Bulgarian Journal of Veterinary Medicine (2011), **14**, No 4, 231–237

RELATIONSHIP BETWEEN BLOOD MALONDIALDEHYDE AND CATALASE CONCENTRATIONS AND THE TIME OF OCCURRENCE OF NON-FIXED LONG BONE FRACTURES IN DOGS

M. PASKALEV

Department of Veterinary Surgery, Faculty of Veterinary Medicine,
Trakia University, Stara Zagora, Bulgaria

Summary

Paskalev, M., 2011. Relationship between blood malondialdehyde and catalase concentrations and the time of occurrence of non-fixed long bone fractures in dogs. *Bulg. J. Vet. Med.*, **14**, No 4, 231–237.

The purpose of the present study was to follow out the blood concentrations of malondialdehyde (MDA), an end product of lipid peroxidation and the activity of one of antioxidant defense enzymes – catalase (CAT), in dogs with long bone fractures. Forty eight dogs with single fractures of the humerus, radius and ulna, femur and tibia were included. Depending on the time elapsed from the trauma to the referral in the clinic and blood sample collection, 7 time periods were formed: less than 12 h; 12–24 h; 24–72 h; 3–6 days; 1–2 weeks; 2–4 weeks; 4–8 weeks. Blood samples were obtained during the initial examination and before the surgery. Another 36 clinically healthy adult dogs aged 2 to 6 years served as controls. Statistically significantly higher MDA concentrations were observed in fractures with < 12 h elapsed from the accident. They remained elevated in dogs with bones fractured up to 2 weeks ago ($p<0.001$). In dogs with 2 to 4 weeks old fractures, they were lower and increased again in fractures occurring 4–8 weeks ago ($P<0.05$). The activity of catalase was significantly higher at time from fracture periods 12–24 h ($p<0.001$), 3–6 days ($p<0.001$) and 1–2 weeks ($p<0.001$). The results demonstrated high extent of blood lipid peroxidation together with increased catalase activity in dogs with accidental non-fixed fractures of long bones in dogs which occurred up to 2 weeks ago. It could be therefore affirmed that all methods and treatment options that would reduce blood MDA concentrations in the early post fracture period and at the same time, maintain an adequate level of the antioxidant defense, would be beneficial for the timely and proper bone healing.

Key words: bone fracture, catalase, dogs, malondialdehyde

INTRODUCTION

The group of reactive oxygen species (ROS) includes the superoxide radical ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), nitric oxide (NO), peroxynitrite ($ONOO^-$) and hypochloric acid (HOCl). The increased systemic level of ROS or the compromised antioxidant defense causes DNA damage, protein synthesis inhibition, disorganisation of cell structure and functions (Chopra *et al.*, 1998; Patterson *et al.*, 1998). The impaired equilibrium between ROS and the antioxidant defense is termed oxidative stress. The main targets of oxygen-derived radicals are polyunsaturated fatty acids in cellular membrane lipids, causing lipid peroxidation (Marks *et al.*, 1996).

The normal concentrations of malondialdehyde (MDA), glutathione peroxidase (Gpx) and superoxide dismutase

(SOD) activities are considerably higher in older dogs. Elderly dogs, especially males, are more vulnerable to the adverse effect of free radicals and lipid peroxidation than young animals (Vajdovich *et al.*, 1993; Todorova *et al.*, 2005). The physical exercise is also known to produce oxidative stress and to reduce systemic antioxidant potential (Marshall *et al.*, 2002).

The formation and accumulation of ROS and their harmful products after bone fractures, both at the fracture site and in peripheral blood is related to the impaired blood supply in bone ends. Beyond any doubt, it influences the bone healing process. Numerous investigations have reported the occurrence and development of oxidative stress in spontaneous and experimental fractures in rats (Turgut *et al.*, 1999; Cetinus *et al.*, 2005; Yeler *et al.*, 2005) and humans (Wildburger *et al.*, 2000; Prasad *et al.*, 2003; Keskin & Kiziltunc, 2007), and experimental osteotomies in rats (Tasatargil *et al.*, 2006). In experimental model of canine osteoarthritis, Goranov (2007) has demonstrated the relationship between some oxidative stress indices and cartilage collagen destruction.

The scavenging of ROS depends on the activity of enzymatic antioxidant defense including SOD, catalase (CAT), Gpx and paraoxonase. SOD is the first enzyme, catalysing the detoxication of superoxide radical (Gonzales *et al.*, 1984). The formed H_2O_2 is then reduced to water by CAT and Gpx. Normally, CAT is not essential for some cell types, but under oxidative stress it is a very adaptable antioxidant enzyme playing an important role in cell defense from oxidative damage (Mates *et al.,* 1999).

The intricate mechanisms of oxidative stress in bone fractures could not be monitored by conventional clinical and diagnostic imaging methods such as radiography, radioscopy, computed tomography, echography etc. To this end, biochemical assays of blood oxidative stress parameters are particularly useful. Commonly used markers of systemic oxidative stress are the end product of lipid peroxidation MDA, as well as some of enzymes involved in ROS degradation.

The purpose of the present study was to investigate the effect of accidental long bone fractures in dogs on blood malondialdehyde and catalase concentrations.

MATERIALS AND METHODS

Animals

The survey included 48 dogs (31 males and 17 females) with closed non-complicated long-bone fractures as followed: humerus – 10; radius and ulna – 9; femur – 15; tibia – 14. All animals were referred to the Small Animal Clinic at the Faculty of Veterinary Medicine – Stara Zagora, Bulgaria within 2008–2010. They were aged between 4 months and 4 years and were not treated operatively. Depending on the time elapsed from the incident to the physical examination and blood sampling, the dogs were divided into 7 groups: <12 h: 6 dogs; 12–24 h: 6 dogs; 24–72 h: 8 dogs; 3–6 days: 10 dogs; 1–2 weeks: 6 dogs; 2–4 weeks: 6 dogs and 4–8 weeks: 6 dogs. Blood samples were obtained at referral, before the surgery. As controls, 36 clinically healthy dogs (20 males and 16 females), aged 2–6 years, were used.

Blood sampling and analyses

Venous blood samples were collected by cephalic venepuncture in plain tubes. After clotting, blood samples were centrifuged (1000×*g*, room temperature, 10 min) and sera were harvested.

Malondialdehyde assay is based on the formation of a 1:2 red adduct between MDA and 2-thiobarbituric acid in acid medium that is quantitated at 532 nm after extraction with n-butanol (Uchiyama & Michara, 1978; Andreeva *et al.*, 1988). As a MDA standard, 1,1,3,3 tetraethoxypropane (Sigma Aldrich Chemie GmbH, Munich, Germany) was used.

Serum catalase (CAT) concentrations were assayed by the method of Goth (1991) based on the formation of a stable yellow complex between the substrate (hydrogen peroxide in sodium-potassium phosphate buffer, pH 7.4) and ammonium molybdate, quantified at 405 nm.

The results were statistically processed by the non-parametric Mann Whitney U-test.

RESULTS

In non-fixed long bone fractures which occurred less than 12 hours ago blood MDA concentrations were significantly higher ($P<0.001$) compared to those in healthy dogs (Table 1). The same was observed for fractures occurring 12 hours to 2 weeks before. Dogs with bones fractured 2 to 4 weeks before exhibited MDA values similar to those of healthy controls, whereas a statistically significant increase was registered for the group with 4 to 8 weeks elapsed from fractures ($P<0.05$).

The catalase activity in blood was substantially higher in dogs with bones fractured 12 to 24 h ago ($P<0.001$), reduced in the 24–72 h group and elevated in older fractures (3–6 days and 1–2 weeks; $P<0.001$).

When time elapsed from the fracture was more than 2 weeks, the activity of this enzyme was reduced and even lower than control values.

DISCUSSION

Bone fractures occur after a severe mechanical trauma which also damages the surrounding soft tissues. Thus triggers both a local and systemic response via activation of cellular and humoral factors, aimed at repair of injuries (Keel & Trentz, 2005). In several reports, a relationship between bone healing and ROS accumulation at the fracture site was observed (Kozlova *et al.*, 1997; Turgut *et al.*, 1999; Prasad *et al.*, 2003; Petrovich *et al.*, 2004;

Table 1. Blood malondialdehyde and catalase concentrations in dogs with long-bone fractures according to the time elapsed from the incident. Data are presented as mean ± SEM

Time elapsed from fracture	Number of dogs	Malondialdehyde, μmol/L	Catalase, kU/L
Healthy controls	36	16.17±0.76	22.44±1.53
< 12 hours	6	24.66±2.83 ***	29.00±6.47
12 to 24 hours	6	27.87±3.43 ***	49.33±6.38 ***
24 to 72 hours	8	25.10±1.61 ***	29.87±7.78
3 to 6 days	10	26.04±1.95 ***	32.20±3.90 ***
1 to 2 weeks	6	26.75±5.70 ***	83.66±14.04 ***
2 to 4 weeks	6	18.41±2.09	22.83±6.21
4 to 8 weeks	6	20.57±2.76 *	15.66±4.54

* $P<0.05$; *** $P<0.001$ vs controls.

Yeler *et al.*, 2005). MDA is the end product of lipid peroxidation and its concentrations at the site of injury, blood serum and urine are believed to indicate developing oxidative stress (Durak *et al.*, 1996; Cetinus *et al.*, 2005; Yeler *et al.*, 2005; Goranov, 2007).

The time course of oxidative stress parameters in bone fractures depends also on whether they are fixed operatively or not. After intramedullary osteosynthesis of the tibia in rats, Turgut *et al.* (1999) reported statistically significantly higher MDA concentrations at the fracture site by the 7th and the 14th day in comparison with intact tibia with inserted Kirschner nail.

The type of osteosynthesis is also important. In previous studies of ours, we established that experimental canine osteotomies fixed by intramedullary pinning, blood serum MDA concentrations were the highest by the end of post operative weeks 2 and 4. In osteotomies fixed by a plate and screws, MDA increased gradually but less markedly and attained a peak by the end of the first month (Paskalev, 2009). Similar results were obtained in humans by Prasad *et al.* (2003), and in rats (Petrovich *et al.*, 2004; Cetinus *et al.*, 2005; Yeler *et al.*, 2005).

What is happening when a closed bone fracture is not treated surgically? In this study, we have established significantly higher MDA levels up to the 12th h after the trauma. It is acknowledged that the inflammation stage during normal bone healing occurs during the first 24–48 h and continues until the appearance of new bone vessels, granulation and cartilage tissue. During this stage, an influx of polymorphonuclear leukocytes, macrophages and mastocytes at the fracture site is observed (Cornell & Lane, 1992). Activated leukocytes produce reactive oxygen species, causing lipid peroxidation (Reilly *et al.*, 1991). Other researchers assume that oxidative stress occurs at a later stage (2–3 weeks) after the fracture (Prasad *et al.*, 2003; Petrovich *et al.*, 2004; Cetinus *et al.*, 2005; Yeler *et al.*, 2005). A third group claims that the first three days of bone healing could be compared to the period of ischaemia, while the second and the third week – to the period of reperfusion (Turgut *et al.*, 1999). All cited opinions are, in our view, correct. The oxidative stress products are accumulated at the site of fracture during the inflammatory stage, and after the revascularization they appear into the circulation. The very early increase in MDA observed in dogs with fractures occurring less than 12 h ago could be attributed to the stress of the trauma itself and the accompanying haemodynamic changes. Afterwards, they are due to oxidative stress developing in soft tissues and on a later stage, to events occurring in bone ends. The reason is the effect of ischemia-reperfusion mechanism, which occurs later in bone and is in the background of oxidative stress. An interesting fact was the finding that in accidental non-fixed long-bone canine fractures, blood MDA concentrations remained statistically significantly higher than those in healthy controls even in fractures having occurred up to two weeks ago.

It was experimentally proved that when lipid peroxidation products could not be neutralized by endogenous defense mechanisms, they disturb and slow down the bone healing in rats (Göktürk *et al.*, 1995; Duygulu *et al.*, 2007). Catalase (CAT) is a part of systemic endogenous antioxidant defense, together with SOD and Gpx. The three enzymes are involved in a chain of reactions aimed at detoxification of free oxygen radicals. The initially formed superoxide radical is not

stable and is converted to H_2O_2 and water, either spontaneously or by the action of SOD. The hydrogen peroxide could be scavenged by two mechanisms. First, in the presence of iron in aqueous medium, highly destructive hydroxyl radicals could be generated (Fenton's reaction). Alternatively, it could be converted to water and molecular oxygen by catalase (Lantz, 1995).

In the studied dogs, catalase activity was found to be elevated in fractures occurring 12 to 24 h ago, then was lower in those happening 24 to 72 h ago and increased when the time elapsed after the bone fracture was between 3 and 14 days. It could be inferred that, due to the negative feedback, these higher activities are provoked by respective high H_2O_2 concentrations. Similar changes were observed in an earlier study of ours in operatively fixed experimental tibial osteotomies in dogs (Paskalev, 2009). In fractures older than 2 weeks, the activity of CAT in blood decreased and in patients referred 4 to 8 weeks after the fracture, was even lower than the average level in controls.

In conclusion, accidental non-fixed long-bone fractures in dogs occurring up to 2 weeks ago were accompanied with high blood MDA concentrations. Blood CAT activities were also statistically significantly higher from the 12^{th} to the 24^{th} hour after the incident. These findings confirmed once again the need of rapid and adequate intervention, in accordance with coexistent circumstances. It could be therefore affirmed that all methods and treatment options that would reduce blood MDA concentrations in the early post fracture period and at the same time, maintain an adequate level of the antioxidant defense, would be especially beneficial for the timely and proper bone healing.

REFERENCES

Andreeva, L. I., L. A. Kozhemyakin & A. A. Kishkun, 1988. A modified thiobarbituric acid test for measuring lipid peroxidation. *Laboratornoe Delo*, **11**, 41–43 (RU).

Cetinus, E., M. Kiline, M. Uzel, F. Inanc, E. B. Kurutas, E. Bilgic & A. Karauguz, 2005. Does long-term ischemia affect the oxidant status during fracture healing? *Archives of Orthopaedic and Trauma Surgery*, **125**, 376–380.

Chopra, S. & H. M. Wallace, 1998. Induction of spermidine/spermine N1-acetyltransferase in human cancer cells in response to increased production of reactive oxygen species. *Biochemical Pharmacology*, **55**, 1119–1123.

Cornell, C. N. & J. M. Lane, 1992. Newest factors in fracture healing. *Clinical Orthopedics*, **277**, 297–311.

Durak, K., O. F. Bilgen, T. Kaleli, P. Tuncel, R. Ozbek & K. Turan, 1996. Antioxidant effect of alpha-tocopherol on fracture haematoma in rabbits. *Journal of International Medical Research*, **24**, 419–424.

Duygulu, F., B. Yakan, S. Karauglu, R. Kutlubay, O. I. Karahan & A. Ozturk, 2007. The effect of zymosan and the protective effect of various antioxidants on fracture healing in rats. *Archives of Orthopaedic and Trauma Surgery*, **127**, 493–501.

Göktürk, E., A. Turgut, C. Bayçu, I. Günal, S. Seber & Z. Gülbas, 1995. Oxygen free radicals impair fracture healing in rats. *Acta Orthopaedica Scandinavica*, **66**, 473–475.

Gonzales, R., C. Auclair, E. Voisin, H. Gautero, D. Dhermy & P. Boivin, 1984. Superoxide dismutase, catalase and glutathione peroxydase in red blood cells from patients with malignant diseases. *Cancer Research*, **44**, 4137–4139.

Goranov, N. V., 2007. Serum markers of lipid peroxidation, antioxidant enzymatic defense and collagen degradation in an experimental (Pond-Nuki) canine model of

osteoarthritis. *Veterinary Clinical Pathology*, **36**, 192–195.

Goth, L., 1991. A simple method for determination of serum catalase activity and revision of reference range. *Clinica Chimica Acta*, **196**,143–151.

Keel, M. & O. Trentz, 2005. Pathophysiology of polytrauma. *Injury*, **36**, 691–709.

Keskin, D. & A. Kiziltunc, 2007. Time-dependent changes in serum nitric oxide levels after long bone fracture. *Tohoku Journal of Experimental Medicine*, **213**, 283–289.

Kozlova, M. V., V. N. Ivanov, I. S. Pinelis & I. A. Petrovich, 1997. The effect of selenium on free-radical oxidation processes in the bone regenerate after a fracture. *Pathophysiology and Experimental Therapy* (Russia), **2**, 35–37 (RU).

Lantz, G. C., 1995. Oxigen free radicals and reperfusion injury. Special therapy. In: *Kirk's Current Veterinary Therapy XII. Small Animal Practice*, ed J. D. Bonagura, W.B. Saunders, Philadelphia, pp. 64–67.

Marks, D. B, A. D. Marks & C. M. Smith, 1996. Oxygen metabolism and oxygen toxicity. In: *Basic Medical Biochemistry. A Clinical Approach,* ed J. Velker, Williams & Wilkins Baltimore, Maryland, pp. 327–340.

Marshall, R. J., K. C. Scott, R. C. Hill, D. D. Lewis, D. Sundstrom, G. L. Jones & J. Harper, 2002. Supplemental vitamin C appears to slow racing greyhounds. *Journal of Nutrtion*, **132** (Suppl 2), 1616–1621.

Mates, J. M., 1999. Antioxidant enzymes and human disease. *Clinical Biochemistry*, **32**, 593–603.

Paskalev, M., 2009. Time course of serum malondialdehyde concentrations as a marker of oxidative stress in experimental canine osteotomies, fixed by two different techniques. *Comparative Clinical Pathology*, **18**, 265–268.

Patterson, R. A. & D. S. Leacke, 1998. Human serum, cysteine and histidine inhibit the oxidation of low density lipoprotein less at acidic pH. *FEBS Letters*, **434**, 317–321.

Petrovich, Y. A., R. P. Podorozhnaya, S. M. Kichenko & M. V. Kozlova, 2004. Effects of selenium-containing compounds and their metabolism in intact rats and in animals with bone fractures. *Bulletin of Experimental Biology and Medicine*, **137**, 74–77.

Prasad, G., M. S. Dhillon, M. Khullar & O. N. Nagi, 2003. Evaluation of oxidative stress after fractures. A preliminary study. *Acta Orthopaedica Belgica*, **69**, 546–551.

Reilly, P. M., H. J. Schiller & G. B. Bulkley, 1991. Pharmacologic approach to tissue injury mediated by free radicals and other reactive oxygen metabolites. *American Journal of Surgery*, **161**, 488–503.

Tasatargil, A., B. Cadir, S. Dalaklioglu, E. Yurdakonar, S. Caglar & C. Turkay, 2006. Effects of vitamin K1 supplementation on vascular responsiveness and oxidative stress in a rat femoral osteotomy model. *Cell Biochemistry and Function*, **25**, 485–490.

Todorova, I., G. Simeonova, D. Kyuchukova, D. Dinev & V. Gadjeva, 2005. Reference values of oxidative stress parameters (MDA, SOD, CAT) in dogs and cats. *Comparative Clinical Pathology*, **13**, 190–194.

Turgut, A.,E. Gokturk, N. Kose, M. Kacmaz, H. S. Ozturk, S. Seber & S. Acar, 1999. Oxidant status increased during fracture healing in rats. *Acta Orthopaedica Scandinavica*, **70**, 487–490.

Uchiyama, M. & M. Michara, 1978. Determination of malondialdehyde precursor in tissues by thiobarbituric acid test. *Analytical Biochemistry*, **86**, 271-278.

Vaidovich, P., T. Gaal & A. Szilagil, 1993. Changes of lipid peroxidation parameters in dogs with alloxan diabetes. *Acta Physiologica Hungarica*, **81**, 317–326.

Wildburger, R., S. Borovic, N. Zarkovic & F. Tatzber, 2000. Post-traumatic dynamic changes in the antibody titer against

oxidized low density lipoproteins. *Wiener Klinische Wochenschrift*, **112**, 798–803.

Yeler, H., F. Tahtabas & F. Candan, 2005. Investigation of oxidative stress during fracture healing in the rats. *Cell Biochemistry and Function*, **23**, 137–139.

Paper received 29.07.2011; accepted for publication 11.11.2011

Correspondence:

Assoc. Prof. M. Paskalev, PhD
Department of Veterinary Surgery,
Faculty of Veterinary Medicine,
Student's Campus,
6000 Stara Zagora, Bulgaria
e-mail: paskalev@uni-sz.bg