Evaluation of osteoprotegerin and tumor necrosis factor-α changes in synovial fluid and serum in dogs with osteoarthritis: An experimental study

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Abstract
Osteoarthritis (OA) is a progressive and degenerative condition of the articular cartilage and other joints’ structures. It is essential to diagnose this condition as early as possible. The present study was conducted to measure the Osteoprotegerin (OPG) and Tumor Necrosis Factor-α (TNF-α) levels in synovial fluid and serum samples of dogs with experimental cruciate ligament rupture as a model of OA. In the present study, four adult (~20 months), large (weighing ~18 kg), mixed breed, male clinically healthy dogs were selected to investigate the effect of experimental OA, on OPG and TNF-α as a way of early detection of OA. OPG and TNF-α were measured in synovial fluid and serum on days 0, 14, 28, 90 and 180 after the surgical transaction of the cranial cruciate ligament in one stifle joint. Statistical analysis of the results showed that there was a significant increase in the concentrations of TNF-α in both synovial fluid and serum. Serum level of OPG showed a reduction before and two weeks after surgery and remained steady for the rest of the study period. Synovial fluid levels of OPG had no wide fluctuation throughout the study. OPG had constant levels at the beginning of experiment and increased at final stage. In conclusion, TNF-α could be used in both synovial fluid and serum as a way of early detection of OA.

Key words: Osteoarthritis, Osteoprotegerin, Tumor necrosis factor α, Synovial fluid

Introduction
Osteoarthritis (OA) is a progressive degenerative disease of joints which mainly originates from articular cartilage and extends to other periarticular structures. It affects both subchondral bones and peripheral soft tissues. Articular cartilage has no vessel or nerves (Lubar et al.2010). OA has a silent progressive nature because that it is limited to the cartilage. In the time that there are symptoms, subchondral bones are exposed and the cartilage is extensively lost (Lorenz and Richter2006) and the other hand, articular cartilage repair capability is very weak (Goldring and Goldring2006).
Early diagnosis of OA- particularly when there is not extensive damage to the articular cartilage- has been a topic of concern and research. Many attempts have been made to find ways to diagnose OA as early as possible, using different methods of diagnosis such as radiography, magnetic resonance imaging and different biochemical tests on synovial fluid and serum parameters (Lorenz and Richter2006, Goldring and Goldring 2006, Stannus et al. 2010). In osteoarthritic joints there is a concurrent process of osteogenesis and osteolysis, which are often radiographically diagnosed as periarticular osteophyte formation and bone remodeling in chronic cases. A glycoprotein regulates bone resorption and absorption. The protein, termed Osteoprotegerin (OPG) is a novel member of the TNF receptor superfamily that activates receptor activator of nuclear factor-kappa β ligand and has a role in osteoclast activation and differentiation (Pilichou et al.2008). Because of the role that osteoclasts play in osteolysis and remodeling, measurement of OPG activity may lead to early diagnosis of OA. Osteoprotegerin (OPG) reduces the development of pain behavior and joint pathology in a model of osteoarthritis (Spahni et al. 2009). Moreover, in OA, TNF-α contributes to the instability of the articular cartilage matrix by inhibiting proteoglycan synthesis and up-regulating matrix metalloproteinase (MMP) enzymes capable of degradation (Fernandes et al., 2002). TNF-α is reported to have a strong stimulatory effect on MMP-3 and induce a 10-fold up-regulation of MMP-3 activities (Kammermann et al.1996).

Therefore, the present study was conducted to measure OPG and TNF-α levels in synovial fluid and serum samples of dogs with experimental cruciate ligament rupture as a model of OA. The present study was elucidating the changes of OPG and TNF-α levels in osteolysis and articular cartilage degradation, and was showing if measurement of these values can be used as a way of early detection of OA.

Materials and Methods

Animals

Four adult (~ 20 months), large (weighing ~ 18 kg), mixed breed, male dogs, were used for this study. All dogs were healthy based on clinical examinations, orthopedic, and laboratory findings before surgery. Two conventional radiographic views were obtained before surgery. Surgeries and sample collections were carried out under aseptic conditions. The protocol of anesthesia, surgical procedures, post-operative care and sacrifice were the same for all animals and were under the supervision of Animal Care Ethics Committee of Shiraz School of Veterinary Medicine. This study was part of a larger investigation to characterize the development of OA due to experimental stifles joint instability in dogs. During the experiments, the animals were housed one per cage and were allowed to move freely 4 h a day in a wide fenced area but were not forced to exercise. A group including 4 adult dogs was used as control group.

Animal ethics

All animal experiments were approved by the State Committee on Animal Ethics, Shiraz University, Shiraz, Iran (IACUC no: 4687/63). The recommendations of European Council Directive (86/609/EC) of November 24, 1986, regarding the standards in the protection of animals used for experimental purposes, were also followed.

Experimental protocol and samplings

The stifle region was aseptically prepared for surgery. The animals were sedated with Acepromazine (0.1 mg/kg IM) and the whole stifle region was aseptically prepared for surgery. Anesthesia was induced by a combination of Ketamine (5 mg/kg IV) and Diazepam (0.2 mg/kg IV). The animals were placed in dorsal recumbency, and the
CCL of the left knees was transected through a 4 mm parapatellar stab incision as previously described for dogs (Pond and Nuki 1973). The animals were given appropriate analgesics for three days postoperatively. Synovial fluid was aspirated from the stifle joints once before surgery –as a baseline- and on days 14, 28, 90 and 180. The medial parapatellar approach was used for arthrocentesis (Clements2006). All synovial samples were centrifuged at 4°C, 4000 g, for 10 minutes to separate cells and debris. Supernatants were kept at -70°C until assay (Fujita et al.2006). Blood samples were collected from the cephalic vein of the dogs into vacutainers, and serum was separated by centrifugation at 750g for 15 min and kept in a freezer at −20 °C until assessment. For radiography, grades of OA were measured based on Kellgren and Lawrence (1957).

**OPG and TNF-α measurements**

Osteoprotegrin (OPG) was measured in serum and synovial fluid using a solid phase sandwich enzyme linked immunoassay (ELISA) method (Canine ELISA kit, Shanghai Crystal Day Biotech Co., LTD, Shanghai, China). The sensitivity of OPG kit was 1.4 pg/ml. The intra-assay precision and inter-assay precision of OPG kit were CV <10% and CV <12%, respectively.

Tumor necrosis factor α (TNF-α) was measured in serum and synovial fluid using a solid phase sandwich enzyme linked immunoassay (ELISA) method (Canine ELISA kit, Shanghai Crystal Day Biotech Co., LTD, Shanghai, China). The sensitivity of TNF-α kit was 0.039 pg/mL. The intra-assay precision and inter-assay precision of TNF-α kit were CV < 8% and CV < 10%, respectively.

**Statistical Analysis**

Significant differences for each parameter, between different times were evaluated using Repeated Measures Analysis of Variance (ANOVA) and Tukey multiple comparisons test as post-hoc. All values were expressed as mean and standard error (SE), and P<0.05 was considered as statistically significant. All data were analyzed using computer software GraphPad Prism for windows version 5.01 (GraphPad Software, Inc. 2007).

**Results**

All the operated joints showed OA based on radiographical assessments. Results of OPG and TNF-α are illustrated in serum samples and synovial fluid in Fig. 1 and 2. As shown in Fig 1, serum level of OPG showed a reduction before and two weeks after surgery and remained steady for the rest of the study period. Synovial fluid levels of OPG had no wide fluctuation throughout the study. It had constant levels at the beginning of experiment and increased at final stage. Except for the difference between control and day 14 in serum OPG, all the differences were insignificant for OPG. TNF-α showed a significant increase in study period, in both serum and synovial fluid samples.
Fig. 1. Data of measured OPG in serum samples showed a significant decrease between control samples and afterward. However, from two weeks after operation and after that, the measures remained relatively unchanged. OPG level measured in synovial fluid showed no significant changes throughout the study. OPG had constant levels at the beginning of experiment and increased at final stage. The maximum levels were seen on day 90 post surgery.

Fig. 2. There was a significant increase in the concentrations of TNF-α in both synovial fluid and serum. Serum and synovial fluid concentrations of TNF-α showed a significant increase throughout the study period.

Discussion
In this study, we found that TNF-α showed a rising pattern in both serum and synovial fluid, indicating the progressive degradation of the articular cartilage and other joints’ structures. Based on its role in both cartilage and bone destruction, degradation in either or both could be expected. This suggests that there is mild inflammation in the process of degradation of the joints’ structures which can be tracked and measured as an early sign of OA. However, since TNF-α is a non-specific marker, other systemic coexisting diseases should be ruled out. TNF-α plays a key role in cartilage degradation and bone resorption (Stannus et al. 2010). It can induce the production of other cytokines (e.g. IL-6), MMP and prostaglandins (Pelletier et al. 2001), and prevents the synthesis of proteoglycans and type II collagen (Goldring and Goldring 2004). A Dutch dog group showed that high innate ex vivo synthesis of TNF-α in blood assay upon lipopolysaccharide stimulation was
not associated with an increased risk of OA (Riyazi et al. 2005). However, longitudinally, patients in the highest quartile of TNF-α production had a 6-fold increased risk of joint space narrowing (JSN) progression compared to those in the lowest quartile over 2 years (Botha-Scheepers et al. 2006). In human osteoarthritis, increased concentration of tumor necrosis factor-α was reported (Amin 1999). Our findings showed in accordance with Stannus et al. (2010), that reported a low level of inflammation in knee OA in older people by measuring TNF-α. They also correlate their values with JSN. It is not possible to evaluate JSN unless radiography is taken in weight bearing position which is seldom possible in animals because they have to be radiographed under general anesthesia or deep sedation in a non-weight bearing fashion. This finding is also in accordance with recent findings that have shown the elevation of IL-6 in dogs with experimental CCL ruptures (Nikahval et al. 2013).

Changes of the subchondral bone are hallmarks of OA. The involvement of the bones beneath the cartilage was first suggested by Radin and Rose (1986). The integrity of cartilage could depend on the subchondral bones. Kadri et al. (2008) found that cartilage degradation was prevented by administration of OPG in an experimental model of OA in mice; however, the same results were not achieved in the in vivo part of their experiment on cartilage (Kadri et al. 2008). In the present study, there was a decrease in OPG levels in both synovial fluid and serum two weeks after OA induction and despite some fluctuations in serum levels, there were no significant changes afterward. Although it is not possible to differentiate the exact reason of OPG reduction, and further studies should be conducted, it might be partly because of initiation of cartilage degradation or osteolysis. Surgical models of OA in animals have suggested that early OA is related to an increase in osteolysis of adjacent bones (Ding et al. 2003, Pelletier et al. 2004, Hayami et al. 2006). OA signs can even be less severe by administration of OPG (Kadri et al. 2008), despite the fact that little is reported about the effects of OPG on OA. In one study which is similar to the present study, daily local injections of OPG have shown to prevent cartilage degradation and reduce chondrocyte apoptosis (Shimizu et al. 2007). However, Kadri et al. (2008) observed no effect of OPG on proteoglycan release in cartilage explants, which could indicate an effect within bones. These findings also suggest the contribution of bone close to the cartilage and possible interaction via bone cytokines. The inhibition of mechanically induced cartilage degradation might be related to a direct effect on bone. Also, it has been speculated that increased bone resorption might be responsible in part for initiating cartilage destruction under stressful conditions (Felson and Neogi 2004). Spahni et al. (2009) reported that in elbow dysplasia, a surplus of molecules promoting osteoclastogenesis was evident and is indicative of an imbalance between the mediators regulating bone resorption and bone formation. Therefore, OPG has the potential to counterbalance bone resorption. Whatever the exact reason(s) are, OPG stayed relatively the same from two weeks after surgery and afterward in our study. Thus, it has low value in diagnosis or confirmation of OA in early stages. Particularly in clinical cases where there is no data prior to trauma. On the other hand, since serum levels of OPG are correlated to general bone status, it should be taken into account that other systemic or general bony changes or abnormalities can greatly influence the OPG levels. Thus, other pre-existing bone diseases should be excluded before measuring OPG as an indicator for OA.

In the present study, TNF-α and OPG showed different patterns of change in serum and synovial fluid. Time dependent
changes in TNF-α concentration in serum and synovial fluid samples was observed. Serum and synovial fluid levels of TNF-α showed a significant increase in study period. Serum level of OPG showed a reduction before and two weeks after surgery and remained steady for the rest of the study period. Synovial fluid levels of OPG had no wide fluctuation throughout the study. OPG had constant levels at the beginning of experiment and increased at final stage. We concluded that TNF-α could be diagnostic in early stages of OA, however, OPG concentrations in serum and synovial fluid are of low value in OA prediction.

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Conflict of interest
Authors declare that he/she have no conflict of interest.

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