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Original article

Effect of low-energy laser irradiation and antioxidant supplementation on cell apoptosis during skeletal muscle post-injury regeneration in pigs

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Abstract

The aim of this study was to evaluate the effect of low-energy laser irradiation, coenzyme Q10 and vitamin E supplementation on the apoptosis of macrophages and muscle precursor cells during skeletal muscle regeneration after bupivacaine-induced injury. The experiment was conducted on 75 gilts, divided into 5 experimental groups: I – control, II – low-energy laser irradiation, III – coenzyme Q10, IV – coenzyme Q10 and vitamin E, V – vitamin E. Muscle necrosis was induced by injection of 0.5% bupivacaine hydrochloride. The animals were euthanized on subsequent days after injury. Samples were formalin fixed and processed routinely for histopathology. Apoptosis was detected using the TUNEL method. The obtained results indicate that low-energy laser irradiation has a beneficial effect on macrophages and muscle precursor cell activity during muscle post-injury regeneration and protects these cells against apoptosis. Vitamin E has a slightly lower protective effect, limited mainly to the macrophages. Coenzyme Q10 co-supplemented with vitamin E increases the activity of macrophages and muscle precursor cells, myotube and young muscle formation. Importantly, muscle precursor cells seem to be more sensitive to apoptosis than macrophages in the environment of regenerating damaged muscle.

Key words: apoptosis, skeletal muscle, regeneration, laser irradiation, coenzyme Q10, vitamin E, pig

Introduction

Apoptosis, i.e. programmed cell death, is an important process that accompanies physiological and pathological conditions, maintaining tissue homeostasis in the body. Apoptotic mechanisms are the subject of numerous *in vivo* and *in vitro* studies. Knowledge about programmed cell death in mononuclear cells is quite extensive, but mechanisms of apoptosis in multinuclear cells continue to be the subject of debate. Apoptosis plays a vital role in skeletal muscles, and is intensified in various physiological and

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pathological states (Otrocka-Domagała 2011). Apoptosis also regulates the regeneration of muscle fibre. Muscle fibre regeneration, irrespective of the type of damage-inducing factor, occurs according to a regular pattern and includes cleaning up of the damaged muscle debris by phagocytic cells, the activation and proliferation of muscle precursor cells followed by their fusion and myotube formation, and finally production of young fibres (Allbrook 1981, Akiyama et al. 1992). The rate and intensity of regeneration is determined by the right balance between proliferation and apoptosis of the muscle precursor cells (mpcs: satellite cells, myoblasts), phagocytic cells, and also myotubes and young muscle fibre formation (Kami and Senba 2002, Shaltouki et al. 2007). Macrophages play a significant role in the phagocytosis of the myocyte remnants, as well as in the regulation of mpcs proliferation and death during post-injury regeneration of skeletal muscle (Sonnet et al. 2006). *In vitro* studies have also shown that macrophages can stimulate mpcs proliferation through soluble mitogenic factors and prevent their apoptosis through direct cell-cell contacts involving adhesion molecules expressed by both types of cells (Chazaud et al. 2003, Sonnet et al. 2006). In addition, Chazaud et al. (2003) showed that mpcs through soluble factors attract monocytes to the site of the post-injury regeneration and use macrophages as a support to escape apoptosis. Furthermore, macrophages also attract myogenic cells from the vicinity of the regeneration areas, which migrate along muscle fibres or even across the basal lamina toward the injury area (Chazaud et al. 2003). Moreover, it is possible that macrophages, via adhesion molecules, can rescue differentiating mpcs from myoblast-fusion-associated apoptosis, and they can help myotubes achieve structural stabilization during elongation through additional myoblast fusion (Chazud et al. 2003, Sonnet et al. 2006). On the other hand, the phagocytic activity of macrophages is associated with the release of superoxide radicals and hydrolytic enzymes responsible for peroxidation of phospholipids, followed by damage to plasma membranes of myocytes and endothelial cells, production of new free superoxide radicals, which contribute to oxidative stress, activation of transcription factors and apoptosis (Matés and Sánchez-Jimenez 2000). Therefore, for the proper course of muscle regeneration, it is essential to maintain the correct balance between the production of reactive oxygen species and the antioxidant defense system. The appropriate level of the radical-scavenging substances in the environment of the regenerating muscle fibres is important for the protection of mpcs from apoptosis. Vitamin E (α-tocopherol) and coenzyme Q10 (CoQ10) as antioxidants and stabilizers of plasma membranes have a beneficial effect on the course of muscle fibre regeneration (Bliznakov et al. 1970, Otrocka-Domagała et al. 2004). Both substances enhanced the phagocytic activity of macrophages, which contributed to the rapid cleaning of myocyte debris, and evoked a positive effect on the proliferative activity of myogenic cells, myotube formation and maturation of young muscle fibres (Otrocka-Domagała et al. 2004). Therefore, due to these properties, examination of the impact of α-tocopherol and CoQ10 on apoptosis during skeletal muscle regeneration is needed.

Low-energy laser irradiation (LELI) has been shown to promote skeletal muscle regeneration at the level of phagocytosis of the necrotic remnants, as well as activation and proliferation of mpcs, especially satellite cells (Shefer et al. 2001, Podbielski et al. 2006). Nevertheless, it seems to have a less favorable effect on myoblast differentiation and maturation of newly formed muscle fibres (Shefer et al. 2001, Rodrigues et al. 2014). The main mechanism of LELI is based on the stimulation of the cell cycle entry by the mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK) pathway (Shefer et al. 2001). LELI also plays the role of structural antioxidant, preventing cellular structures from injury caused by reactive oxygen species and stimulating the activity of many antioxidant enzymes (Shefer et al. 2001, Bulyakova et al. 2011). Therefore, LELI also has a beneficial effect on the plasma membrane status, mitochondrial respiration and ATP synthesis, reduction of the post-injury inflammatory process and stimulation of new blood vessel formation (Shefer et al. 2001, Bulyakova et al. 2011, Rennó et al. 2014). It was also suggested that LELI can reduce apoptosis of myonuclei and myogenic cells during skeletal muscle disorders (Shefer et al. 2002).

The model of skeletal muscle injury using injection of bupivacaine hydrochloride (BPVC), a long-acting local anesthetic, is reliable in research on muscle fibre regeneration. Muscle fibers damaged by BPVC regenerate within 14 days, and this process involves phagocyte infiltration, the removal of necrotized myocytes, the activation of myogenic cells, and the formation of myotubes and young muscle fibers, in a manner which occurs during many muscular disorders (Allbrook 1981, Akiyama et al. 1992).

The aim of this study was to evaluate the effect of LELI, coenzyme Q10 and vitamin E on apoptosis of macrophages and mpcs during skeletal muscle regeneration after bupivacaine-induced injury on the pig model.

Materials and Methods

Study design

The study, approved by the Local Commission of Ethics (Decision No. 24/2006/N), was conducted on 75 gilts (Large Polish breed), aged 10 weeks (weighing ca 20 kg), fed commercial grower feed and with free access to water. The animals were divided into 5 experimental groups. Group I (control) included 15 gilts with bupivacaine-induced skeletal muscle injury, receiving no treatment. The 0.5% BPVC solution (Marcain; Astra Zeneca, UK) was injected into the *longissimus lumborum* muscle, 10ml on both sides of the spine. The site of injection was marked with a tattoo ink. Group II included 15 gilts with bupivacaine-induced skeletal muscle injury, treated with LELI. The therapy used a 830 nm laser (CTL-1106 MX, Laser Instruments Ltd.; Poland) with the following parameters: power 100 mW, exposition area 4.9 cm^2, energy 4J/cm^2, exposition time 3.16s. Punctual contact irradiation was performed above the injured area. The first treatment was performed 18h after inducing the muscle injury. The following 4 treatments were performed every 24h. Group III included 15 gilts with bupivacaine-induced skeletal muscle injury, treated with coenzyme Q10 (Jemo-Pharm A/S; Denmark) for the following 5 days, with a daily dose of 120 mg. The drug was administered orally once a day, immediately after feeding. The first dose was administered 8h after inducing the muscle injury. Group IV included 15 gilts with bupivacaine-induced skeletal muscle injury, treated with coenzyme Q10 (protocol as in group III) and vitamin E (Tocopherolum aceticum, Polfa; Poland) for the following 5 days, with a daily dose of 150 mg (intramuscular injections). The first dose was administered 18h after inducing the muscle injury. Group V included 15 gilts with bupivacaine-induced skeletal muscle injury, treated with vitamin E (protocol as in group IV). The animals were euthanized by intravenous injection of pentobarbital sodium salt (Morbital, Biowet; Poland) 24h after injury (one gilt from each group) and on days 2, 3, 4, 5, 7, 10, 14 after injury (two gilts from each group).

Tissue preparation

Injured *longissimus lumborum* muscles taken from all animals of each group, were sampled immediately after euthanasia, fixed in neutralized 10% formalin, paraffin wax- embedded and cut into 3 μm sections. The longitudinal and transverse muscle sections were stained with Mayer's haematoxylin and eosin for histopathological examination. Apoptosis in tissue sections was detected using the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) method, using commercially available staining kit (2TdT-DAB In Situ Apoptosis Detection Kit, Trevigen, USA) according to the manufacturer's instructions. After deparafinization and rehydration, sections were incubated in proteinase K solution for 1h at 37°C. After quenching the tissue-specific peroxidase activity in 3% hydrogen peroxide methanol solution (5min at room temperature), sections were incubated in terminal deoxynucleotidyl transferase (TdT) labelling buffer (5min at room temperature). Incubation of sections in labelling reaction mix, containing TdT labelling buffer, TdT enzyme, biotynylated nucleotides and Co^{2+} (1h, 37°C), was followed by stopping the reaction in TdT stop buffer (5min at room temperature). The TdT enzyme was omitted from the labelling reaction mix for negative control sample, and TACS-nuclease was added to the labelling reaction mix for the positive control sample. Subsequently, sections were incubated with streptavidin-horseradish peroxidase conjugate solution for 10 min at 37°C, stained with DAB solution and counterstained with Mayer's haematoxylin. Brown precipitates in cell nuclei were regarded as a positive reaction. Apoptosis was evaluated in macrophages and mpcs and counted at 400x magnification in 10 fields of muscular injury area for each section. Apoptotic index was expressed as a percentage of positively labelled cells in the necrotic area.

Statistical analysis

For each group, the mean apoptotic index (together with standard deviation) was calculated. Statistical analysis was conducted using ANOVA F-test. The differences between evaluated groups, as well as between days post-injury within each group, were estimated using the SNK (Student-Newman-Keuls) test. Differences were considered significant when $p<0.05$.

Results

Histopathological examination revealed in all evaluated groups, 24h post injury, the presence of locally extensive coagulative necrosis of the *longissimus lumborum* muscle and infiltration of numerous macrophages. Necrosis and phagocytic cells were observed up to the 4th day in all examined groups (Fig. 1). Necrotic fibres were not observed in the group II (LELI) from the 5th day, and in the group IV (CoQ10 and vitamin E) from the 7th day in all examined samples. In groups III (CoQ10) and V (vitamin E)

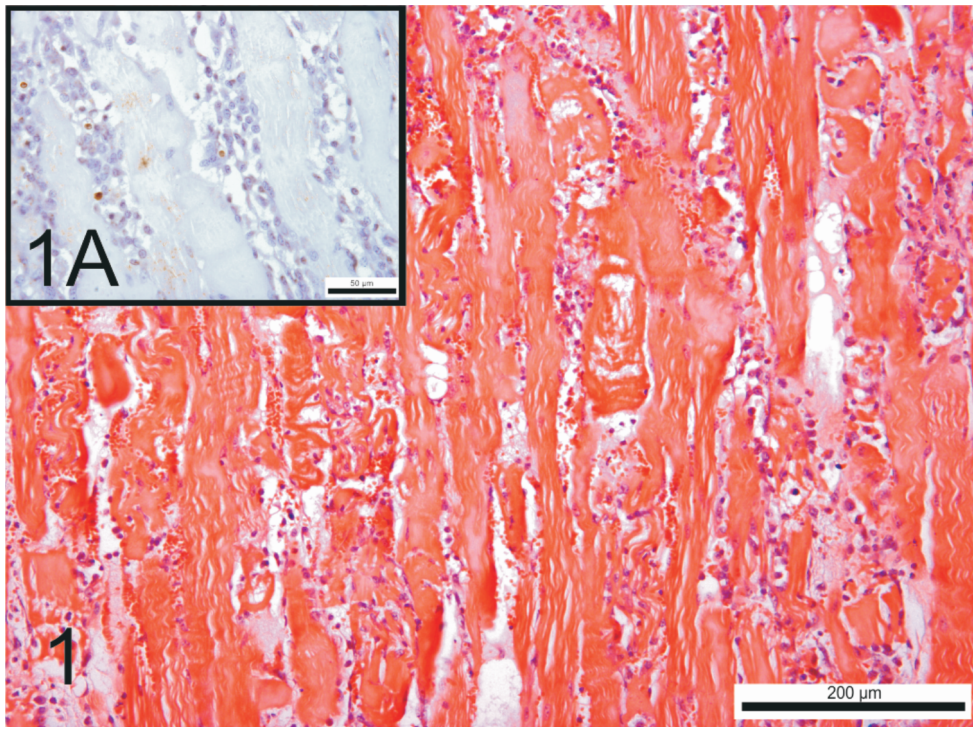

Fig. 1. Group II (LELI), 2nd day. Multifocal segmental coagulative necrosis of the muscle fibres with macrophage infiltration and single mpcs. HE. Inset (1A): Single macrophages undergoing apoptosis show brown nuclear staining. TUNEL.

segmental necrosis of single fibres was noticed only in half of the examined samples at the end of the research. In the control group in all muscle samples necrotic fibres were observed up to the 14th day. Macrophages were numerous until the 5th day in all examined muscles of each group. In subsequent days, their number decreased and in groups II and IV phagocytic cells were observed only in a single sample, in groups III and V in half of examined samples but in the control group in all muscle samples until the 14th day. MPCs were noticed in the injured area from the 2nd day in all examined samples of each group, but in group I (control) mpcs number was lower. On the 3rd day mpcs were numerous in the necrotic area in all examined muscle and their contribution in the regeneration of the injured muscle samples gradually decreased until the 10th day of the experiment. On the 14th day single mpcs were noticed in every examined muscle of each group. The first myotubes appeared on the 3rd day in groups II, III, IV and V. In groups II and IV myotubes were noted in half of the samples and in groups III and V were not observed only in one muscle. In the control group myotubes were first observed on the 4th day. In all groups the number of myotubes increased until the 7th day of the research. On the 10th day myotubes were not observed in group I, but in groups II, III, IV and V they were observed in a single muscle sample of each group. On the 14th day myotubes were not observed in any of the examined samples of each group. The first young muscle fibres appeared on the 4th day in all examined samples of groups II, III, IV and V, but they were most numerous in the group treated with coenzyme Q10 and vitamin E. In the control group, on the 4th day only single young fibres were observed in half of the samples. Their number increased until the 14th day of the experiment in all groups and they were numerous in groups III, IV and V on day 14. In groups I and II on the 14th day single young fibers were noticed in all samples.

The apoptotic index, calculated on the basis of TdT/TUNEL positive cells localized in the area of muscular injury in all evaluated groups during the following days of the research is presented in table 1. In the area of muscular damage, 24 hours after injury, TdT positive cells consisted prevalently of macrophages in all evaluated groups. Two days after injury, positive labelling cells in the area of muscular injury consisted of macrophages and mpcs in groups II, III and V (Fig. 1A). In the control group, TdT positive cells consisted mainly of macrophages but in group IV positive reaction was observed only among mpcs. On the following days, TdT positive cells in all evaluated groups consisted mainly of mpcs, but in the group treated with vitamin E only mpcs were affected. In groups I-IV positive TdT labelling was also observed in macrophages, but in the control group it was additionally observed in fibroblasts and endothelial cells.

Table 1. TdT/TUNEL positive cells undergoing apoptosis in the area of muscular injury together with apoptotic index (mean with standard deviation) in groups I-V.

Group	I	II*	III	IV	V*
24 h post injury	macrophages 3.55 ± 1.34	macrophages 1.89 ± 0.24	macrophages 2.56 ± 0.23	macrophages 2.9 ± 0.51	macrophages 2.43 ± 0.94
2 days post injury	macrophages 3.56 ± 0.25	macrophages, myogenic cells 1.45 ± 0.13	macrophages, myogenic cells 3.65 ± 1.09	myogenic cells 3.43 ± 0.4	macrophages, myogenic cells 2.54 ± 0.8
3 days post injury	macrophages, myogenic cells 4.76 ± 0.51	macrophages, myogenic cells 2.11 ± 0.7	macrophages, myogenic cells 3.89 ± 1.2	myogenic cells 4.67 ± 0.91	macrophages, myogenic cells 2.65 ± 0.59
4 days post injury	macrophages, myogenic cells 4.8 ± 0.57	myogenic cells 1.97 ± 0.52	myogenic cells 5.75[a] ± 0.87	macrophages, myogenic cells 5.76[a] ± 1.07	myogenic cells 3.24 ± 0.5
5 days post injury	macrophages, myogenic cells 5.32[a] ± 0.46	myogenic cells 2.45 ± 0.31	myogenic cells, macrophages 4.49 ± 1.1	myogenic cells 4.31 ± 0.76	myogenic cells 3.56 ± 0.91
7 days post injury	myogenic cells 7.98[a] ± 0.57	myogenic cells, macrophages 3.43 ± 0.47	myogenic cells 6.84 ± 1.34	myogenic cells 6.84 ± 0.98	myogenic cells 5.34[a] ± 0.73
10 days post injury	myogenic cells, fibroblasts, endothelial cells 7.35[a] ± 2.6	myotubes, myogenic cells 3.56 ± 0.53	macrophages, myogenic cells 7.34[a] ± 2.11	myogenic cells 6.35 ± 2.41	myogenic cells 4.26 ± 1.54
14 days post injury	myogenic cells, fibroblasts, macrophages 3.59[b] ± 1.21	myogenic cells 4.56[a] ± 0.63	macrophages, myogenic cells 6.56[a] ± 0.45	myogenic cells, macrophages, satellite cells 4.56 ± 0.31	myogenic cells 3.67 ± 1.37

* statistically significant differences between evaluated Groups II-V and control group (I) ($p<0.05$)
a statistically significant increase of the apoptotic index within the group ($p<0.05$)
b statistically significant decrease of the apoptotic index within the group ($p<0.05$)

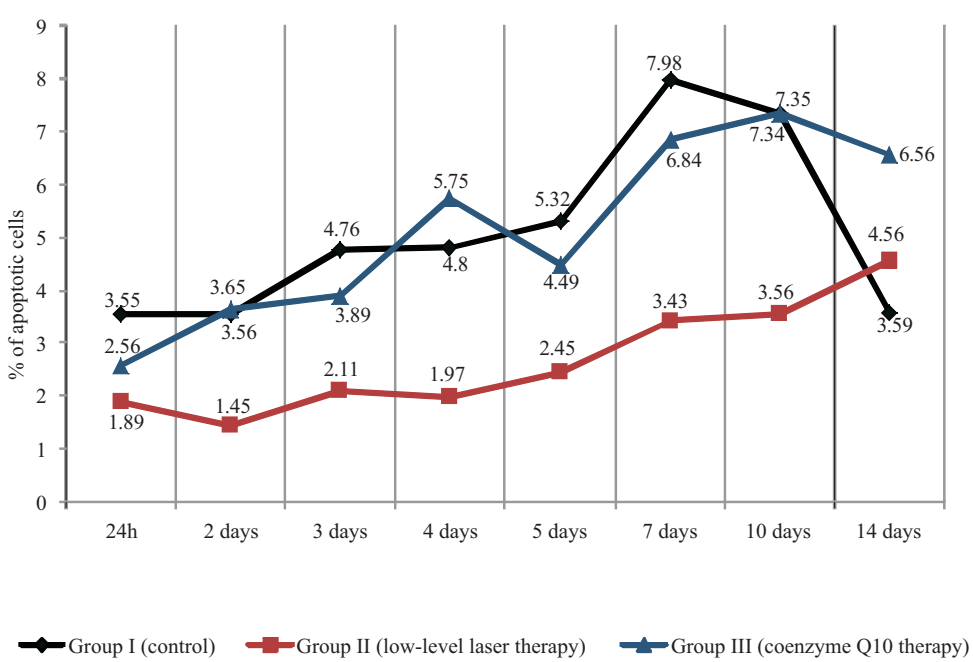

Fig. 2. Apoptotic index of cells localized in the area of muscular injury in control group, in group II (low-level laser therapy) and in group III (coenzyme Q10 therapy).

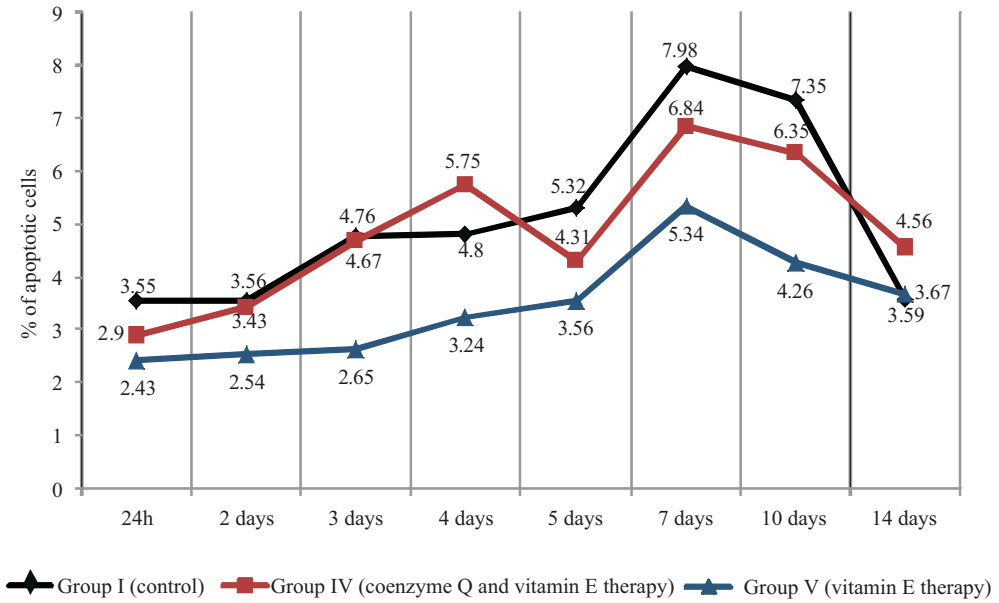

Fig. 3. Apoptotic index of cells localized in the area of muscular injury in control group, in group IV (coenzyme Q10 and vitamin E therapy) and in group V (vitamin E therapy).

Furthermore, in the group treated with LELI on the 10th and 14th day positive reaction was observed in myotubes. Detailed qualitative analysis of TdT/TUNEL positive cells undergoing apoptosis in the area of muscular injury in consecutive days post injury is presented in table 1. The statistical analysis revealed that in group I the apoptotic index increased on the 5th, 7th and 10th day post injury, and decreased on the 14th day (Fig. 2, 3). In group II, the apoptotic index increased on the 14th day post injury (Fig. 2). In group III, the apoptotic index increased on the 4th, 10th and 14th day post injury (Fig. 2). In groups IV and V the apoptotic index increased on the 7th day post injury (Fig. 3)

The statistical analysis revealed that the apoptotic index was significantly lower in groups II and V, compared to the control group (I).

Discussion

A single bupivacaine injection into the *longissimus lumborum* muscle caused, in all examined samples, locally extensive coagulative necrosis followed by phagocytic cell infiltration, activation and proliferation, myotubes and young muscle fibre formation, which was consistent with the results of other research (Akiyama et al. 1992, Otrocka-Domagała et al. 2004, Podbielski et al. 2006). In the present study, necrotic fibres were removed faster in groups II (LELI) and IV (coenzyme Q10 and vitamin E), compared to other evaluated groups, which was probably associated with increased activity of phagocytic cells. This confirmed the results obtained by other authors, demonstrating the positive impact of the LELI on muscle regeneration by increasing the metabolic activity of macrophages (Weiss and Oron 1992, Bibikova and Oron 1994, Podbielski et al. 2006). On the other hand, LELI has an ability to modulate the inflammatory process and to optimize tissue repair by inhibiting some inflammatory mediators: prostaglandin, arachidonic acid and cyclooxygenase-2 (Rennó et al. 2014). This property of inflammatory phase shortening could affect the rapid disappearance of the macrophages in the necrotic area in the LELI treated group in our study. α-Tocopherol and CoQ10 as antioxidants and stabilizers of plasma membranes also enhance the phagocytic activity of macrophages which contribute to the rapid cleaning up of myocytes debris (Bliznakov et al. 1970, Otrocka-Domagała et al. 2004). Our studies demonstrated that this beneficial effect is higher after their co-supplementation, as in groups III (CoQ10) and V (vitamin E) cleaning up of damaged fibres was slower and necrosis and macrophages were observed in half of the examined samples at the end of the research. The increase of functional effectiveness of α-tocopherol and coenzyme Q10 during their co-supplementation was also confirmed by other authors and in our own previous research (Thomas et al. 1996, Otrocka-Domagała et al. 2004, Ognjanović et al. 2006). Moreover, it is considered that CoQ10 has a beneficial effect on vitamin E regeneration by reactivation of α-tocopheroxyl radicals, and therefore, some researchers believe that the protection of α-tocopherol is the main function of coenzyme Q10 (Kagan et at. 1990, Stoyanowski et al. 1995).

MPCs were noticed in the regeneration area from the 2nd day, and on this day they were more numerous in all treated groups, compared to the control group. Similar results were obtained by Podbielski et. al (2006) in research on LELI influence on muscle fibre regeneration in swine, when the participation of mpcs on the 2nd day was higher in the laser treated group compared to the untreated control group. This is consistent with the results obtained by other authors, that laser irradiation accelerated the activation and proliferation of mpcs during regeneration (Shefer et al. 2001, Bulyakova et al. 2011). The beneficial effect of the coenzyme Q10 on the metabolism and proliferation acitivity of different cells has been confirmed in our own earlier study (Otrocka-Domagała et al. 2003). In our previous study, we also observed higher proliferation activity of mpcs during skeletal muscle regeneration after bupivacaine-induced injury accompanied by CoQ10 supplementation (Otrocka-Domagała et al. 2004). The influence of vitamin E on cell proliferation is controversial. Several studies have shown that α-tocopherol, independently of its antioxidative effect, can inhibit cells proliferation (Azzi and Stocker 2000, Yano et al. 2000). However, in another study this property was not confirmed (Li et al. 2011). In our previous research on myofibre regeneration in pigs during coenzyme Q10 and vitamin E supplementation, we observed that α-tocopherol does not affect mpcs proliferation when administered alone. The increase of mpcs was noticed only after coenzyme Q10 and vitamin E co-supplementation (Otrocka-Domagała et al. 2004).

MPCs differentiation and fusion with myotube formation was observed from the 3rd day in all treated groups. However, in groups treated with vitamin E separately or with CoQ10 co-supplementation, myotube participation in muscle regeneration was more pronounced compared with other treated groups. Likewise, young muscle fibre formation observed from the 4th day in all groups was more extensive in the group treated with CoQ10 and vitamin E co-supplementation. The beneficial effect of vitamin E and coenzyme Q on the myotube and young muscle formation may result from their antioxidant defense, improvement of energy metabolism and membrane stabilization of mpcs. Similar results were obtained in our previous study (Otrocka-Domagała et al. 2004). The less favorable effect of the LELI at this stage of muscle regeneration observed in our study, confirmed other results that laser irradiation can inhibit cell differentiation and fusion, and does not affect the dynamics of myofibre maturation (Shefer et al. 2001, Podbielski et al. 2006).

Apoptosis in the muscle regeneration area was noticed from the 1st day in all examined samples of each group and affected only macrophages, the main cell population observed in sites of the injury on this day. During the following days apoptosis concerned macrophages and/or mpcs depending on the group. The apoptotic index on the 1st day, and during following days, was significantly lower in the LELI treated group, compared to other evaluated groups, and macrophages seem to be the most protected from apoptosis in this group. MPCs were more affected by apoptosis compared to macrophages in this group, but considering the low apoptotic index of all cell populations during LELI treatment, it should be assumed that mpcs were also protected against apoptosis, but not on the same level as macrophages. Shefer et. al (2002) in research on cultured myogenic cells showed that the LELI has a protective effect on mpcs apoptosis resulting from enhanced activity of anti-apoptotic protein Bcl-2 and the reduction of pro-apoptotic protein BAX. The limitation of these studies is that they were carried out on isolated fibres without evaluation of other cells taking part in *vivo* regenerating muscles. On the other hand, protection of proliferating and differentiating mpcs from apoptosis during regeneration in the LELI group could be the result of the anti-apoptotic, protective effect of macrophages, which was confirmed in other research (Chazaud et al. 2003, Sonnet et al. 2006).

The apoptotic index was also low in the vitamin E treated group throughout the experiment and macrophages also seem to be the most protected population. The anti-apoptotic activity of α-tocopherol has been confirmed in several studies (Haendeler et al. 1996, Ramanathan et al. 2005, Guney et al. 2007). The protective effect of vitamin E on cell death is the result of its free radical scavenging property, membrane stabilizing action and regulation of the expression of the number of genes and signal regulatory pathways (Allen and Tresini 2000, Ramanathan et al. 2005). Consequently, this antioxidant can protect and stabilize mitochondrial membrane permeability, and block subsequent release of cytochrome c and caspase activation and finally cell death (Ramanathan et al. 2005). Vitamin E can also reduce the level of tumor necrosis factor-α (TNF-α), primarily produced by activated macrophages, involved in the activation of external apoptosis pathway. Furthermore, the anti-apototic activity of vitamin E is also connected with increasing the function of the anti-apoptotic Bcl-2 protein (Haendeler et al. 1996, Ramanathan et al. 2005). Due to the fact that the main anti-apoptotic action of α-tocopherol is connected with membrane stabilization and cell protection against oxidative stress-induced apoptosis, and macrophages are the main source of superoxide radicals and hydrolytic enzymes during muscle regeneration, macrophages seem to be

the most apoptosis-protected population, which was confirmed in our results. Interestingly, in a group treated with vitamin E, necrotic debris of the fibre were observed during whole experiment, and perhaps protection of macrophages against apoptosis did not reflect on the rate of cleaning up of damaged muscles. However, protection of macrophages against death during muscle regeneration can influence, according to their properties, the stimulation of mpcs proliferation, prevention of mpcs apoptosis, rescue of differentiating mpcs from myoblast-fusion-associated apoptosis, and stabilization of myotube structure during elongation through additional myoblast fusion (Chazaud et al. 2003, Sonnet et al. 2006). On the other hand, protection of macrophages against apoptosis is reflected in the increase in their number in the regenerating area and the release of more superoxide radicals and hydrolytic enzymes followed by damage to mpcs and their apoptosis (Matés and Sánchez-Jiménez 2000). Therefore, as observed especially in the groups treated with LELI and vitamin E, myogenic cell apoptosis may be rather the result of oxidative stress rather than a lack of protective action of these two agents.

The administration of coenzyme Q10 and co-supplementation with vitamin E did not affect the incidence of apoptosis in the regeneration area. This implies that Co10 exhibits less anti-apoptotic effect compared with other factors used in the experiment. Protective coenzyme Q10 action against apoptosis seems to be the result of preservation of mitochondrial membrane lipids against peroxidation and DNA damage (Kagan et al.1999). However, this action is not cell specific and can affect all cell populations involved in muscle fibre regeneration. Furthermore, apoptosis during muscle recovery can be activated by both external and internal stimuli, which promote a different, mitochondrial-dependent and mitochondrial-independent apoptotic pathway, due to which stabilization of the mitochondrial membrane and its enzyme protection may be less important in the prevention of apoptosis during skeletal muscle post-injury regeneration. On the other hand, CoQ10 inhibits the activity and expression of caspases 2 and 3, crucial during the early phases of apoptosis, but has no effect on the late apoptosis phase, associated with DNA fragmentation (Groneberg et al. 2005, Chen et al. 2011). In our study, we detected the late apoptosis stage using the TUNEL method, and we could not show a protective CoQ10 inhibitory effect on caspase activation; therefore, future investigations are needed. Recently, attention has been drawn to the role of CoQ10 in the autophagy, suggesting its protective action against this phenomenon (Rodriguez-Hernández et al. 2009). Apoptosis and autophagy, as functionally related processes undoubtedly play an important role in maintaining the proper course of skeletal muscle regeneration and in many muscle disorders (Rodriguez-Hernýndez et al. 2009, Otrocka-Domagała 2011, Mariño et al. 2014). However, further investigations concerning correlations of these two phenomena in the course of the regeneration of skeletal muscle are required. It is difficult to explain the weak anti-apoptotic effect of CoQ10 and vitamin E co-supplementation during muscle regeneration, considering the low apoptotic index in the α-tocopherol treated group. With regard to the protective role of coenzyme Q on vitamin E, the decrease of the apoptotic index during their co-supplementation was expected. The explanation for this could be that the main role of these substances is concentrated on plasma membrane stabilization and antioxidative action which accelerate macrophages activity, and therefore the apoptotic cells were rapidly removed from the tissue.

The obtained results indicate that LELI has a beneficial effect on skeletal muscle post-injury regeneration by its influence on macrophage phagocytic activity, and mpcs activation and proliferation. Furthermore, LELI inhibits apoptosis of macrophages and mpcs and protects them against oxidative stress damage. Vitamin E showed similar anti-apoptotic action, but its protective effect was limited mainly to macrophage activity followed by rapid clearing up of miocyte remnants. Coenzyme Q10 does not significantly affect the late stage of apoptosis, even with vitamin E co-supplementation, but both substances have a synergistic effect on increasing macrophage and mpcs activity, myotube and young muscle formation. Importantly, mpcs seem to be more sensitive to apoptosis in the environment of regenerating damaged muscle. The obtained results extend knowledge about the apoptosis phenomenon during skeletal muscle post-injury regeneration, especially in the context of sensitivity of individual cell populations on this process and the possibility of influencing the modulation of damaged skeletal muscle regeneration.

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