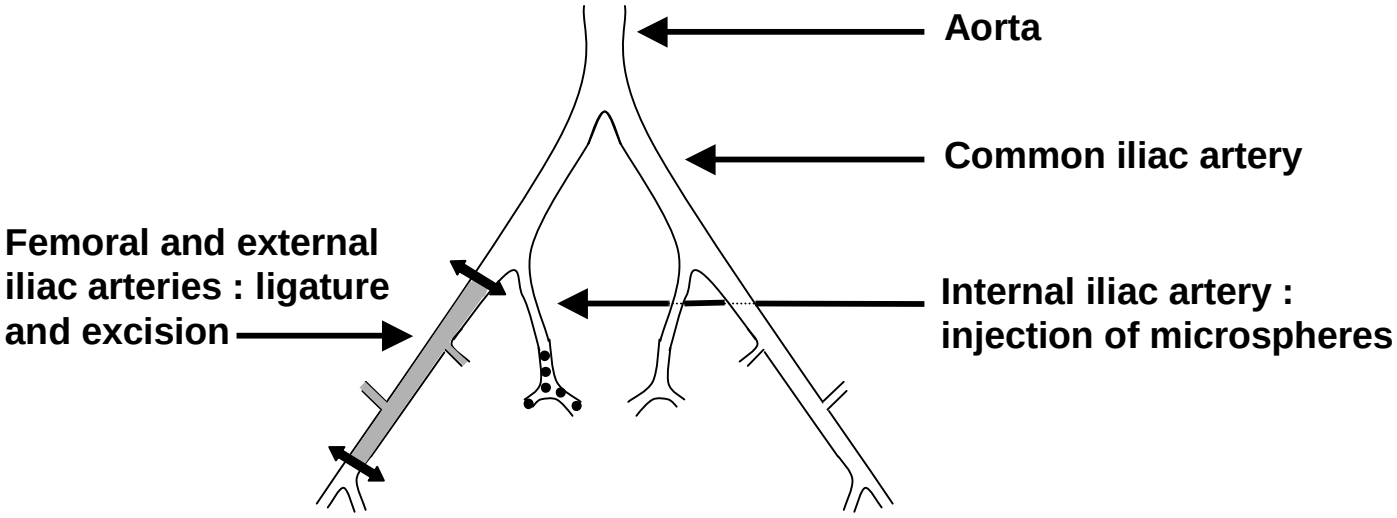

Figure 2

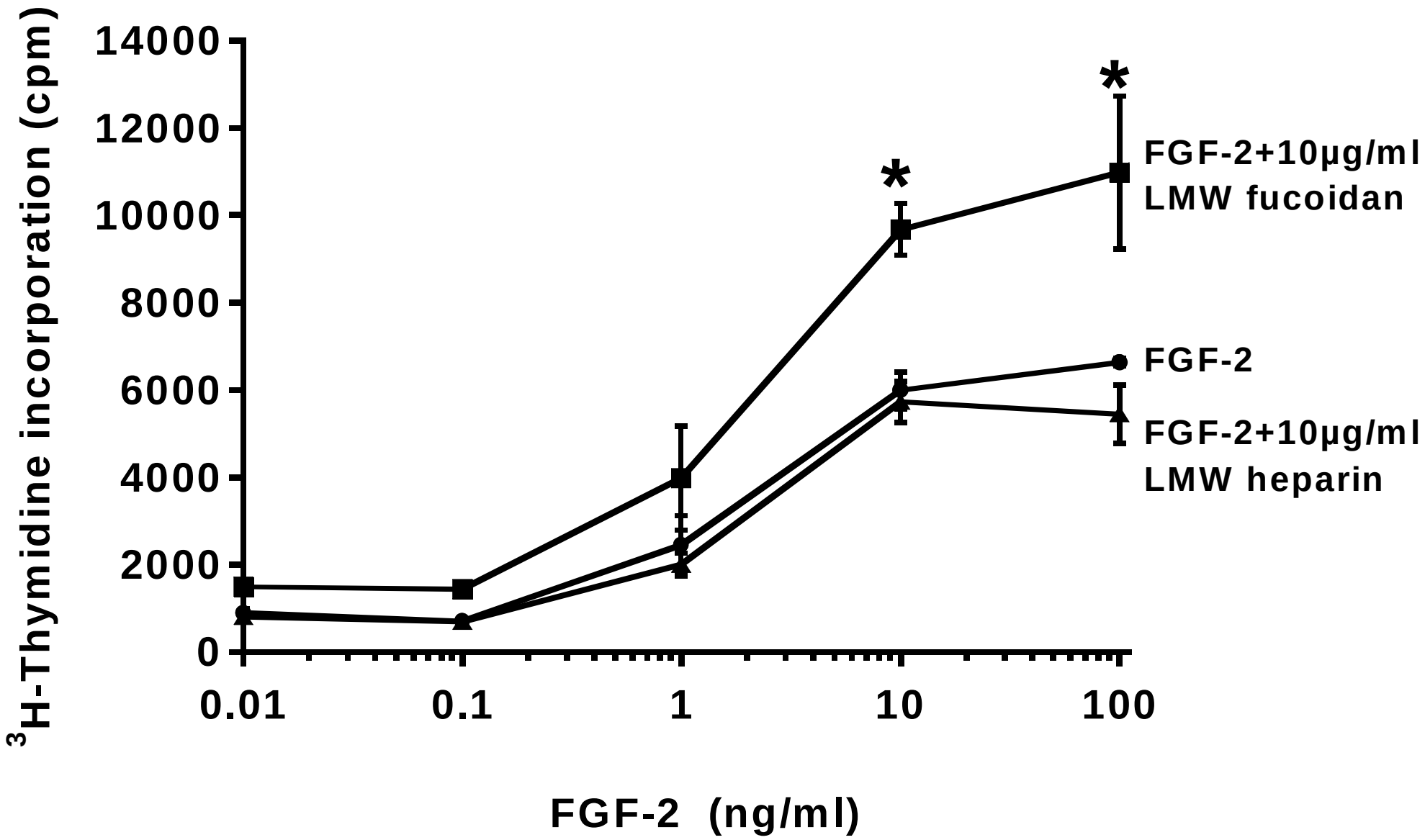

Figure 3

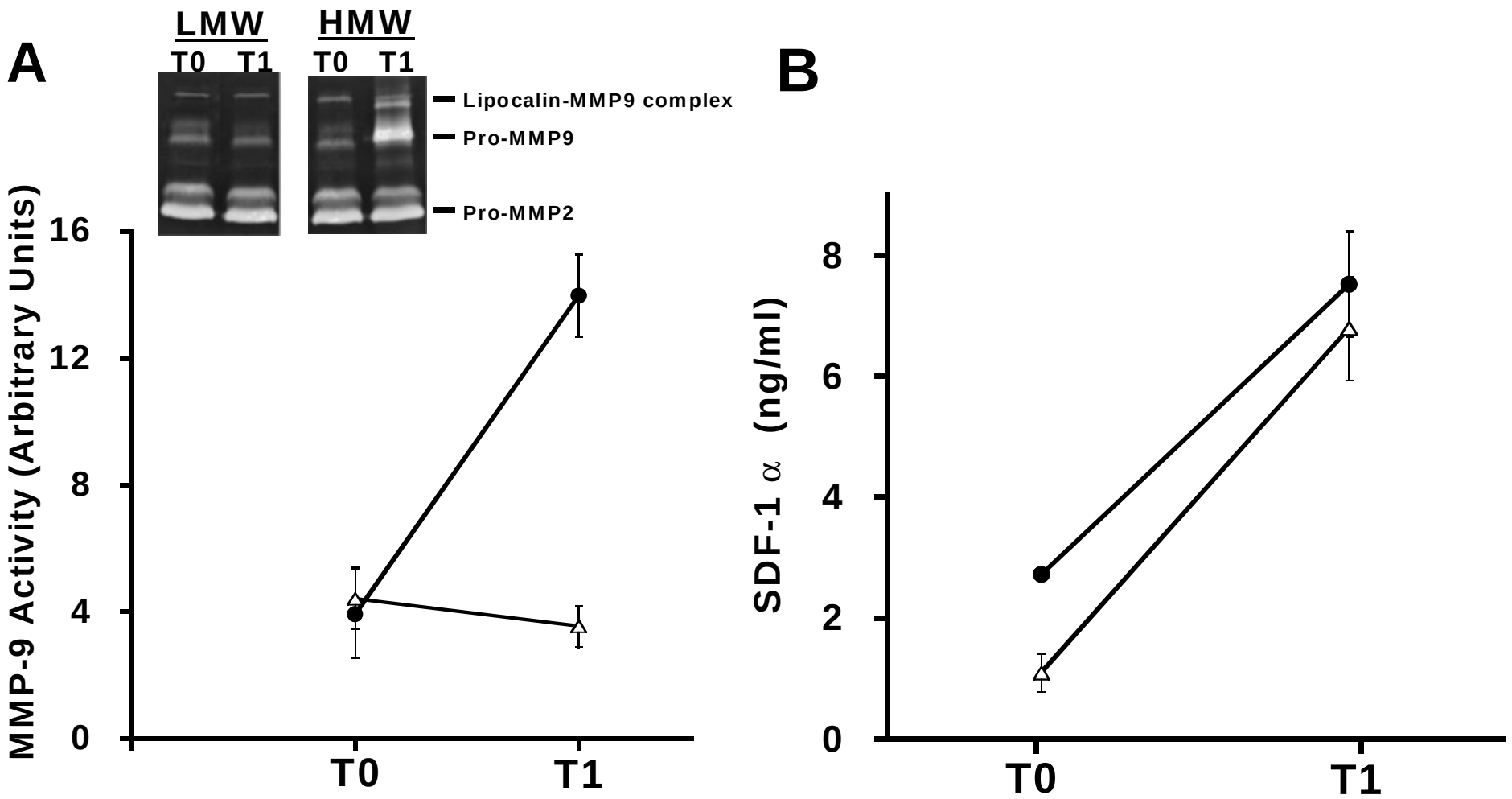

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Figure 4

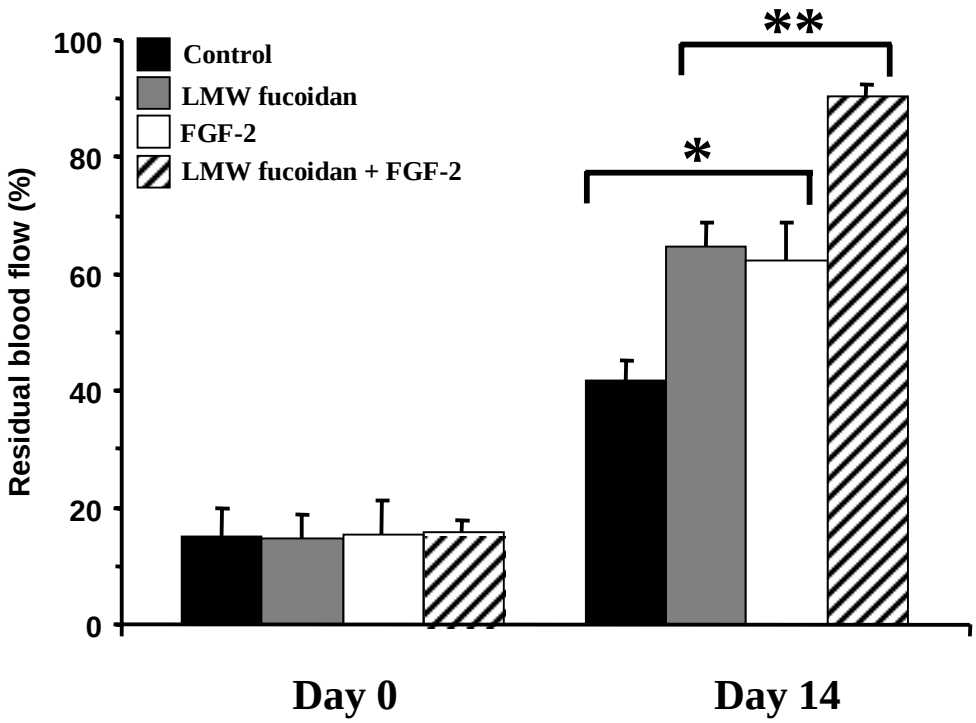

Figure 5

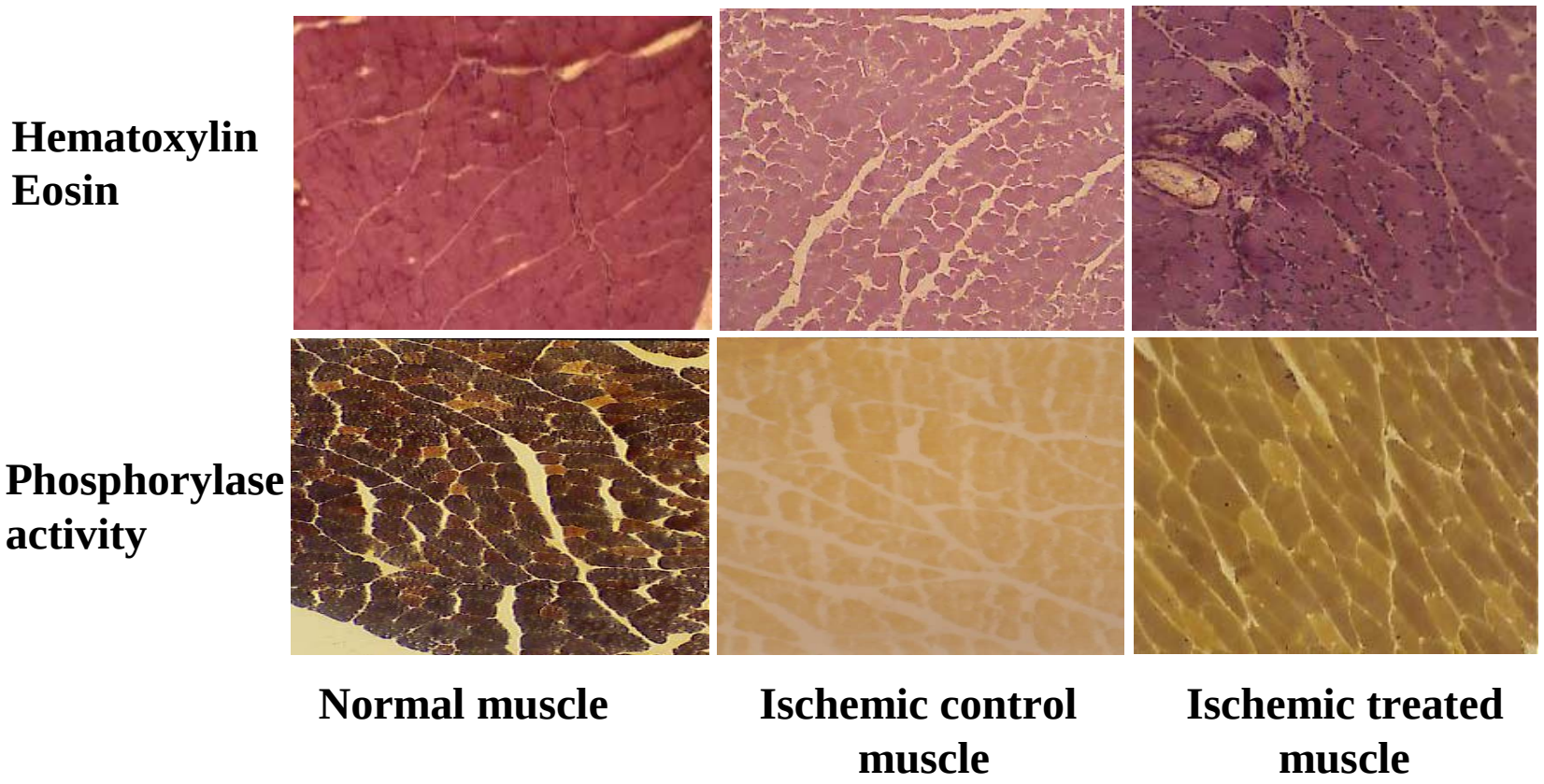

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Figure 6