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BRIEF REPORT

Low-power laser irradiation fails to improve liver regeneration in elderly rats at 48 h after 70 % resection

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Abstract The liver regeneration is an important clinical issue after major hepatectomies. Unfortunately, many organs (including the liver) exhibit age-related impairments regarding their regenerative capacity. Recent studies found that low-power laser irradiation (LPLI) has a stimulatory effect on the liver regeneration process. However, its effects in elderly remain unknown. Thus, this study aimed to investigate the main molecular mechanisms involved in liver regeneration of partially hepatectomized elderly rats exposed to LPLI. The effects of 15 min of LPLI (wavelength of 632.8 nm; fluence of 0.97 J/cm^2; total energy delivered of 3.6 J) were evaluated in hepatectomized elderly *Wistar* male rats. Afterwards, through immunoblotting approaches, the protein expression and phosphorylation levels of hepatocyte growth factor (HGF), Met, Akt and Erk 1/2 signaling pathways as well as the proliferating cell nuclear antigen (PCNA) were investigated. It was observed that LPLI was not able to improve liver regeneration in elderly rats as evidenced by the lack of improvement of HGF and PCNA protein expression or phosphorylation levels of Met, Akt and Erk 1/2 in the remnant livers. In sum, this study demonstrated that the main molecular pathway, i.e. HGF/Met→Akt and Erk 1/2→PCNA, involved in the hepatic regeneration process was not improved by LPLI in elderly hepatectomized rats, which in turn rules out LPLI as an adjuvant therapy, as observed in this protocol of liver regeneration evaluation (i.e. at 48 h after 70 % resection), in elderly.

Keywords Hepatectomy · Laser · Liver regeneration · Aging · Elderly · HGF · Met · Proliferation

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Introduction

Currently, liver surgery has achieved standards that were not imaginable in the past, especially in terms of the amount of parenchyma that can be resected. Indeed, this procedure is now being performed more safely due to progress in surgical techniques and instruments [1–3]. The capacity of the liver to regenerate is an important clinical issue after major hepatectomies and certainly makes the difference between life and death in some cases of postoperative malfunction when the liver remnant is too small or has an impaired regenerative capacity [1–6]. In this sense, several approaches have been tested to stimulate liver regeneration after a massive resection and in situations in which the liver remnant may be too small; however, they have produced controversial results [4, 5, 7–9].

Recently, an important review demonstrated that low-power laser irradiation (LPLI), particularly red and near infrared light, plays an important role by inducing regeneration in various tissues, especially by stimulating cell proliferation [10]. LPLI has been presented as a simple, easy, safe, and effective adjunctive tool for improving the capacity of liver regeneration [11–16]. A recent study by our group

demonstrated the main molecular mechanisms associated with the abovementioned effect, since it demonstrated that LPLI acts by increasing hepatocyte growth factor (HGF) protein expression and the phosphorylation levels of Met, Akt and Erk 1/2, accompanied by higher levels of proliferating cell nuclear antigen (PCNA) and Ki-67 (established proliferating markers) in the remnant liver of partially hepatectomized rats [16].

It is now recognized that many organs exhibit important age-related impairments and accordingly, a modest amount of information is available regarding the behaviour of the cellular and molecular mechanisms that regulate liver regeneration during the aging process [17]. Furthermore, it is important to study techniques for conditioning the liver remnant that can be used to improve this age-related deficiency. As far as we know, there are no studies that demonstrate how the abovementioned molecular mechanisms involved in laser-induced hepatic regeneration behave during aging. Indeed, it is unknown whether the well-characterized laser-induced liver regeneration occurs in the elderly. For this reason, the purpose of this brief report is to present the age-related effect of LPLI on the main molecular mechanisms involved in liver regeneration in elderly hepatectomized rats (70 % hepatectomy).

Materials and methods

Animals

Fifty-week-old male *Wistar* rats weighing 550–600 g were obtained from the State University of Campinas Central Breeding Centre. The animals were maintained under a controlled room temperature (23±2 °C) under a 12/12 h light and dark cycle and were fed standard laboratory (rodent) chow and water ad libitum. All experimental protocols were approved by the Animal Care and Use Committee at the State University of Campinas (no. 1962-1) and were in accordance with the guidelines for the Care and Use of Laboratory Animals.

All aged animals were randomly assigned to three groups, each comprising six rats: A-sham (sham-operated controls), A-PHx (subjected to partial hepatectomy (70 %)), and A-PHx+laser (subjected to partial hepatectomy (70 %) and laser therapy).

70 % partial hepatectomy (Higgins procedure)

Animals were anaesthetized with ketamine 5 % (30 mg/kg) and xylazine 2 % (30 mg/kg) intraperitoneally. Under strict sterile conditions, two thirds partial hepatectomy was performed according to the method of Higgins and Anderson [18]. In sham-operated controls that were anaesthetized as described above, the livers were briefly removed from the peritoneal cavity, but were not tied or excised.

During surgery, the animals were kept in the same conditions as previously described [16].

Laser treatment

Before closing the surgical wound, the laser group was treated by direct irradiation of the remnant liver with a He-Ne laser model 3184H V1.0 (Hughes® Aircraft Company Electron Dynamics Division, Culver City, CA, USA). All LPLI characteristics are shown in Table 1. The parameters of laser irradiation and the anatomical points were selected based on earlier study [16]. The laser beam was optically expanded to match the entire size of the remaining liver to guarantee uniform exposure. Recordings from the thermocouple placed under the irradiated area showed no elevation of temperature in the abdomen of the rats.

Tissue extraction and immunoblotting

Forty-eight hours after the partial hepatectomy and laser exposure, rats were anaesthetized and used 10–15 min later, as soon as anaesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened and any remaining liver tissue was removed and homogenized immediately in extraction buffer at 4 °C as described previously [16, 19]. After centrifugation, the whole tissue extracts were subjected to SDS-PAGE and immunoblotted according to a previous report [16, 19]. The homogeneity of gel loading was evaluated by blotting the membranes with antibodies against β-actin, Met, Akt, PCNA and Erk 1/2 as appropriate.

Materials

As described previously, all antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), with the exception of phospho (Tyr1234/1235)-Met, Met, phospho (Ser 473)-Akt and Akt, which were obtained from Cell Signaling Technology (Beverly, MA, USA) [16]. Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless specified otherwise.

Statistical analysis

Data are displayed as mean±standard error of the mean (S.E.M.). The results of blots are presented as direct comparisons of bands or spots in autoradiographs and quantified by optical densitometry (UN SCAN IT gel®, Silk Scientific Inc., Orem, UT, USA). Multiple comparisons were tested by one-way ANOVA, followed by Tukey's post hoc test, with the significance level set at $p<0.05$ using SPSS software (SPSS for Windows, version 16.0, Chicago, IL, USA).

Table 1 Parameters of the laser used in the experimental procedures

Parameters of laser (He-Ne)	Values
Wavelength	632.8 nm
Frequency	Continuous wave (CW)
Optical power output	4 mW
Spot diameter	1.6 cm
Fluence	0.97 J/cm^2
Total energy delivered	3.6 J
Distance	10 cm
Irradiation time	15 min

Application mode: a single application of laser was used in the A-PHx laser group; the laser beam irradiation was directed to the remaining liver with a 90° angle

Results

The HGF/Met axis does not respond to stimulation with laser in elderly hepatectomized rats

As expected, HGF protein expression in the remnant liver of the A-PHx group was augmented (approximately two-fold) in comparison to the A-sham group, but surprisingly, any additional improvement in HGF expression in the A-PHx+laser group when compared with the A-PHx group (Fig. 1a) was not observed. The phosphorylation levels of the HGF receptor (Met) were also evaluated, and unsurprisingly, the A-PHx group showed an increase in tyrosine phosphorylation levels of Met (~3.5-fold) in comparison with the A-sham group (Fig. 1b). On the other hand, the elderly hepatectomized rats exposed to laser showed no improvement in activation of Met compared with the A-PHx group (Fig. 1b). Additionally, no changes in Met total protein expression were observed among any of the groups (Fig. 1b; lower panel).

Proliferation and growth-related pathways showed no improvement in elderly hepatectomized rats exposed to LPLI

The results showed that the expression of phospho-Akt protein in the A-PHx+laser group was similar to that in the PHx group (Fig. 2a). Likewise, expression of the phospho-Erk 1/2 protein in the A-PHx+laser group was unchanged in comparison with the A-PHx group (Fig. 2b). Indeed, no changes in Akt and Erk 1/2 total protein expression were observed among any of the groups (Fig. 2a, b; lower panels). It was also observed that the absence of improvement on activation of Akt and Erk 1/2 was closely related to the lack of changes in PCNA expression in the A-PHx+laser group when compared with the PHx group (Fig. 2c).

Discussion

It has previously been reported that there is a three to fivefold higher rate of deaths from liver disease in those aged over 65 years compared with those under 45 years [20]. Moreover, there are data showing that the decline in the rate of hepatic regeneration following partial resection (hepatectomy) or chemically induced injury is the main clinical age-related deficiency in liver regeneration [17]. Also, it has been demonstrated in the clinical setting that elderly patients may have an impaired capacity for recovery after major hepatectomies due to primary or metastatic tumours [17]. In the face of these difficulties, this paper demonstrated the effects of LPLI exposure (i.e. a technique for conditioning the liver remnant) in elderly hepatectomized rats, mainly by studying some of the molecular mechanisms involved in liver regeneration (i.e. HGF/Met system and their downstream signaling).

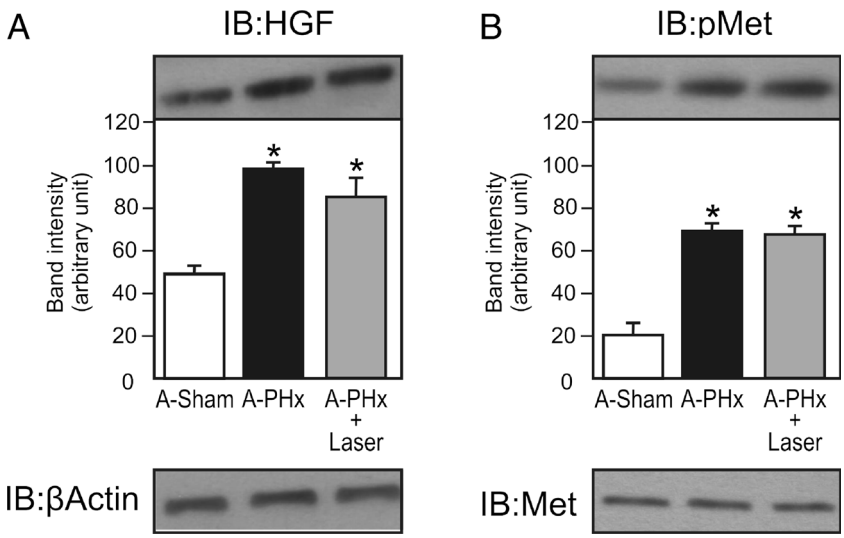

Fig. 1 Representative blottings show HGF expression (**a**) and Met phosphorylation (**b**) levels in remaining livers of partial elderly-hepatectomized rats exposed or nonexposed to the laser. Total protein expression of Met (**b** *lower panel*). Western blots were quantified after standardization with β-actin. Data were representative of three independent experiments. The values represent the mean±SEM (n=6). *p<0.05 vs. A-sham. *IB* immunoblot

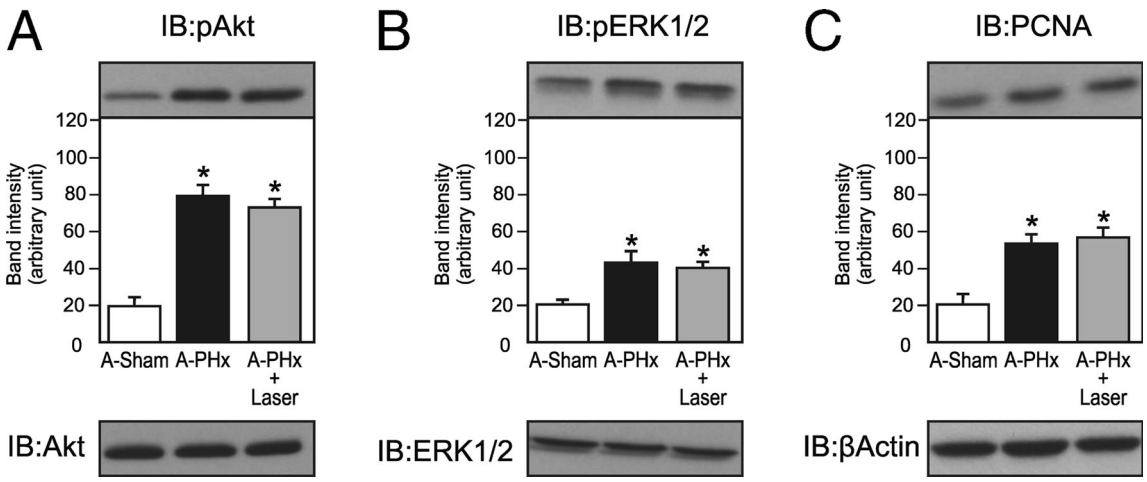

Fig. 2 Representative blottings show Akt (**a**) and Erk 1/2 (**b**) activation as well as PCNA content (**c**) in remaining livers of partial elderly-hepatectomized rats exposed or nonexposed to the laser. Total protein expression of Akt and Erk 1/2 (**a** and **b** *low panel*, respectively). β-actin was used as a normalization housekeeping protein. Data were representative of three independent experiments. The values represent the mean± SEM (n=6). *p<0.05 vs. A-sham. *IB* immunoblot

Initially, this study was based on a well-established model known as 'Higgins procedure', which mimics the hepatic regeneration process, and previous studies using animals and hepatocytes culture that found that the molecular mechanisms associated with the initiation and control of proliferative events after partial hepatectomy procedure are closely related to HGF signaling [16, 21]. It is worth to mention that the hepatocytes initiate mitosis 6–8 h after the surgery, reaching its maximum 48 h later [21]. Therefore, it was chosen to study the remaining livers 48 h after partial hepatectomy and exposure to laser surgery. Indeed, the HGF pathway is the most important contributor to hepatic regeneration [16, 21]. With this in mind and also given that our group has previously demonstrated the key role of this pathway in the improvement of liver regeneration following treatment with LPLI [16], we decided to study this pathway in elderly hepatectomized rats exposed to LPLI. Surprisingly, the current research demonstrated that in elderly hepatectomized rats, LPLI did not improve the expression of HGF or phosphorylation levels of Met in the remnant liver. Therefore, these data conflicted with previous study in which laser exposure induced an improvement in the HGF/Met system during liver regeneration in young rats [16]. In this way, this study identified the influence of age on the effect of lasers on the hepatic regeneration process. This was reinforced by the fact that the downstream proteins of Met, such as Akt and Erk 1/2, which are associated with the cellular process of differentiation, proliferation, growth and apoptosis, unlike in young rats [16], were not upregulated in the remnant liver of old rats that were exposed to laser. Moreover, we observed that the expression of PCNA (a well-established proliferation marker) following laser irradiation showed the same behaviour as the other proteins mentioned above in the remnant liver of aged rats.

In order to discuss the effects of LPLI on the main molecular mechanisms involved in liver regeneration in elderly hepatectomized rats in more detail, the following points should be taken into consideration. Without doubt, the reactive oxygen species (ROS) is a collaborator in this lack of

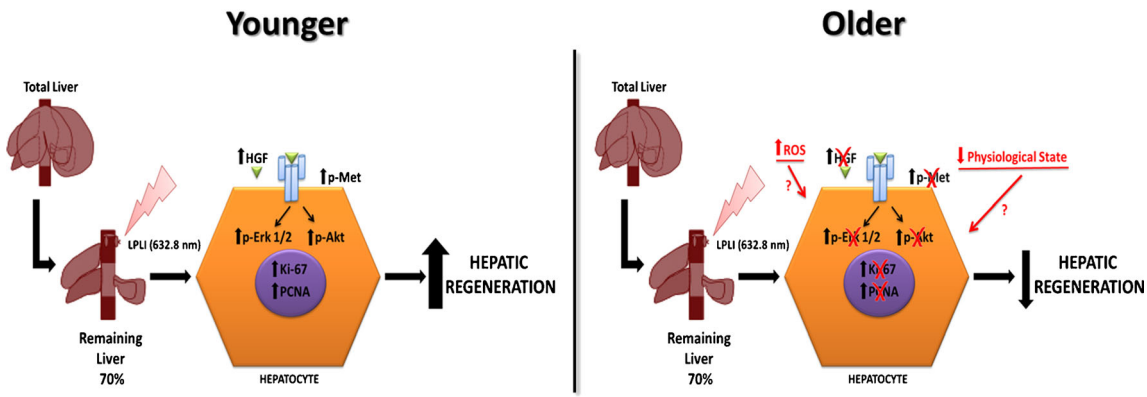

Fig. 3 Schematic representation shows a comparison of the effects of LPLI on liver regeneration in younger versus older animals. Furthermore, it is suggested the mechanisms behind the ineffectiveness of the laser to increase liver regeneration in aging

effect of laser irradiation, since an increase in ROS generation accompanied by the initiation of apoptosis in hepatocytes was observed after partial hepatectomy in elderly mice [22]. Indeed, another study proposed that decreased activation of Erk in old rodents following partial hepatectomy is also an important contributor [23]. Furthermore, it has been shown that the lag period (the interval between two related phenomena) for cell division correlates with the age of the animal [24]. Following this line of reasoning, a previous in vitro study demonstrated through satellite cells that the activation and proliferation induced by laser irradiation decreased with age and also suggested that this reduction in the effect of the laser is related to the longer lag time in satellite cells derived from older rats [25]. In other words, the older cells remain dormant and do not become active, while the younger cells immediately respond to the stimulus. In this sense, a previous hypothesis helps to explain these findings by stating that the 'physiological state' of the cells is the determining factor of the effects of laser irradiation [10].

In summary, unlike in our previous study conducted in younger hepatectomized rats, we have demonstrated that the main molecular pathway, i.e. HGF/Met→Akt and Erk 1/2→PCNA, involved in the hepatic regeneration process is not improved by LPLI in elderly hepatectomized rats (Fig. 3). Thus, based on the data of this study, we concluded that LPLI is not suitable as an adjuvant therapy at least in this investigated protocol of liver regeneration, i.e. at 48 h after 70 % resection, in elderly. Finally, it is clear that the development of tools that are able to improve liver regeneration during aging should be an issue of extreme interest to the scientific community.

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Conflict of interest No potential conflicts of interest relevant to this article were reported.

References

1. van den Broek MA, Olde Damink SW, Dejong CH, Lang H, Malago M, Jalan R, Saner FH (2008) Liver failure after partial hepatic resection: definition, pathophysiology, risk factors and treatment. Liver Int 28(6):767–780
2. Hammond JS, Guha IN, Beckingham IJ, Lobo DN (2011) Prediction, prevention and management of postresection liver failure. Br J Surg 98(9):1188–1200
3. Clavien PA, Petrowsky H, DeOliveira ML, Graf R (2007) Strategies for safer liver surgery and partial liver transplantation. N Engl J Med 356(15):1545–1559
4. Yigitler C, Farges O, Kianmanesh R, Regimbeau JM, Abdalla EK, Belghiti J (2003) The small remnant liver after major liver resection: how common and how relevant? Liver Transpl 9(9):S18–S25
5. Di Domenico S, Santori G, Traverso N, Balbis E, Furfaro A, Grillo F, Gentile R, Bocca B, Gelli M, Andorno E, Dahame A, Cottalasso D, Valente U (2011) Early effects of portal flow modulation after extended liver resection in rat. Dig Liver Dis 43(10):814–822
6. Court FG, Wemyss-Holden SA, Dennison AR, Maddern GJ (2002) The mystery of liver regeneration. Br J Surg 89(9):1089–1095
7. Teixeira AR, Molan NT, Bellodi-Privato M, Coelho AM, Leite KR, Seguro AC, Bacchella T, Machado MC (2008) Rosiglitazone-enriched diet did not protect liver ischemia-reperfusion injury in a rat model. Acta Cir Bras 23(4):378–383
8. Teixeira AR, Molan NT, Kubrusly MS, Bellodi-Privato M, Coelho AM, Leite KR, Machado MA, Bacchella T, Machado MC (2009) Postconditioning ameliorates lipid peroxidation in liver ischemia-reperfusion injury in rats. Acta Cir Bras 24(1):52–56
9. Seyama Y, Imamura H, Inagaki Y, Matsuyama Y, Tang W, Makuuchi M, Kokudo N (2012) Intermittent clamping is superior to ischemic preconditioning and its effect is more marked with shorter clamping cycles in the rat liver. J Gastroenterol 48:115–124
10. Gao X, Xing D (2009) Molecular mechanisms of cell proliferation induced by low power laser irradiation. J Biomed Sci 16:4
11. Oron U, Maltz L, Tuby H, Sorin V, Czerniak A (2010) Enhanced liver regeneration following acute hepatectomy by low-level laser therapy. Photomed Laser Surg 28(5):675–678
12. Castro-e-Silva O Jr, Zucoloto S, Marcassa LG, Marcassa J, Kurachi C, Melo CA, Ramalho FS, Ramalho LN, Bagnato VS (2003) Spectral response for laser enhancement in hepatic regeneration for hepatectomized rats. Lasers Surg Med 32(1):50–53
13. de Castro e Silva O, Zucoloto S, Menegazzo LA, Granato RG, Marcassa LG, Bagnato VS (2001) Laser enhancement in hepatic regeneration for partially hepatectomized rats. Lasers Surg Med 29(1):73–77
14. Oliveira AF, Silva TC, Sankarankutty AK, Pacheco EG, Ferreira J, Bagnato VS, Zucoloto S, Silva Ode C (2006) The effect of laser on remanescent liver tissue after 90 % hepatectomy in rats. Acta Cir Bras 21(Suppl 1):29–32
15. Barbosa AJ, Santana AC, Castro e Silva T, Kurachi C, Inada N, Bagnato VS, Silva Ode C Jr (2011) Effect of laser on the remnant liver after the first 24 hours following 70 % hepatectomy in rats. Acta Cir Bras 26(6):470–474
16. Araujo TG, de Oliveira AG, Tobar N, Saad MJ, Moreira LR, Reis ER, Nicola EM, de Jorge GL, Dos Tartaro RR, Boin IF, Teixeira AR (2013) Liver regeneration following partial hepatectomy is improved by enhancing the HGF/Met axis and Akt and Erk pathways after low-power laser irradiation in rats. Lasers Med Sci 28(6):1511–1517
17. Schmucker DL, Sanchez H (2011) Liver regeneration and aging: a current perspective. Curr Gerontol Geriatr Res 2011:526379
18. Higgins GM, Anderson RM (1931) Experimental pathology of the liver I. Restoration of the liver of the white rat following surgical removal. Arch Pathol 12(4):186–206
19. Araujo TG, Oliveira AG, Carvalho BM, Guadagnini D, Protzek AO, Carvalheira JB, Boschero AC, Saad MJ (2012) Hepatocyte growth factor plays a key role in insulin resistance-associated compensatory mechanisms. Endocrinology 153(12):5760–5769
20. Regev A, Schiff ER (2001) Liver disease in the elderly. Gastroenterol Clin N Am 30(2):547–563, x-xi
21. Michalopoulos GK (2010) Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. Am J Pathol 176(1):2–13
22. Haga S, Morita N, Irani K, Fujiyoshi M, Ogino T, Ozawa T, Ozaki M (2010) p66(Shc) has a pivotal function in impaired liver regeneration in aged mice by a redox-dependent mechanism. Lab Investig 90(12):1718–1726

23. Palmer HJ, Tuzon CT, Paulson KE (1999) Age-dependent decline in mitogenic stimulation of hepatocytes. Reduced association between Shc and the epidermal growth factor receptor is coupled to decreased activation of Raf and extracellular signal-regulated kinases. J Biol Chem 274(16): 11424–11430
24. Schultz E, Lipton BH (1982) Skeletal muscle satellite cells: changes in proliferation potential as a function of age. Mech Ageing Dev 20(4):377–383
25. Ben-Dov N, Shefer G, Irintchev A, Wernig A, Oron U, Halevy O (1999) Low-energy laser irradiation affects satellite cell proliferation and differentiation in vitro. Biochim Biophys Acta 1448(3):372–380