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Evaluation of treatment with bone marrow derived monocytes of minipigs in muscle fibres after exposure to Bisphenol-A

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ABSTRACT

Bisphenol-A (BPA) has been described as an endocrine disruptor that impairs human and animal health. As BPA is present in food and drink, due to leaching from polycarbonate containers or aluminum cans, humans are constantly exposed to it. Due to the importance for public health of unconscious exposure to BPA, we conducted a study of its rapid action on muscle tissue and its possible treatment with bone marrow monocytes. For this purpose, 16 minipigs were randomly distributed (n = 4) to one of the following experimental groups: the control group treated with BPA (for 4 months), the BPA-withdrawal group (treated for 2 months with BPA and two months without), and the bone marrow monocytes (MN) group (treated for 2 months with BPA, after which it was withdrawn and MN treatment began lasting 2 months). At the end of the treatments, biopsy of the longissimus lumbaris (LL) was carried out. The samples were analysed by optical and electron microscopy. In our study, it was observed that after continuous exposure to BPA some alterations appeared both in the muscle fibres and in the collagen fibres of their sheaths. The group from which BPA was withdrawn for two months exhibited a reversible degenerative action, with a partial recovery of both the muscle and the collagen fibres. In the last study group, we evaluated the action of the bone marrow monocytes on the modified muscle tissue, and observed that they presented a notable recovery action since practically all the muscle and collagen fibre alterations which had appeared were reversed.

Key words: Bisphenol A; minipig; muscle; bone marrow monocytes

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Introduction

Numerous environment pollutants can act as endocrine disruptors (EDCs), modifying the equilibrium of the endocrine system, and also the muscular-skeletal system. Therefore, the number of scientific studies focusing on the investigation of the toxic action mechanisms of these compounds, and their repercussions on the environment and health has multiplied (MANDICH et al., 2007; MOLINA et al., 2013; MORTAZAVI et al., 2013; GIMÉNEZ et al., 2016; LORA et al., 2016; BARASONA et al., 2017; MOLINA et al., 2018).

The effects of most of these substances with endocrine activity are caused as a result of their action due to their structural similarity to steroid hormones. They are capable of triggering a similar response in target cells to those of endogenous hormones, or of inhibiting that response by exerting an antagonistic effect. So, the compounds most studied are those with the capacity to mimic the effects of androgens and oestrogens on the organism.

Bisphenol A (BPA) is a known EDC that is present in a great variety of consumer products, a large number of which come into direct contact with food by means of drink or food containers, in the protective inside coating of the tins of conserved foods, among others. They are also used in multiple non-alimentary products such as dental sealants, medical equipment and materials, which could result in creating a public health problem (VANDERBERG et al., 2007; MILEVA et al., 2014).

Due to the beneficial action produced, in general and in muscles in particular, in the degenerative processes of the paracrine substances in bone marrow monocytes, these compounds are being employed in the treatment of muscle pathologies. Monocytes are applied at high doses via the bloodstream or in direct treatment of the area that is the focus of study, and both treatments are also recommended simultaneously. The effects of BPA on muscle fibres may be reversible, so that the use of treatments with monocytes for the recovery of fibres could be applicable (CARMONA et al., 2017).

The current research consists of two different phases. First, with a daily dose of BPA, we studied the possible alterations caused by EDCs in muscle fibres, and, in parallel, we analysed the modifications occurring in fibrocytes and collagen fibres. Second, once the BPA action had been verified, we evaluated whether the paracrine treatment factors, which act directly on muscle and connective fibres, could provoke a recovery of the latter.

Materials and methods

Animals. Sixteen two-month-old "minipigs" were used, from the breeding colony of the Experimental Animal Service at the University of Córdoba, where they were stabled during the whole experiment, following the conditions specified in the guidelines relative to the housing and care of animals (ANONYM., 2013). All the experiment protocols were

approved by the Córdoba University Committee of Bioethics. The animals were fed once a day during the study (Nantaunic, Nantaporc PI®) and distributed randomly to one of the four experiment groups: Control Group (GC), (n = 4); the group treated with BPA (n = 4) orally 1 mg/kg bw/day (Sigma Aldrich®, St. Luis, EE.UU) for four months; the third group - BPA-withdrawal (n = 4) which, after two months of treatment, was taken off BPA for two more months up until their biopsy; and a fourth group, treated with monocytes (MNCs) (n = 4). The animals of this group were treated with BPA for 2 months, then it was withdrawn and they began treatment with MNCs for 2 months. In all the treated groups, BPA was administered orally by a syringe. At the end of the different periods of exposure, treatment and/or withdrawal, depending on the study group, samples were collected by means of a biopsy of the longissimus lumbaris (LL) muscle, for subsequent study.

Isolation of mononucleate cells (MNC) derived from the bone marrow (BM). The MNCs were extracted from the BM proceeding from the femur and tibia of the minipigs. The medullary cavity of both bones was repeatedly washed with 10mL of saline solution. The suspension obtained was collected in sterile tubes, filtered and subsequently centrifuged at 4 ºC for 5 minutes. The remaining supernatant was discarded, and the pellet with the cell concentrate of the BM from both bones was re-suspended in 1 mL saline.

The erythrocytic fraction and the granulocytes were eliminated from the medullary cell for enrichment of the MNC fraction by density gradient centrifugation for 20 minutes at 1500 g and 20 ºC, using Lymphoprep™ (Oslo, Norway). After centrifugation, the MNC concentrate was collected. This MNC suspension was washed adding 5mL of PBS, phosphate-buffer saline, to completely eliminate the Ficoll (branched hydrophilic polysaccharides) remains. The resulting pellet was resuspended in a protein medium (FBS, foetal bovine serum) at a concentration of 5×10^6MNCs/mL. Cell viability was determined by the Trypan blue exclusion method (Lonza, Verviers, Belgium) and the cells were counted in a Neubauer camera. This cell suspension was employed as treatment in the *in vivo* study.

Histological study. Light microscopy: For structural evaluation, the samples were routinely processed for paraffin sections by fixing in 10% formaldehyde, dehydrating in graded series of ethanol, immersing in xylol and embedding in paraffin wax. Every tenth section (4 µm thick) of each block was stained with haematoxylin and eosin, and used for the morphological study. Additionally, muscle sections were subjected to Masson's trichrome stain, which stains collagen blue, providing an excellent colour contrast differentiating it from other structures.

Electron microscopy. For the ultrastructural study, small randomly selected samples were primarily fixed in a 2% glutaldehyde solution in 0.1M phosphate buffer (pH 7.4) overnight at 4 ºC, and then refixed in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.4) for 30 min. After dehydration in graded ethanol series and embedding in Araldite,

semi-thin and ultra-thin sections were cut on an LKB ultramicrotome. Semi-thin sections were stained with toluidine blue, whereas ultra-thin sections were double-stained with uranyl acetate and lead citrate. Ultra-thin sections were viewed and photographed under a JEM 1400 transmission electron microscope (TEM; JEOL, Ltd.).

In order to avoid individual variations, structural and ultra-structural evaluations were carried out by the same pathologist.

Results

In the control group, a close relationship was evidenced under the light microscope between the muscle fibres and the endomysium, perimysium and epimysium, all of these sheaths being relatively thin. The muscle fibre is a multinucleate cell with its nucleus arranged in a peripheric position, and its contractile material is spread longitudinally over so-called myofibrils, with highly evident transversal striae, especially the A and I bands. The relationship of the muscle fibres with the collagen fibre of the endomysium was detected using the trichrome technique. However, certain muscle areas were observed in which that activity appeared to be accentuated or diminished (Figs 1A, B, D).

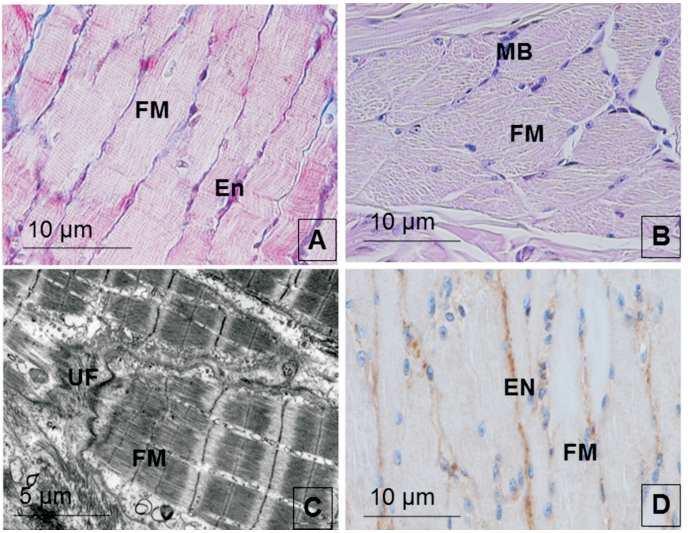

Fig. 1. Histopathological changes in muscle. Control Group. A. Image of the muscle fibre section under light microscope. Masson's trichrome technique. Muscle fibres (MF) surrounded by a thin endomysium (En). B. Image under light microscope. Apparently normal muscle fibres (MF), and area of myoblast association (MB). C. Ultrastructural image. Apparently normal muscle fibres (MF), with area of fibrous unions between them (FU). D: Image of the muscle fibre section under light microscope. Immunocitochemical technique, anti-collagen serum l. The muscle fibres can be seen (MF), surrounded by a thin endomysium (En), giving a positive reaction to staining.

The ultrastructure they presented was classical; the nuclei were arranged next to the plasma membrane, which, in turn, was related to a highly dense microenvironment, alongside the existence of invaginations configured by the "T" tubules (Fig. 1C).

In the group treated with BPA we observed, first, very marked fibrillar pleomorphism, with a prominent diminution in fatty tissue and endomysial connective tissue. Inside the marked pleomorphism there was clear fibrillar hypertrophy, and also some fibres of a smaller size. The fibrillar hypertrophy was accompanied by modifications in its components. There were alterations in the nucleus. Although the muscle fibres were multinucleate and arranged peripherically, a large amount of their components being "centralized", *i.e.* they had lost their localization and became arranged towards the centre of the sarcoplasm. The principal alteration produced by BPA in muscle fibres was undoubtedly cell hypertrophy which, both in the transversal and longitudinal sections, displayed a notable increase in sarcoplasma and myofibrils (Fig. 2).

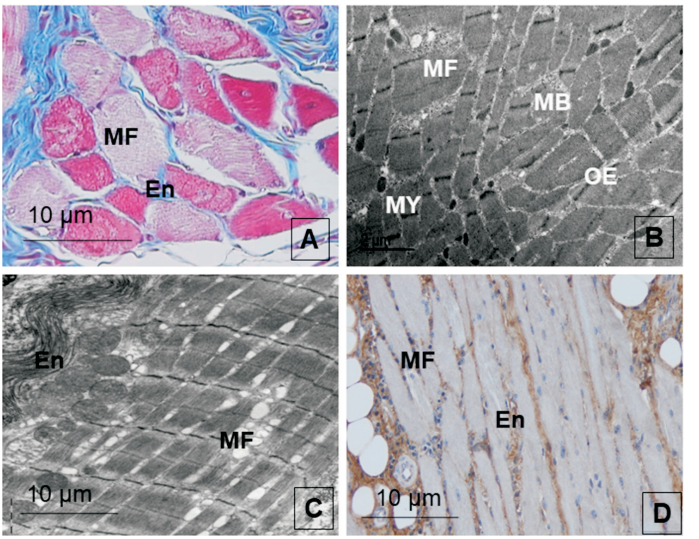

Fig. 2. Histopathological changes in muscle. Group treated with BPA. A. Image of the muscle fibre section. under the light microscope Masson trichrome technique. Detail of muscle fibres, degenerated and hypertrophic (MF), surrounded by a thick endomysium (En). B. Ultrastructural image. Detail of muscle fibres (MF), arranged irregularly on the myofibrils (MY). Detail of muscle fibre (MF), in relation to degenerated myoblasts (MB), surrounded by an endomysium with oedema (OE). D. Ultrastructural image. Vacuolized muscle fibres (MF), surrounded by a fibrous band of endomysium (En). E: Image under the light microscope of the muscle fibre section. Immunocitochemical technique, anticollagen serum l. The muscle fibres (MF) were noted surrounded by thick bands of endomysium (En) giving a positive reaction to staining.

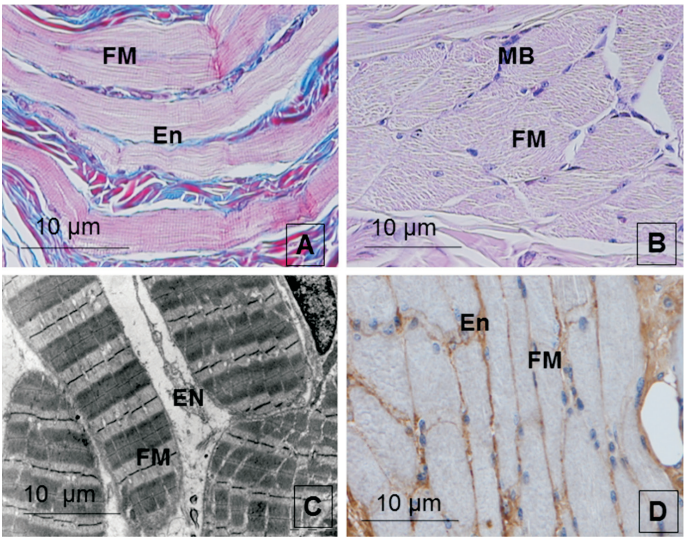

Fig. 3. Histopathological changes in muscle. Group with BPA withdrawal. A. Image under the light microscope of the muscle fibre section. Masson trichrome technique. Hypertrophic muscle fibres (MF) surrounded by a thick endomysium (En). B. Image under the light microscope-. Hypertrophied muscle fibres stood out (MF) associated with myoblasts (MB). C. Ultrastructural image. Detail of muscle fibres showing apparently normal striae (MF) surrounded by thick fibrous endomysia and with oedema (OE). D. Image under light microscope of the muscle fibre section. Immunocitochemical technique. Anti-collagen serum l. The muscle fibres are seen (MF) surrounded by thick bands of endomysium (En) with a positive reaction to staining.

With regard to the group exposed to BPA for two months, and the two groups in which it was withdrawn, under the light microscope the fibres showed prominent pleomorphism, visible hypertrophy, with images of nuclei centralization, loss of striae and homogenization, and, above all, "fingerprint" degenerations and canalizations in the sarcoplasma. What undoubtedly and specifically stood out in this experimentation group was the presence of generalized oedema or anasarca; this oedema was manifested by separation between fibres and the interstitial tissue of the endomysium, leading to the loss of its normal texture and it appeared clear and homogenized (Fig. 3A, B, D).

In the electron microscope studies, the muscle fibres showed modifications were fundamentally characterized by an increase and centralization in the nuclei, and especially a disproportionate increase in myofibrils, which resulted in the internal disorganization of the myofilaments studied in the group treated with BPA. To be specific, a generalized oedema was detected, and, inside the fibres, dilatations in the "T" tubules and in the

endoplasmic reticulum were noted. In the connective interstitium, wide, very clear areas were evidenced, with the loss of the structure of its connective components (Fig. 3C).

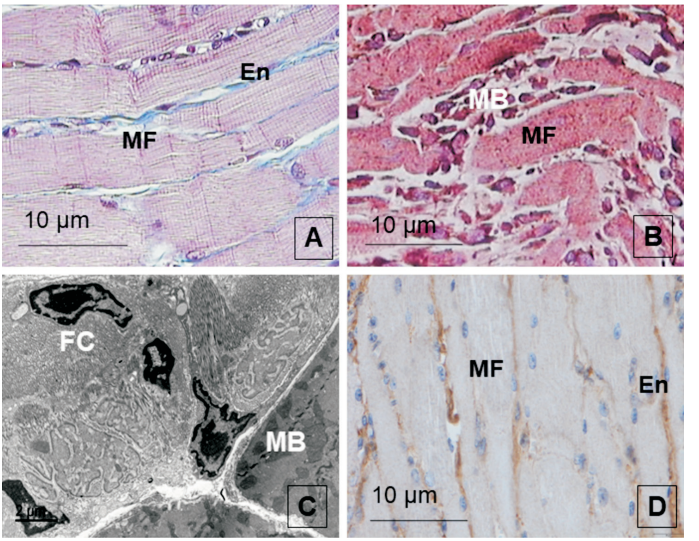

Fig. 4. Histopathological changes in muscle. Group treated with monocytes. A. Image of the muscle fibre section under the light microscope - Masson trichrome technique. Muscle fibres (MF) surrounded by a thin endomysium (En). B. Image under the light microscope. Mature muscle fibres (MF) and presence of myoblast accumulation (MB). C. Ultrastructural image. Presence of myoblasts noted (MB) and fibrocytes (FC). D. Image of the muscle fibre section under the light microscope. Immunocitochemical technique, anti-collagen serum l. There were muscle fibres (MF) surrounded by a thin endomysium (En) that had a positive reaction to staining.

In the group treated with monocytes, the muscle displayed a normal proportion between its tissue and the connective tissue. The muscle fibre was seen to be multinucleate, with peripheric nuclei, and its fibrils organized themselves with normal striae. With the trichrome technique it was possible to confirm an obvious relationship between all their components (Fig. 4D).

The ultrastructure presented by the fibres was a relatively classic one. The nuclei were arranged close to the plasma membrane, which, in turn, was related to a highly dense microenvironment, with the existence of invaginations configuring the "T" tubules. The most important elements were the myofibrils. The first thing to note is that these contractile fibrils were arranged parallel to each other and spread longitudinally over the whole cell volume, and at no time did they change direction (Fig. 4C).

In the group treated with monocytes, under the light microscope groups of small nucleate cells stood out, that were identified with satellite cells or myoblasts. There were some cells with irregular edges in their ultrastructure, and it was even difficult to identify those edges. What was notable was the presence of numerous nuclei and, more important still, contractile material of myosin and actin, forming myofibrils (Fig. 4 A, B).

Discussion

The low-dose effects of endocrine-disrupting chemicals such as BPA are mediated by endocrine-signalling pathways that have evolved to act as powerful amplifiers, with the result that important changes in cell function can occur in response to extremely low concentrations (WELSHONS et al., 2003). In June 2017, the European Chemical Agency (ECHA) classified BPA as a "substance of very high concern" because of its endocrine-disrupting properties, which have serious effects on human health.

The action of BPA as a neuroendocrine disruptor has been widely studied (MOLINA et al., 2013; VANDERBERG et al., 2013; JEDEON et al., 2016; LORA et al., 2016; BARASONA et al., 2017). However, its effect as an xenoestrogen on muscle tissue has not been evaluated in the same way (INDUMATHI et al., 2013; MORTAZAVI et al., 2013; WANG et al., 2013; GIMENEZ et al., 2016). Due to its importance as an environmental pollutant and the possible exposure to it through diet, the objective of this study was to establish an evaluation of the possible effects of continuous exposure to BPA on muscle tissue, as well as the latter's capacity to recover after withdrawing the treatment. We also studied whether a treatment with monocytes, applied in muscle pathologies of other aetiologies, could be effective for lesions caused by EDCs.

Very many studies have evaluated the toxicity of BPA, using rodents or fish as biomodels (HATEF et al., 2012; LÓPEZ-CASAS et al., 2012; WANG et al., 2013), and there are a few references to work employing other experimentation animal species (CHOI and JEUNG, 2003; GAO et al., 2010; INDUMATHI et al., 2013). In our study it was observed that the hypertrophy of muscle fibres resulting from BPA action was mainly due to an increase in contractile material. This phenomenon was detected even under a light microscope, due to the increase in the size of the fibres, but, without any doubt, it was observed best under an electron microscope. The myofibrils presented in a larger number and showed serious alterations. For that reason, under both the light and electron microscopes, images in which the myofibrils were arranged in arcades were detected, with fingerprint degenerations being formed. These modifications could even show canalizations in the cytoplasm, and this type of lesion was previously described by GIMÉNEZ et al. (2016) using the same biomodel, BPA dose and exposure time, as well as the action of other substances, that could act as xenoestrogens (PELLEGRINO et al., 2004; DOUILLARD et al., 2011).

On withdrawing the BPA, some degeneration images were detected under both the light and electron microscopes, although they were less marked than those in the group treated continuously with BPA, which would demonstrate a certain level of recovery and the reversibility of the muscle lesions.

In the group treated with monocytes, the myoblasts acted following two patterns. First, together with the increase in the number of nuclei, the organoids participating in the synthesis of contractile proteins became more evident, with extensive development of the transfer vesicle. This increase in activity led, in this first phase, to myosin and actin filaments beginning to be produced indiscriminately. Then, subsequently, the new filaments, in the second phase, organized themselves, forming the sarcomeres of the myofibrils. As observed in the group treated with paracrine substances, the satellite cells that were in the activation phase were maintained within the basement membrane of the muscle fibre, associating themselves with capillaries. On one hand, these satellite cells increased their metabolism, and then they subsequently re-organized themselves in parallel to the fibres, even forming new multinucleate and contractile ones. These signs of recovery were similar to data observed by GIMÉNEZ et al. (2016), who treated muscle lesions caused by BPA exposure with platelet-derived growth factors. At present, there are no references related to paracrine treatment after exposure to BPA, so what was observed in our study is highly novel and leads us to believe that this type of treatment could be used in cases of chronic exposure to oestrogenizing substances with severe effects, such as those being used in other pathologies.

One of the most important processes in the recovery of the muscle fibres was the state in which the endomysium was left. From that point on, some important differences were found between the recovery group with no treatment, the group with only the withdrawal of BPA, and the group with subsequent paracrine treatment after BPA withdrawal. In the withdrawal group, the muscle fibres acquired an acceptable degree of recovery of the myofibrils, as described when studying the sarcomeres. The same happened in the fibres treated with paracrine factors, so that in both cases it could be considered that the fibres had recovered their vitality and function. It was observed that in the endomysium of the fibres with no paracrine treatment, heavy condensation of collagen fibre was produced. However, in those treated with monocytes, the collagen was practically non-existent and only its capillaries persisted, coinciding with what was reported by GIMÉNEZ et al. (2016) in mini-pigs treated with platelet-rich plasma. This phenomenon indicated that recovery in the cases of monocyte treatment was good both in the fibre and in the endomysium, whereas, in the withdrawal group, the fibre recovered its functionality, but at an endomysium level the massive appearance of the collagen fibres would trigger fibrosis processes, which would make these muscles susceptible to subsequent tears.

In conclusion, we established a model for necrosis and degeneration of muscle fibres by exposure to BPA, that served as a starting-point of treatments for their recovery from the activation of their myoblasts and of the muscle fibres themselves. It was seen how, after BPA withdrawal, there was a partial recovery of the histological modifications appearing in the muscle. Also, the treatment with monocytes showed the potential regenerating capacity of these compounds in a damaged muscle after exposure to an xenoestrogen.

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SAŽETAK

Bisfenol A (BPA) uzrokuje endokrine poremećaje koji narušavaju zdravlje ljudi i životinja. Kako se BPA, uz svoju prisutnost u polikarbonatnim posudama i aluminijskim limenkama, nalazi i u hrani i piću, ljudi su mu stalno izloženi. Zbog važnosti u javnome zdravstvu i nepostojanja svijesti o izloženosti BPA-u, istražili

smo njegovo brzo djelovanje na mišićna tkiva i mogućnosti liječenja monocitima iz koštane srži. U tu je svrhu 16 minisvinja nasumično raspoređeno u četiri skupine (n = 4): 1. kontrolnu skupinu, 2. skupinu koja je bila izložena BPA-u četiri mjeseca, 3. skupinu koja je dva mjeseca bila izložena BPA-u, a zatim joj je uskraćen, 4. skupinu koja je dva mjeseca bila izložena BPA-u, a zatim je dva mjeseca liječena monocitima iz koštane srži. Na kraju liječenja učinjena je biopsija longissimus lumborum (LL). Uzorci su analizirani optičkom i elektronskom mikroskopijom. Istraživanje je pokazalo da nakon kontinuirane izloženosti BPA-u postoje promjene i u mišićnim i u kolagenskim vlaknima. Skupina kojoj je BPA uskraćen nakon dva mjeseca pokazala je reverzibilni degenerativni učinak, s djelomičnim oporavkom i mišićnih i kolagenskih vlakana. U posljednjoj ispitivanoj skupini procijenili smo učinak monocita iz koštane srži na promijenjena mišićna tkiva i promatrali ima li znatnijeg oporavka s obzirom na to da su se gotovo sva promijenjena mišićna i kolagenska vlakna regenerirala.

Ključne riječi: bisfenol A; minisvinja; monociti iz koštane srži