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COMPARATIVE INVESTIGATION ON BLOOD BONE MARKERS IN NORMALLY HEALING AND INFECTED BONE FRACTURE MODELS IN DOGS

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Summary

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The purpose of the present study was to monitor the time course of serum bone markers concentrations in experimental models of normally healing and complicated fractures of the canine femur. Twelve dogs were divided into two groups: group I (six dogs) with experimental normally healing (NH) fractures and group II (six dogs) with experimentally induced post fracture osteomyelitis of the femur (OM group). Clinical (heart rate, respiratory rate, body temperature) and radiological investigations were carried out over two months after the surgery. By the end of the 1st, 2nd, 3rd and 4th post operative weeks, blood was sampled for analysis of bone formation (total and bone alkaline phosphatase, osteocalcin) and bone resorption (carboxy-terminal telopeptide of type I collagen – ICTP) markers. In the NH group, serum ICTP concentrations were statistically significantly increased by the 1st and 2nd post operative weeks and then returned to normal levels. In the OM group, ICTP was increased considerably by the end of the second week but remained high until the end of the 2nd month. The initial increase occurred before the appearance of specific radiological signs. In the OM group, bone alkaline phosphatase and osteocalcin were also significantly higher, but rapidly declined to baseline concentrations.

Key words: blood biochemical bone markers, dogs, fractures

INTRODUCTION

Biochemical bone markers are widely used in human medical practice for control of therapeutic schedules and monitoring of cell activity in metabolic bone disorders and related diseases (Okazaki *et al.*, 1997). They are an excellent alternative in the study of animal models of osteoporosis, tumour metastasis in bones and some forms of arthritis (Kippo *et al.*, 1998; Tamura *et al.*, 1999; Chavassieux *et al.*, 2001).

Despite that there is a strong correlation between blood and urine bone markers and histomorphometric indices of bone remodeling (Eastell *et al.*, 1988), the definitive diagnosis in osteoporosis and Paget's disease is put after densitometry and/or biopsy.

In animals, bone markers were used to determine the differences in bone formation and resorption levels in horses (Lepage *et al.*, 1991; Price *et al.*, 1995) and cats (DeLaurier *et al.*, 2002; 2004) at different ages. In dogs, bone markers were investigated in detail but only with regard to differences related to breed, age or other factors (Allen *et al.*, 1998; 2000a, b; Ladlow *et al.*, 2002; Breur *et al.*, 2004).

Their clinical use in cases of osteosarcomas and osteoarthritis (Ehrhart *et al.*, 1998; Garzotto *et al.*, 2000; Fox & Cook, 2001), radial osteoctomy (Francis & Millis, 2002), distraction osteogenesis monitoring (Lammens *et al.*, 1998), monitoring of long bone delayed and non-unions (Komnenou *et al.*, 2005) is also reported.

Some of bone resorption markers are either non-specific as urine hydroxyproline, or require tedious HPLC analysis as is the case with pyridinoline crosslinks of collagen (Delmas *et al.*, 1983). These analytes are however considered as non-specific for collagen type I that accounts for about 90% of organic bone matrix (Uebelhart, 1992). Thus, carboxyterminal telopeptide of type I collagen was introduced to monitor bone resorption activity (Risteli *et al.*, 1993). In men, serum ICTP was elevated in a number of diseases accompanied by generalized or local bone destruction as multiple myeloma (Elomaa *et al.*, 1992), and rheumatoid arthritis (Risteli *et al.*, 1993).

Data about the alterations in blood biochemical bone markers over the healing of fractures or the development of posttraumatic osteomyelitis in dogs are rather limited. Therefore, the purpose of this study was to follow out the changes in some serum markers of bone metabolism occurring in the early stage of normal bone healing (within one month) and to compare them to those occurring in canine posttraumatic osteomyelitis.

MATERIALS AND METHODS

Animals

The experiments were carried out in 2003–2005 with 12 mixed-breed male dogs aged 1–3 years. They were divided in two groups of 6 dogs: group I, with normally healing fractures (NH group) and group II, where an experimental model of posttraumatic (post fracture) osteomyelitis was reproduced (OM group). Dogs were housed in individual boxes, maintained on dry canine food for adult dogs (Jambo-dog®, Gallisman-94 S.A., Bulgaria) and had a free access to drinking water. Prior to the experiment, all dogs were treated against ectoparasites (Fipronil, Frontline®, Mérial, France) and endoparasites with praziquantel/pyrantel pamoate (Azipyrin®, Balkanpharma, Bulgaria).

Anaesthetic protocol

The anaesthetic protocol included premedication with 0.02 mg/kg atropine sulphate (Sopharma, Bulgaria) subcutaneously, and 0.05 mg/kg acepromazine maleate (Neurotranq, Alfasan, Woerden, Holland) intramuscularly, followed 15 min later by intraveous induction with 6 mg/kg 2.5% thiopentone sodium (Thiopental, Biochemie GmbH, Kudl, Austria), intubation and maintenance of general anaesthesia with 2.5 vol% halothane (Narcotan, Spofa, Czech Republic).

Experimental design

In all dogs, after aseptic preparation and lateral approach, transperiosteal osteotomies of one femoral bone diaphyse were performed.

In the NH group, osteotomies were fixed with a Kuntscher nail, inserted in the medullary canal through the intertrochanteric fossa (intramedullary osteosynthesis). In the OM group, osteotomies were also fixed with a Kuntscher nail. Prior to fixation, 0.5 mL mixed microbial culture consisting of equal parts *E. coli* 026 and *Ps. aeruginosa* with density of 10^5 cfu/mL was applied in each bone fragment.

The soft tissues were sutured routinely in both groups. In the post operative period, clinical (rectal body temperature, heart and respiratory rates) and radiological examinations were carried out over 2 months to monitor bone healing in the NH group or the development of osteomyelitis in the OM group. Blood laboratory analyses were performed on a weekly basis until the end of the 4th week.

Blood samples were collected between 7.30 and 8.00 AM (to eliminate any circadian effects) prior to surgery and by the end of the 1st, 2nd, 3rd and 4th post operative weeks. Mediolateral radiographies of operated limbs were done at same time intervals and by the end of the 2nd month.

Serum concentrations of the following bone markers were assayed: 1) total and bone alkaline phosphatase activities – by a commercial colorimetric kit (Biotrol, France); 2) serum osteocalcin – by radioimmunoassay kit (Henning Berlin GmbH, Germany); 3) serum carboxy-terminal telopeptide of type I collagen (ICTP) – by radioimmunoassay kit (Orion Diagnostica, Finland).

All samples tested in RIA were run in duplicate and the mean of both values was retained.

The results were statistically processed by one-way analysis of variance.

RESULTS

No complications were observed throughout the physical examination of dogs from the NH group. In the OM group, a marked, warm and painful swelling of the operated thighs was observed. The rectal body temperature was elevated in all animals from this group (from 39.8 to 40.7 ^{o}C). Within 3 to 6 days, the sutures in all six dogs suppurated, the swelling opened spontaneously and a purulent discharge has appeared. Up to the 20th day, poorly exudating fistulas formed at various areas. In four dogs, crepitation at the osteotomy site was seen, a sign of destabilization of osteosynthesis. The lameness was considerable until the end of the experiment.

In both groups, there were no radiologically visible changes in operated bones by the end of the 2nd week. Femoral radiographs of dogs from the osteomyelitis group (Fig. 1) showed lucency areas in adjacent soft tissues due to accumulated purulent exudate. By the end of the 3rd week, fissures and a fine periosteal reaction of a various extent, sign of beginning sequestration, could be seen. By the 4th week, sequestra were well differentiated in all six dogs, the periosteal reaction was clearly visible, but no union of both bone fragments was achieved. Newly formed bone tissue (periosteal callus) surrounded the sequestra. One month later, by the end of the 2nd month, osteosclerotic events around the sequestra were clearly visible. Periosteal callus was obvious both distally and proximally, without bridging the fracture line. Fixation nails were loosened due to adjacent osteolytic processes.

The results from blood biochemical investigations are summarized in Table 1. In the NH group, serum ICTP concentrations increased from initial values of 3.78±0.28 ng/mL to 4.62±0.21 ng/mL by post operative week 1 ($P<0.05$). This bone marker maintained significantly high levels by the end of the 2nd week too ($P<0.05$) and returned to baseline values by the 4th week. Total and bone alkaline phosphatase activities decreased by the end of the first week whereas serum osteocalcin increased, but not statistically significantly. Until the end of the experimental period, they varied insignificantly.

Total alkaline phosphatase in the OM group increased statistically insignificantly whereas the bone isoenzyme was significant elevated as early as the end of the first week (7.68 U/L; P<0.01) and particularly by the end of the 2nd week (12.54 U/L; P<0.01).

The time course of serum ICTP in this group was characterized with continuous increase from 3.77 ng/mL at baseline to

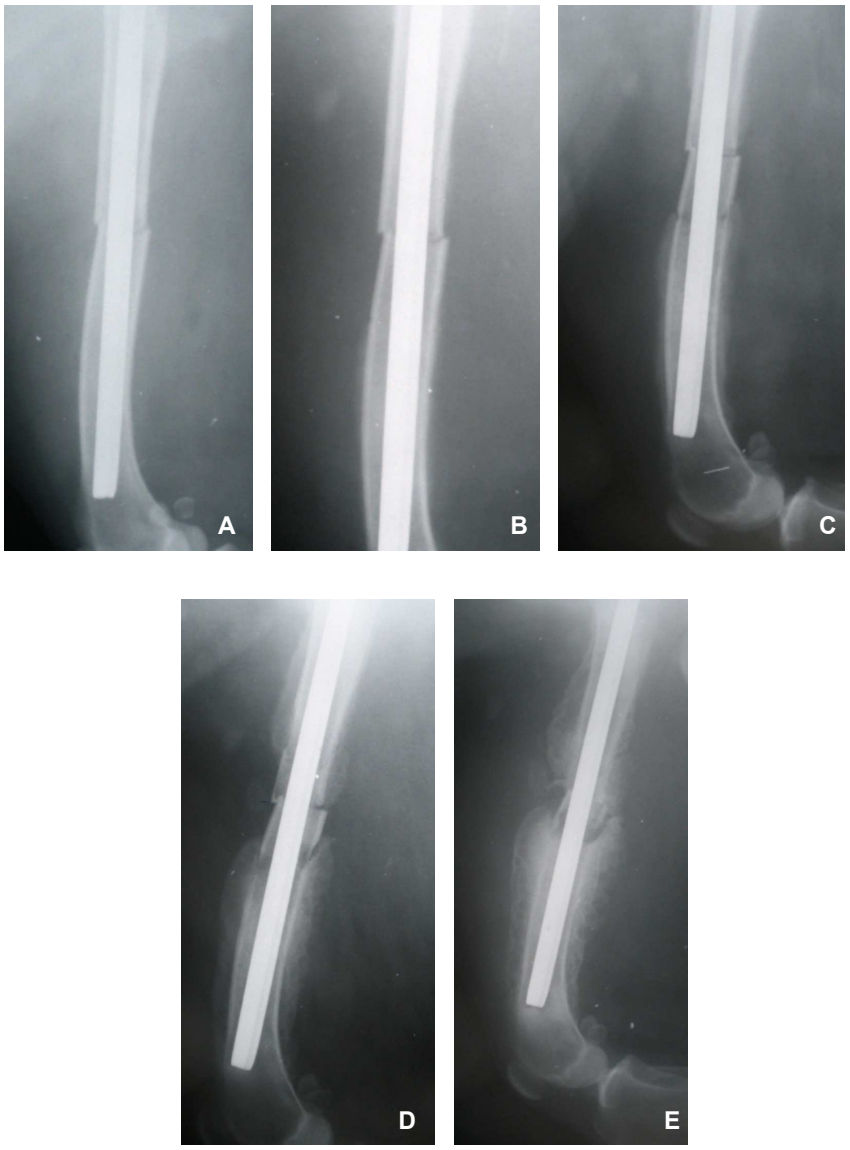

Fig. 1. Mediolateral radiographs of the femur in a dog from the osteomyelitis group by the 1st, 2nd, 3rd, 4th and 8th post operative weeks (from A to E, respectively).

Table 1. Time course of some blood serum bone markers in dogs with normally healing fracture (NH) and experimental osteomyelitis (OM). Data are presented as mean ± SEM (n=6)

Group	Prior to surgery	Weeks after the osteosynthesis			
		1	2	3	4
Total alkaline phosphatase, U/L					
NH	38.33±2.59	28.51±4.20	33.74±3.68	30.83±7.31	32.20±6.74
OM	45.02±8.13	125.09±36.01*[a]	73.04±24.04	57.01±22.00	46.07±7.02
Bone alkaline phosphatase, U/L					
NH	12.74±2.99	9.30±1.73	9.31±3.03	7.72±3.20	6.22±0.82
OM	4.02±0.90	7.68±1.11**	12.54±2.81**[a]	6.30±0.54	5.30±0.73
Osteocalcin, ng/mL					
NH	3.90±0.38	4.50±0.54	4.42±0.51	3.95±0.24	4.38±0.48
OM	6.23±0.91	30.34±14.50*	46.23±9.92** [c]	7.13±2.20	4.71±0.83
Carboxyterminal telopeptide of type I collagen, ng/mL					
NH	3.78±0.28	4.62±0.21*	4.61±0.31*	4.10±0.25	3.85±0.19
OM	3.77±1.85	5.30±2.88	8.80±1.50*** [a]	15.70±12.10*[c]	15.50±6.49**[c]

* P<0.05; ** P<0.01; *** P<0.001 vs preoperative values; [a] P<0.05; [c] P < 0.001 between groups.

8.80 ng/mL (p<0.001) by the end of the 2nd week, 15.70 ng/mL (P<0.05) by the 3rd week and 15.50 ng/mL (p<0.01) by the first month.

Statistically significant differences between groups were observed by the first week in total alkaline phosphatase activities (P<0.05); by the second week for serum osteocalcin (P<0.001) and for ICTP by the 2nd (P<0.05), 3rd and 4th weeks (P<0.001).

DISCUSSION

Bone fracture healing is a local process that reflects upon the systemic mineral homeostasis. This exchange involves vitamins, hormones, enzyme systems etc. Apart radiology, callus formation could be successfully monitored by several bone markers. This event is characterized by intricate interaction of bone resorption and bone formation and that is why in this study, we selected blood biochemical markers of both processes.

Bone metabolism markers are influenced by factors such as age, nutrition, physical exercise (Souberbielle *et al.*, 1999; Watts, 1999) and time of sample collection (Liesegang *et al.*, 1999; Ladlow *et al.*, 2002). The dogs included in this experiment had completed their growth, were fed uniformly and housed individually, and blood samples were obtained only in the morning to exclude circadian influences. Although the dogs were not purebred, breed and body weight was reported to be of no significance for analyzed biochemical markers (Breur *et al.*, 2004).

Kurdy (2000) has followed out the role of type I and III collagen propeptides and bone alkaline phosphatase (bAP) in poorly healing tibial diaphyseal fractures in men and found out that there were no statistically significant reduction in bAP

and propeptide concentrations up to the 10th week after the trauma, with increased levels of type III collagen propeptide. Data about deficient osteoblastic response were however present after the 12th week. On the contrary, in normally healing canine fractures in this study, bAP decreased insignificantly.

Having investigated bAP, osteocalcin and telopeptide concentrations, Emami *et al.* (1999) established that in slowly healing tibial fractures in men, bone resorption rate was similar to that in normal healing up to the 4th week, whereas bone formation occurred later (about the 10th–16th week). In our study on femoral fractures in dogs, the bone resorption marker (ICTP) was statistically significantly increased during the second week and returned to normal values by the end of the 4th week. This corresponded to osteolysis events around the bone ends that probably occurred earlier in dogs. The information about bAP and osteocalcin was confirmed, i.e. during the period of the survey, they either decreased (bAP) or did not change (osteocalcin) although fractures healed without complications. Thus, our results are similar to those reported by Akesson *et al.* (2005) about serious disturbances of bone metabolism in wedge-shaped tibial ostectomies and more difficult monitoring of osteoporosis. By serum and urine bone markers, the authors provide evidence that bone resorption rate was higher than bone formation rate in the post ostectomy (post fracture) period.

For slowly healing fractures in humans, osteocalcin could be an earlier marker as it remains unchanged after the 4th week, whereas in normally healing fractures it is statistically significantly elevated (Herrmann *et al.*, 2002). In a similar setting, Nyman *et al.* (1991) observed a considerable increase in osteocalcin and bAP levels by the 6th week in both groups, followed by reduced concentrations in the group of normally healing fractures and persistence of high activities, although with insignificant differences between groups. The findings for normally healing fractures are confirmed in this experiment too (variations in serum osteocalcin levels were minor and bAP was reduced almost twice).

Komnenou *et al.* (2005) reported a correlation between serum total alkaline phosphatase (tAP) and the healing of spontaneous long bone fractures in dogs. As early as the 10th day post fracture, tAP increased statistically significantly and returned to usual values by the 30th day. In our opinion, it is more appropriate to monitor not only tAP, but also bAP that is a more sensitive marker of bone metabolism. In our experiments, bAP activities decreased as early as the first post operative week on the background of preserved tAP concentrations. This corresponds to data reported by Akesson *et al.*, (2005) about higher bone resorption rate compared to bone formation rate in the early stages after tibial ostectomy.

After the first week, the OM group showed a steady tendency towards elevation in serum ICTP concentrations over the end of the 1st month, accompanied by enhanced bone formation during the first two weeks (evidenced by statistically significantly higher bAP and osteocalcin levels). In contrast to this group, dogs with normally healing fractures showed highest bone resorption rates during the first two weeks that rapidly returned to baseline levels afterwards. These differences could be attributed to more extensive osteolytic processes in osteomyelitis, accompanied by a strong periosteal reaction in the beginning, whereas the

resorption stage in normally healing fractures was short at the background of a relatively constant bone formation rate.

Osteomyelitis is an infectious inflammatory bone disease, with chronic and progressive course, without tendency for spontaneous recovery (Hulin, 1970). The specific alterations of bone, consequently to infections, are local destructions and reactive new bone formation.

The time course of studied serum bone markers showed in general increased concentrations of bone formation parameters by the end of weeks 1–2. This could be explained by speeding up of biological events in bone or the so-called regional acceleratory phenomenon (RAP) (Woodard & Riser, 1991). The typical for osteomyelitis bone destruction is visualized by radiography only by the end of the experimental period, i.e. when about 30% of mineral substances of bone were resorbed (Waldvogel *et al.*, 1970), whereas serum ICTP levels were statistically significantly elevated as early as the end of the 2nd week, and then almost doubled over the next week. In the view of Gillespie (1990), osteomyelitic bone lesions could be due to release of lysosomal enzymes by polymorphonuclear leukocytes, but activation of bone macrophages such as osteoclasts was also possible. The sharp increase in serum telopeptide, a degradation product of type I (bone) collagen, supports the second assumption.

It should be emphasized that the peaks in serum concentrations of bone formation and bone resorption markers did not occur simultaneously. The most enhanced bone formation (as shown by highest bone alkaline phosphatase activity) preceded the most extensive bone resorption (higher ICTP levels) by one week. These alternating periodical fluctuations in rates of the two principal bone remodeling events explain the typical mosaic-like pattern of bone trabeculae.

When discussing the changes in blood biochemical indices of bone remodeling throughout the development of osteomyelitis, the possible effect of one more factor should be considered, although its role is not quite clear – the immobilization after the surgery and bone infection and resulting osteoporosis. Data are reported that in men, osteoporosis resulted in higher C-terminal telopeptide concentrations too (Eastell *et al.*, 1988; Akesson *et al.*, 2005). The role of immobilization in acute osteomyelitis, in our view, is not important. For its explication, further studies are needed, that are beyond the subject of this investigation.

In conclusion, serum concentrations of C-terminal telopeptide of type I collagen in dogs with normally healing fractures of the femur were statistically significantly elevated by the first week, remained higher until the end of the second week and then returned to baseline values. In dogs with experimental osteomyelitis, this bone marker increased considerably by the second week and persisted elevated until the end of the fourth week. The initial increase occurred before the process could be radiologically seen.

As the early radiological diagnosis of osteomyelitis is difficult or not possible, the assay of biochemical markers of bone resorption is recommended when bone infection or postoperative complications are suspected. During the first and the second week of osteomyelitis development, bone alkaline phosphatase and osteocalcin were statistically significantly higher, but rapidly returned to usual concentrations.

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