MODULATION OF NITRIC OXIDE PRODUCTION BY THERAPEUTIC PULSED ULTRASOUND IN A CANINE TIBIA FRACTURE MODEL

Mihail Paskalev, Nikolay Goranov
Department of Veterinary Surgery, Faculty of Veterinary Medicine, Trakia University, 6000 Stara Zagora, Bulgaria
E-mail: paskalev@uni-sz.bg

Abstract. The purpose of the present experiment was to monitor the effect of therapeutic ultrasound application on the time course of blood nitric oxide concentrations in an experimental fracture model in dogs. In 24 male mixed-breed adult dogs, transperiosteal osteotomies of right tibia and fibula were performed. After being operated, dogs were allotted to 4 groups: in Group 1 (IMO control; n=6) the osteotomies were fixed with a Kuntscher nail (intramedullar osteosynthesis; IMO). In Group 2 (PLO control; n=6), the osteotomies were fixed with a plate and 6 cortical screws. In dogs from Group 3 (IMO+U; n=6), fractures were fixed with Kuntscher nails and a low-intensity ultrasound therapy was applied at the fracture site. Osteotomies of dogs from Group 4 (PLO+U; n=6) were fixed by plate osteosynthesis and submitted to ultrasound therapy. Blood serum nitric oxide concentrations were assayed prior to the surgery, by post operative weeks 1, 2 and post operative months 1, 2 and 3. In control groups (IMO and PLO), serum NO increased statistically significantly as early as the first week and persisted high until the end of the second week. In the groups treated with ultrasound, the increase was significant by the end of the second post-operative week for both osteosynthesis techniques used. For the PLO+U group only, they remained higher vs preoperative values until the end of the second month. Serum NO concentrations differed considerably between ultrasound-treated and control groups by the first post-operative week ($p<0.01$ in both methods of osteosynthesis) and by the end of the 2nd and the 3rd months ($p<0.01$ for PLO groups). In the early fracture healing stage, NO levels were higher in control groups, whereas in late stages – in ultrasound-treated groups. In conclusion, despite the osteosynthesis technique used, the application of therapeutic ultrasound reduced the production of nitric oxide during the early inflammation stage of fracture healing but stimulated its formation at later stages of callus formation. It could therefore be successfully utilized as a physical therapeutic adjunct to operative treatment of such traumas in a clinical setting.

Keywords: dogs, osteosynthesis, therapeutic ultrasound, nitric oxide.

ULTRAGARSO EFEKTAS ŠUNŲ EKSPERIMENTINIO BLAUZDIKAULIO LŪŽIMO ATVEJAIS ESANT SKIRTINGAI AZOTO OKSIDO KONCENTRACIJAI

Mihail Paskalev, Nikolaj Goranov
Chirurgijos skyrius, Veterinarinės medicinos fakultetas, Trakijos universitetas
6000 Stara Zagora, Bulgarija
el. paštas: paskalev@uni-sz.bg

Santrauka. Atliktas bandymas norint ištirti ultragarso poveikį šunų eksperimentinio blauzdikaulio lūžimo atvejais esant skirtingai azoto oksido (AO) koncentracijai. Tyrimas atliktas su 24 šunų patinais mišrūnais, kuriems buvo atlikta dešiniojo blauzdikaulio ir šeivikaulio osteotomija. Po operacijos patinai mišrūnai suskirstyti į 4 grupes: I grupė (IMO kontrolė; n=6) – šunys, kuriems osteotomija atlikta naudojant Kuntčerio geležtę (intramedulinė osteosintezė; IMO); II grupė – šunys (PLO kontrolė; n=6), kuriems osteotomijai atlikti naudota plokštelė ir šeši kortikaliniai sraigtai; III grupė – šunys (IMO+U; n=6), kurių lūžiai fiksuoti Kuntčerio geležte ir taikyta ultragarso terapija; IV grupė (PLO+U; n=6) – šunys, kuriems osteotomijos fiksuotos plokštele ir taikyta ultragarso terapija.

Azoto oksido koncentracija kraujo serume buvo matuojama prieš operaciją, 1–2 savaites ir 1, 2 bei 3 mėnesius po operacijos. I ir II grupės šunų AO koncentracija serume statistiškai ženkliai padidėjo jau pirmąją bandymo savaitę ir tokia išliko iki antros savaitės pabaigos. III ir IV grupės šunims, kuriems buvo taikomas ultragarsas, statistiškai ženklus AO padidėjimas nustatytas nuo antros pooperacinės savaitės. Padidėjusi AO koncentracija kraujo serume IV grupės šunims nustatyta iki antro mėnesio po operacijos pabaigos. Azoto oksido koncentracija buvo statistiškai ženkliai skirtinga tarp ultragarsu gydytų šunų (III ir IV grupės) ir negydytų gyvūnų (I ir II grupės) ($p<0,01$ osteosintezės metu) ir antro bei trečio mėnesio po operacijos metu ($p<0,01$ palyginti su kontrole). Ankstyvoje lūžio gijimo stadijoje AO koncentracija didesnė buvo kontrolinės I ir II grupės šunų kraujo serume, o III ir IV grupės šunų, gydytų ultragarsu, – vėlesnėse gijimo stadijose. Nustatyta, kad, nepaisant taikyto osteosintezės metodo, ultragarso terapija AO koncentraciją kraujo serume sumažino ankstyvose lūžio gijimo stadijose ir stimuliavo kaulo gijimą vėlesnėse gijimo stadijose. Taigi, norint, kad kaulai gytų sparčiau, siūloma papildomai taikyti ultragarso terapiją.

Raktažodžiai: osteosintezė, ultragarso terapija, azoto oksidas, šunys.

Introduction. Nitric oxide (NO) is a free radical gas produced during the conversion of the L-arginine in L-citrulline (Moncada & Higgs, 1993). The reaction is catalyzed by nitric oxide synthase (NOS), existing in

three isoforms: endothelial (eNOS; type III), neuronal (nNOS; type I) and inducible (iNOS; type II) (Alderton et al., 2001). The first two isoenzymes are normally expressed in the respective cells and are calcium-dependent. The third isoenzyme formation is induced by cellular and humoral factors in a number of pathological events. It is calcium-independent and produces a considerable amount of NO (Michel & Feron, 1997).

The role of NO in bone pathology is still disputable. Some authors put it in the group of free radicals and consider that it is involved in lipid peroxidation at a specific stage of oxidative stress development (Stamler, 1994; Viola et al., 2000). Others suggest that depending on the process, NO could act as antioxidant or prooxidant (Andican et al., 2005).

It is reported that the production of NO could regulate the metabolism of both osteoblasts and osteoclasts and thus, a number of metabolic bone events (Collin-Osdoby et al., 1995; Corbett et al., 1999; Diwan et al., 2000). The high NO levels, produced by cytokine-induced iNOS suppresses the growth and differentiation of osteoblasts (van't Hof et al., 2000; Armour et al., 2001), whereas moderate elevation attributed to eNOS activity is necessary for the normal function of osteoblasts and bone healing (Corbett et al., 1999). The role of NO in the differentiation and enzyme activity of osteoblasts and osteoclasts depends on the stage of bone fracture healing (Zhu et al., 2001, 2002; Kdolsky et al., 2005; Sinha & Goel, 2009). Keskin & Kiziltunç (2007) have reported a considerable increase in serum NO concentrations during the first 2 weeks and especially the first 6 hours in single or multiple bone fractures in human patients. Ectopic osteoinduction in rats caused increased blood levels of NO metabolites that confirm its role in cell differentiation in osteogenesis (Bigham et al., 2009).

There are evidence that NOS suppression impairs, while the supplementation of NO at the fracture site or of L-arginine in diet restored and stimulated, respectively, normal bone healing in rats, men and guinea pigs (Diwan et al., 2000; Kdolsky et al., 2005). The role of L-arginine is also confirmed in an experimental rabbit ulna osteotomy model (Sinha & Goel, 2009).

Applied on fractures, ultrasound produces repeated micromechanical stress on bones and surrounding soft tissues by motion of fractured ends, cavitation and acoustic streaming (Mundi et al, 2009). The mechanically stimulated cancellous bone remodeling is enhanced as well as vascular ingrowth.

Numerous studies provide evidence that low-intensity pulsed ultrasound (LIPUS) produces significant osteoinductive effects. The overall mineral density of fractured bone and the density at the site of fracture are increased (Heybeli et al., 2002). The healing process is accelerated (de Sousa et al., 2008) and the bone-bending strength improved (Wang et al., 1994 и Yang et al., 1994; de Albornoz et al., 2011).

Reports on NO release during ultrasound-stimulated fracture healing are very limited. Ultrasound was found to produce a significant increase in both induced nitrite and PGE_2 production in a culture of human mandibular osteoblasts that could explain the healing effect of sonic waves on bone (Reher et al., 2002). There are no available information on the in vivo time course of nitric oxide concentrations in fracture models, when bone healing is stimulated by low-intensity pulsed ultrasound therapy.

The purpose of the present experiment was to monitor the effect of therapeutic ultrasound application on the time course of blood nitric oxide concentrations in an experimental fracture model in dogs.

Material and methods

The experiment was approved by the Committee on Animal Ethics to the National Veterinary Service in Bulgaria (protocol 16/09.06.2010) and performed in strict compliance with Directive 86/609/EEC on the protection of animals used for scientific purposes, the Animal Welfare Act 25/10.06.05 and the Law on Veterinary and Medical Activities.

Experimental animals

Twenty four male mixed-breed adult dogs, born and reared in the kennels of the Faculty of Veterinary Medicine, were used. They were from 2 to 5-year-old and weighed 12-20 kg. After the end of the experiment, the dogs were returned to the shelter or were adopted by citizens and students.

Ten days before the start of the trial, all dogs were treated against endo- and ectoparasites. They were housed in individual boxes, fed with a commercial dry food for adult dogs and received water *ad libitum*.

Anaesthetic and operative protocols

After pre-treatment with 0.02 mg/kg s.c. atropine sulphate® (Sopharma, Bulgaria) and 0.05 mg/kg i.m. acepromazine maleate (Neurotranq®, Alfasan, Woerden, Holland), anaesthesia was induced with 6 mg/kg i.v. 2.5% thiopentone sodium (Thiopental®, Biochemie GmbH, Kudl, Austria) and maintained after intubation with 2.5% halothane (Narcotan®, Spofa, Czech Republic).

After aseptical preparation and medial approach, transperiosteal osteotomies of diaphyses of the right tibia and fibula were performed. After being operated, dogs were allotted to 4 groups as followed: In Group 1(IMO control; n=6) the osteotomies were fixed with a normograde insertion of a Kuntscher nail in the medullar canal (intramedullar osteosynthesis; IMO). In Group 2 (PLO control; n=6), the osteotomies were fixed with a plate (3.5 mm thick and 10 mm wide) and 6 cortical screws (3 in the distal and 3 in the proximal bone fragment; 3.5 mm). In dogs from Group 3 (IMO+U; n=6), fractures were fixed with Kuntscher nails and a low-intensity ultrasound therapy was applied at the fracture site. Osteotomies of dogs from Group 4 (PLO+U; n=6) were fixed by plate osteosynthesis and submitted to ultrasound therapy.

The soft tissues were routinely sutured and protective bandages were placed on operated areas.

Pre- and postoperative pain management was done with butorphanol tartrate (Torbutrol®, Fort Dodge, USA, 0.2 mg/kg s.c., every 6 hours) for 3 days. An intramuscular antibiotic combination of lincomycin and spectinomycin (Linco-Spectin®, Pharmacia N.V./S.A.,

Puurs, Belgium) was administered at 1 mL (50 mg lincomycin and 100 mg spectinomycin) per 5 kg body weight for four days following surgery.

Ultrasound therapy

An ultrasound wave generator BTL-07 (Beauty Beautyline® BTL Czech Republic) was used for the therapy. Daily procedures were performed in groups 3 and 4 (IMO+U and PLO+U) using a 1 cm² semi-stationary transducer, frequency 3 MHz, 50 Hz impulse regimen, power density 1 W/cm² and a pulsed wave duty cycle of 1/2 (impulse time/interval) as recommended by the manufacturer. The procedures were performed during the first 10 post-operative days with duration of 10 min.

Blood sampling and analysis

Venous blood samples were collected by cephalic venepuncture in plain tubes prior to the surgery, on post operative weeks 1, 2 and post operative months 1, 2 and 3 between 7.30 and 8.00 AM for avoiding circadian effects. After clotting, blood samples were centrifuged (1000×g, room temperature, 10 min), and sera were analyzed immediately. Deproteinization was done with acetonitrile (1:1 v/v) (Ghasemi et al., 2007).

All chemicals used – sulfanilamide, N-(1-Naphthyl) ethylendiamine dihydrochloride; acetonitrile, potassium nitrate, vanadium (III) chloride – were purchased from Merck (Germany) and Fluka (Swiss).

Nitric oxide concentrations in deproteinized sera were assayed by the method of Miranda et al. (2001) for simultaneous evaluation of nitrate and nitrite concentrations in biological fluids. The principle of this assay is reduction of nitrate by vanadium(III) and detection by the acidic Griess reaction.

Statistical analysis

The results were statistically processed by repeated measures ANOVA or the non-parametric Friedman's test for two-way repeated measures analysis. Differences were considered as significant at $p<0.05$.

Results

In the post operative period, there were no deviations in the general condition of dogs. The rectal body temperature, respiratory and heart rates were within the reference ranges. The appetite was preserved. Dogs were predominantly lying during the first day after the surgery, and over the next days exhibited weight-bearing lameness grade II to III that disappeared within one week in groups with plate osteosynthesis and within 2–3 weeks in groups with intramedullary osteosynthesis

The results from blood serum NO assays are presented in Table 1. In control groups (IMO and PLO), serum NO increased statistically significantly as early as the first week to 97.66±4.17 μmol/l ($p<0.001$) and 83.83±5.30 μmol/l ($p<0.001$) respectively compared to baseline and persisted high until the end of the second week. In the groups treated with ultrasound, the increase was significant by the end of the second post-operative week (95.16±9.70 μmol/l in the IMO+U group and 88.5±12.61 μmol/l in the PLO+U group). In the group with plate osteosynthesis and ultrasound therapy, high NO levels in blood remained statistically significantly higher than preoperative values until the end of the second month ($p<0.05$).

Table 1. **Time course of blood serum nitric oxide** (μmol/l) **in dogs with experimental tibial osteotomies, fixed by either intramedullary** (IMO control) **or plate osteosynthesis** (PLO control) **without or with application of therapeutic ultrasound** (IMO+U; PLO+U)**. Data are presented as mean±SEM; n=6.**

	IMO control	IMO+U	PLO control	PLO+U
baseline	60.83±4.12	63.66±3.80	38.67±2.12	34.17±1.68
week 1	97.66±4.17***	50.50±2.44 #	83.83±5.30***	59.33±3.25 #
week 2	98.50±2.97***	95.16±9.70**	82.50±2.52***	88.5±12.61*
month 1	60.83±4.11	64.66±5.51	50.66±3.45	83.66±21.65*
month 2	46.33±3.77	56.33±3.55	50.66±4.62	89.16±10.69* #
month 3	43.00±3.84	46±4.04	39.16±4.30	58.33±4.75 [b]

*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs preoperative levels (baseline); # $p < 0.01$ between respective control and experimental groups

Serum NO concentrations differed considerably between ultrasound-treated and control groups by the first post-operative week ($p<0.01$ in both methods of osteosynthesis) and by the end of the 2nd and the 3rd months ($p<0.01$ for PLO groups). By the end of the 1st week, NO levels were higher in control groups ($p<0.01$), whereas by the 2nd and 3rd months - in ultrasound-treated groups.

No variations in blood NO levels were observed in relation to the used method of osteosynthesis.

Discussion

In the experimental canine tibia osteotomy model used, blood serum NO increased statistically significantly as early as the first post operative week, persisted elevated by the end of the 2nd week and returned to baseline values either gradually (when intramedullary osteosynthesis was applied) or rapidly (in fractures fixed by plate and screws). Two mechanisms could be involved in these events. The first, rapidly triggered mechanism is mediated via cytokine-induced inducible nitric oxide synthase in macrophages and monocytes, and the other is slower and acts via endothelial NOS, activated after vascular destruction (Zhu et al., 2001; 2002). Therefore, we could assume the increase blood NO concentrations during the first post fracture week is generated by the first mechanism, whereas the second was responsible for NO

production at later stages.

The nitric oxide produced by iNOS inhibits the growth and differentiation of osteoblasts, and its eNOS-mediated gradual increase supports osteoblastic function (Corbett et al., 1999; van't Hof et al., 2000; Armour et al., 2001). The role of eNOS-produced nitric oxide is essential as once generated, it stimulates the ingrowth of new blood vessels by assisting the formation of the vascular endothelial growth factor (Wang et al., 2004).

In human patients with single and multiple non-fixed fractures, serum NO concentrations exhibited a peak by the 6th hour, then decreased up to the 3rd day and increased again by the end of the 2nd week (Keskin & Kiziltunc, 2007). Similar temporal pattern in NO production is reported by Diwan et al. (2000). On the contrary, Corbett et al. (1999) observed an enhanced eNOS expression by the first day and of iNOS – as late as the 14th day. The results obtained in this study rather support the findings of the first group of researchers because high blood NO levels occurred during the first 2 weeks. The nitric oxide generated after the activation of cytokine-induced iNOS could inhibit osteoblasts' growth and differentiations and stimulate, either directly or via reactive oxygen species, osteoclastic activity (van't Hof et al., 2000; Armour et al., 2001).

An interesting time course of blood NO concentrations was observed when therapeutic ultrasound was applied. During the first week, physical procedures reduced the inflammation and serum NO levels also decreased. It could be assumed that iNOS activity has also decreased and thus provide en explanation for the difference observed between ultrasound-treated and control groups. It was emphasized (Hantes et al., 2004), that the effect of therapeutic ultrasound application in fractured bones was expressed rather during the phase of inflammation and early callus formation, than during the remodeling stage. In experimental groups, treated with ultrasound, blood NO concentrations reached peaks by the end of the 2nd week and remained relatively high for a long time. In the PLO+U group, NO levels remained statistically significantly higher until the end of the 2nd month. Provided that this resulted from the enhanced activity of NOS isoforms (Diwan et al., 2000; Zhu et al., 2002) it could be inferred that therapeutic ultrasound had a beneficial effect on bone healing. We could only make suggestions about the origin of NO as the activity of the three NOS isoforms was not a subject of this study. Nevertheless, the later gradual increase in blood NO levels, the lack of inflammation at the osteotomy site and the better radiological healing scores in groups treated with ultrasound allowed assuming that the produced NO and its beneficial effects were most probably of endothelial origin.

Conclusion

The therapeutic pulse ultrasound (frequency 3 MHz, impulse frequency 50 Hz, power density applied 1 W/cm^2) applied on recent fractures of the long bones in dogs for 10 min over 10 consecutive days, decreased the formation of NO during the early inflammation stage and stimulated NO production during the stage of callus formation. Considering the acknowledged role of nitric oxide during bone healing, it could be assumed that ultrasound would be a useful physical therapeutic adjunct to operative treatment of such traumas in a clinical setting.

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