Revista ARACÊ
ISSN: 2358-2472

EVALUATION OF C-REACTIVE PROTEIN, ACID GLYCOPROTEIN AND ERYTHROCYTE SEDIMENTATION RATE IN DOGS WITH HIP DYSPLASIA

https://doi.org/10.56238/arev6n4-230

Submitted on: 11/14/2024 **Publication date:** 12/14/2024

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ABSTRACT

The objective of this study is to evaluate the inflammatory response in the hip joint in dysplastic dogs to compare laboratory findings of synovial fluid: nucleated cell count and its differential: mononucleated and polymorphonucleated cells, analysis of acute phase proteins: α1-acid glycoprotein, C-reactive protein and erythrocyte sedimentation rate, with radiographic findings of the hip joint in dogs with symptomatic and Asymptomatic. A total of 30 dogs were evaluated in the experiment, arranged in 3 experimental groups. The first group included 10 healthy animals (Control Group - CG), the second 10 animals with symptomatic hip dysplasia (Group 1 - G1) and the third 10 animals with hip dysplasia without symptoms (Group 2 - G2). A significant increase in ESR was observed in the symptomatic group, corroborating the hypothesis that in animals with clinical signs of the disease they present inflammation in the hip joint. ESR is useful in evaluating the inflammatory process in symptomatic dogs, indicating an acute inflammatory process. On the other hand, the measurement of AGP and CRP by immunoturbidimetry do not confer plausible results of inflammation in osteoarthrosis of dysplastic dogs, making it an ineffective biomarker for monitoring the disease. It suggests further studies of other inflammatory biomarkers for the disease studied.

Keywords: Alpha-1 Acid Glycoprotein, Inflammation, Osteoarthritis, C-reactive protein, Erythrocyte sedimentation rate.

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Revista ARACÊ
ISSN: 2358-2472

INTRODUCTION

Hip dysplasia (CDF) is a developmental pathology that affects the femoral head and acetabulum, has a complex etiology that affects a significant portion of domestic animals. This malformation manifests itself through the combination of the individual's genotype, the animal's genetics, nutrition and environmental factors to which it was exposed during its development phase (CARNEIRO *et al.,* 2006; REGONATO *et al.,* 2011).

It is a biomechanical disease in which hip instability in young dogs alters the concentration of forces on the growing femur and acetabulum, affecting bone development and resulting in abnormal joint conformation, joint laxity, and osteoarthrosis, also known as degenerative joint disease (KAPATKIN *et al.,* 2002; SMITH, 2004).

Osteoarthrosis is a degenerative process of the joint capsule of chronic evolution, osteoarthrosis of the hip has initial involvement in the articular cartilage and then the subchondral bone, and formation of osteophytes, characterized by the presence of pain, and reduction in the joint space (COIMBRA *et al*., 2002; VANNI *et al.,* 2008; BENNEL and HINMAN, 2011; PASTORE *et al*., 2013). Its diagnosis is made from radiographic examination, the presence of osteophytes, reduction of joint space, sclerosis of the subchondral bone and presence of bone remodeling (PELLETIER *et al*., 2001).

PFAs can be used as promising biomarkers of inflammation, infection, or trauma, as they have particular characteristics of early increase and easy plasma detection, and are increasingly used for diagnostic purposes in specific diseases (SCHRODL *et al.,* 2016).

Erythrocyte sedimentation rate (ESR) is an exam that evaluates the sedimentation of red blood cells, it is an indirect method for evaluating inflammatory activity, (COLLARES and VIDIGAL, 2004; GUIMARÃES *et al*., 2013).

The objective of this study is to evaluate the inflammatory response of the hip joint through laboratory analysis of synovial fluid and analysis of serum inflammatory biomarkers in dogs with hip dysplasia.

METHODOLOGY

A total of 30 dogs were evaluated in the experiment, arranged in 3 groups, 10 healthy animals in the control group (CG), 10 animals with symptomatic hip dysplasia in group 1 (G1) and 10 animals with hip dysplasia without symptoms in group 2 (G2). The project was approved by the ethics committee on animal experimentation (CEUA) of Uningá, under PM 04/2017.

Revista ARACÊ
ISSN: 2358-2472

All animals underwent anamnesis, complete physical examination and orthopedic examination according to hematological examination and abdominal ultrasonography to rule out other associated diseases.

To assess ambulation, an analog scale of claudication was applied, described in Chart 1 (HUDSON, 2004).

Chart 1 - Analog scale of claudication to ambulation

EVALUATION SCORES	LAMENESS CLASSIFICATION
0	Absent
1	Intermittent
2	Continuous and Unobtrusive
3	Continuous and Moderate
4	Intense

Fonte: HUDSON, 2004.

The inclusion criteria were: Animals aged between 2 and 10 years and weighing more than 17 kg, for the control group. And for the other groups, aged between 6 months and 10 years and weighing more than 17kg, of different races and both sexes. And with the confirmatory radiographic diagnosis of hip dysplasia, using the classification of the *Orthopedic Foundation for Animals* (OFA, 2017).

Pregnant or estrus females were not accepted, due to the possible increase in joint laxity due to hormonal variations, resulting in changes in the results (OFA, 2017). Animals that had been using analgesic and anti-inflammatory medication in the last 15 days and animals that had orthopedic conditions unrelated to DCF. In addition to animals that presented another condition of a systemic, inflammatory and/or infectious nature.

Radiographic images and fluid collection were performed under 6-hour food and fluid fasting. For radiographic positioning, the animals were sedated with acepromazine (0.05mg/kg) and meperidine (5mg/kg), associated with midazolam (0.3mg/kg), all intramuscularly. Intravenous propofol was used to collect synovial fluid at a dose of 2-4mg/kg.

The radiographic positioning for the hip joint followed the technique described by the *American Veterinary Medical Association* (OFA, 2017). All radiographs were performed with a Konica® digitized device and Ezy rad® X-ray, and were calibrated according to Feliciano *et. al.* (2015). The diagnosis of Hip Dysplasia was made with the classification parameter of the degree of dysplasia following the evaluation of the *Orthopedic Foundation for Animals* (OFA, 2017).

Revista ARACÊ
ISSN: 2358-2472

Synovial fluid was collected immediately after the radiographic examination, using the technique described by Piermattei (2006). For its analysis, this was in saline solution in 1:6 of the synovial fluid sample (50uL of saline solution + 10 uL of sample). Such dilution allowed the sample, which was extremely viscous, to acquire a more watery appearance. In this way, it is possible for the cells to sediment more effectively and the cell count becomes much more reliable. The cells (/uL) were counted in four large squares in Neubauer's chamber. The number of cells counted was corrected using the following factors: chamber height (x10), dilution (x6) and number of contact squares (/4).

The differentiation of nucleated cells into polymorphonuclear or mononuclear cells was made by microscopic analysis of smears made by the squash or crushing technique. The smears were stained with Romanowsky-type rapid dye (Instant – Prov, NEWPROV).® 100 leukocytes were identified at 40x magnification under an Eclipse E200 microscope - Nikon®.

Blood was collected by puncture of the jugular vein. 10 ml of blood were collected, 5 ml in a tube with EDTA K3, and 5 ml in a tube with clot-activating gel. All samples were immediately analyzed using Mindray BC-2800Vet® automated equipment. And the differential count of nucleated cells under microscopic analysis.

ESR was measured using the Westergren method, using a specific pipette for the technique, venous blood with EDTA K3 anticoagulant was pipetted in a glass tube 200 mm high and 2.5 mm in diameter, and placed in a vertical column, after 1 hour, the reading was taken corresponding to the distance between the plasma meniscus and the red blood cell column, measured in mm/h, estimating the sedimentation rate of red blood cells in the interval of one hour. The test was performed at room temperature between 20 and 25 °C and in the shelter of sunlight, following the technique described by Epaminondas (2015).

The serum obtained from the tube with clot-activating gel was centrifuged (1,500g/5 minutes) for serum measurement of α1-acid glycoprotein and C-Reactive Protein. The serum was stored in two 2ml eppendorf tubes at –20°C until the test was performed.

Serum biochemical measurements of C-Reactive Protein and α-1 acid glycoprotein were performed in the semi-automatic biochemical device Bioplus BIO200® according to the methodology provided by the manufacturer BioTecnica®. The APG was performed by spectrometry and a calibration curve with the Mean® Multiparameter Control was used. PCR was performed by turbidimetric assay and the level 1® rheumatic control calibrator

Revista ARACÊ
ISSN: 2358-2472

was used. All clinical analyses were performed by the same qualified professional in the area.

For the statistical analysis, the basic assumptions of normality of the residuals (Shapiro-Wilk test; p≤0.05) and homogeneity of variances (Levene test; p≤0.05) were made. The data were compared by analysis of variance (ANOVA) at the level of 5% of significance by the F test, by Tukey's test at 5% of significance. These analyses were performed using the Sisvar® software, version 5.3 (FERREIRA *et al.*, 2014). Pearson's correlation coefficients (STEEL *et al.,* 1997) were estimated among all the variables evaluated, validated by the T test (p≤0.05 and p≤0.01).

RESULTS

A total of 30 dogs participated in the study, 10 animals in the control group, 10 animals in the symptomatic group and 10 in the asymptomatic group. Among them, 63.3% females and 36.6% males of different breeds, aged between 1 and 10 years, the weight ranged from 17 to 46kg with an average of 28.8kg. In these animals, the degree of hip dysplasia according to the *Orthopedic Foundation for Animals* (OFA), the degree of lameness, the synovial fluid collected from the hip joint, the erythrocyte sedimentation rate (ESR) and the inflammatory biomarkers Alpha-1 Acid Glycoprotein (AGP), C-Reactive Protein (CRP) were evaluated. The amount of synovial fluid collected ranged from 20µl to 4ml, with an average of 200µl.

In the study, a significant increase in the Erythrocyte Sedimentation Rate was observed in the group of animals with clinical signs in relation to the control group and the asymptomatic group with a P value of 0.05. The degree of lameness showed a significant difference p ≤0.05 in the symptomatic group in relation to the others. As well as the radiographic grade according to the OFA was also statistically significant, with a higher value in the symptomatic and asymptomatic groups than in the control group.

The values of AGP, total nucleated cell count, and mononucleated and polymorphonucleated cell count in the synovial fluid were not statistically significant between the groups.

The correlation between the biomarkers inflammation, synovial fluid, degree of dysplasia and degree of claudication were evaluated in the 3 groups, correlated two by two. In group 1 (CONTROL), there was a very strong and significant correlation ≤between ESR x PMN (r=0.956 and p=9.228) in group 1 (CONTROL), and between the CELL. NUCL. X

Revista ARACÊ
ISSN: 2358-2472

CRP, which had a strong and significant correlation at p ≤ 0.05 (r=0736 and p=3.077). In group 2 (SYMPTOMATIC), the values of the correlations showed moderate and weak positive correlations, but did not obtain significance by the test. In group 3 (ASYMPTOMATIC), a strong correlation was observed between AGP X OFA, with significance at 5% in the t-test (r=0.684 and p=2.655).

DISCUSSION

Higher levels of ESR were observed in the symptomatic group, suggesting that symptomatic disease promotes systemic inflammatory process, when compared to controls and asymptomatic group. PCR and AGP, in the methodology performed by means of turbidimetry and spectrophotometry, respectively, were not effective with inflammatory biomarkers, obtaining measurements below the reference value.

The CRP values did not obtain reliable measurement, with intervals below the analytical sensitivity of the test, requiring more sensitive and specific techniques, such as the ultrasensitive immunoturbidimetric test, which detects lower values, or the ELISA assay to determine the species-specific canine PCR.

Vecina (2009) in his study evaluated the inflammatory process in several dogs, where CRP was measured by the automated immunoturbidimetry technique and by ELISA. The immunoturbidimetry technique for determining PCR values by automated hematological equipment proved to be inadequate for canine blood samples. There was no reading in any of the samples tested, both in healthy and convalescent dogs (VECINA, 2009). In the measurement with the commercial kit for canines ELISA the ditches were effective and reliable. Yamamoto *et al.* (1992) and Martínez-Subiela *et al*. (2004) state that values lower than 1 µg/ml (1 mg/L) should be interpreted with caution, as they may be influenced by analytical conditions.

The AGP was measured by the immunoturbidimetric method in which antibodies against alpha-1 human acid glycoprotein, form with alpha-1 acid glycoprotein, an insoluble complex giving a turbidity whose intensity is proportional to the concentration of alpha-1 acid glycoprotein in the sample, determined in a spectrophotometer at 340 nm. The measurement of all samples was below the reference value in all samples.

Hayashi *et al.,* (2001) suggested that serum AGP may be a useful marker of acute inflammation in tissue damage, including surgery, and reported that dogs with chronic

Revista ARACÊ
ISSN: 2358-2472

diseases associated with little inflammation have AGP concentrations within the reference range.

Increased values of this protein are observed in cases of hepatitis, parvovirus, distemper, pyometra, filariasis, renal failure, urolithiasis, immune-mediated anemia, trauma, hepatitis, pancreatitis, and hyperadrenocorticism (YUKI *et al.,* 2010), but in hip osteoarthrosis in dogs, this biomarker is not useful for monitoring the disease.

ESR, on the other hand, although it is a nonspecific test, has shown interesting results that, associated with its low cost, speed and ease of execution, can be used to identify an inflammatory process in cases of hip dysplasia. Elevated ESR was observed in 70% of the animals in the symptomatic group. Suggesting an inflammatory process, being the most sensitive marker used in the study.

Differences were observed between the asymptomatic, symptomatic and control groups. It is known that ESR remains elevated during the inflammatory process, as it is influenced by the concentration of PFAs, such as CRP (SCHUTZE, 2000). It can be influenced by other factors such as the presence of anemia and morphological changes in erythrocytes, which does not occur with the concentration of PFAs. The animals used in the research did not present such hematological alterations in any of the groups.

There are some described variations of this method, such as the use of blood anticoagulated with EDTA or under the diluted with sodium citrate or saline (COLLARES, 2004; EPAMINONDAS *et al.,* 2015). In the present study, ESR was performed from blood collected with EDTA anticoagulant, without dilution. Hachem *et al.,* (2010) analyzed the method with undiluted EDTA anticoagulant, the results of this study showed that in the overall average, there was no significant difference between the non-dilution of blood with EDTA in relation to the recommended gold standard method.

Other studies have shown that from values above 30mm/h, ESR values by the undiluted method tend to present higher results than those obtained by the technique with dilution in citrate or saline solution, corroborating the theory that citrate can delay erythrocyte sedimentation (BUCK *et al.,* 2015; MERISIO and ALFF, 2013; EPAMINONDAS *et al*., 2015).

The use of quantification of these inflammatory proteins has increased considerably in the last decade (KJELGAARD-HANSEN and JACOBSEN, 2011; HILLSTRÖM *et al*. 2014), considering that during the inflammatory process, prior to the increase in PFS, there is an increase in the circulating levels of cytokines such as IL-1, TNF-α and IL-6

Revista ARACÊ
ISSN: 2358-2472

(ECKERSALL and BELL, 2010; CRISPE, 2016), one option would be to measure these cytokines as an assessment of the degree of inflammation in animals with hip dysplasia, and thus institute the best treatment for each animal.

The total synovial fluid cell count in osteoarthrosis processes may present normal or slightly increased values, with a predominance of mononucleated cells, and a normal or slightly increased amount of polymorphonucleated cells, with normal or slightly increased neutrophils (TATARUNAS *et al.,* 2004). According to Innes, 2012 healthy dogs have a total of nucleated cells and less than $2x10^9$/L with 94 to 100 % of mononucleated cells and 0-6% of polymorphonucleated cells. In cases of osteoarthrosis, the amount of nucleated cells varies from 2 to $5x10^9$/L, with 88 to 100% of mononucleated cells and 0-12% of polymorphonucleated cells. This study revealed a high amount of nucleated cells in the G1 group (<$6x10^9$/L). When the findings between the groups were compared, there was no significant difference

CONCLUSION

The data obtained from the research proved that ESR can be used to evaluate the inflammatory process of dogs with positive symptoms for hip dysplasia, indicating the presence of an inflammatory process. The same does not occur with the measurement of AGP and PCR by immunoturbidimetry, which show low sensitivity to detect inflammatory processes in osteoarthritis of dysplastic dogs.

Revista ARACÊ
ISSN: 2358-2472

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