





## Correlation of serum acute-phase proteins with bone repair in rabbits (*Oryctolagus cuniculus*) submitted to partial radio osteotomy

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**ABSTRACT:** Bone trauma triggers an acute inflammatory response, which can alter the serum concentration of acute-phase proteins (PFA). We aimed to evaluate the effectiveness of PFA measurement as a laboratory biomarker for inflammation related to bone regeneration. A partial osteotomy of the right radius was performed on 22 young male rabbits. The complete blood count, albumin, ceruloplasmin, haptoglobin, C-reactive protein, and transferrin levels were recorded and correlated with the radiographic evolution of bone healing. Statistically the best correlations with the inflammatory process and radiological findings were reported with haptoglobin and transferrin compared to the other measured PFAs. Haptoglobin and transferrin showed a maximum peak 24 and 36 hours after surgery, respectively. Transferrin displayed signs of decrease in the first 6 hours after surgery, in advance of the leukocyte response. The measurement of acute-phase proteins proved to be viable, considering the conditions in which the present study was carried out. Among all variables studied, haptoglobin and transferrin showed best correlation with the inflammatory process of bone healing. Additional studies are needed to determine the sensitivity of acute-phase proteins as predictors of complications in the treatment of fractures.

## Correlação do proteinograma sérico com a reparação óssea em coelhos (*Oryctolagus cuniculus*) submetidos à osteotomia parcial do rádio

**RESUMO:** Os traumas ósseos desencadeiam resposta inflamatória aguda e conseqüentemente alteram a concentração sérica das proteínas de fase aguda (PFA), podendo essas representarem um parâmetro para avaliação da evolução do processo inflamatório relacionado à cicatrização óssea, assim como complicações. Objetivou-se avaliar a inter-relação da mensuração de PFAs com a reparação óssea em coelhos submetidos à osteotomia parcial do rádio. Foram utilizados 22 coelhos, machos, jovens (160 a 180 dias) e peso médio (3,8 ± 0,3). Para acompanhamento da evolução do processo inflamatório foram realizados hemograma completo e mensuração de albumina, ceruloplasmina, haptoglobina, proteína C reativa e transferrina, além do acompanhamento radiológico. Foi observado a melhor correlação da haptoglobina e da transferrina junto ao processo inflamatório e achados radiológicos frente às demais PFAs mensuradas. A haptoglobina apresentou pico máximo 24 horas do pós-operatório e a transferrina após 36 horas, entretanto, essa última já mostrou indícios de diminuição nas primeiras 6 horas do pós-cirúrgico, antecipadamente à resposta leucocitária. Nas condições em que o presente estudo foi realizado, a mensuração das proteínas de fase aguda mostrou-se factível. Das proteínas estudadas, a haptoglobina e transferrina foram as que mostraram maior correlação com o processo inflamatório da cicatrização óssea. Estudos adicionais são necessários para determinar a sensibilidade das proteínas de fase aguda como previsores de complicações do tratamento das fraturas.

**Palavras-chave:** cicatrização, biomarcador, fraturas ósseas, inflamação, proteína de fase aguda.

## INTRODUCTION

Physical injury and bone regeneration have been widely studied so as to improve the treatment of fractures and other bone disorders (MARSELL & EINHORN, 2011). Despite the established need to investigate implants and new techniques to improve bone healing capacity, researchers around the world have been looking for ways to standardize markers capable of predicting complications in the treatment of orthopedic conditions. Currently, early clinical

and radiographic signs are used to identify potential complications. However, in most cases, the process is already established. Although, a complete blood count is considered to be the reference standard for postoperative follow-up, there is a need for biomarkers that can anticipate the development of complications, allowing for earlier intervention and better results (GOVAERT et al., 2020).

Bone repair involves an inflammatory process with the intense action of pro-inflammatory cytokines, growth factors, and angiogenic factors

released by platelets, macrophages, neutrophils, lymphocytes, and mesenchymal cells present in the primary fracture hematoma (PENG et al., 2005; GERSTENFELD et al., 2006). These pro-inflammatory factors also act on specific proteins, called acute-phase proteins (PFA), which guide the development of the inflammatory process (CRUVINEL et al., 2010; ECKERSALL & SCHMIDT, 2014).

PFA can be classified as positive or negative when their serum concentration increases or decreases, respectively, in the face of homeostasis disorders (CERÓN et al., 2005; MARNELL et al., 2005). Each species has a specific type of PFA, which has a concentration between 100 and 1000 times greater in the face of an inflammatory process and is called major PFA. This hampers a comparative analysis being conducted between species (KJELGAARD-HANSEN & JACOBSEN, 2011; CÉRON et al., 2017).

PFA have proven to be useful in monitoring pathological conditions, favoring the early identification of subclinical stages of the disease. In a study with dogs that were experimentally inoculated with *Leishmania* spp., they tended to show an early serum response compared to the leukocyte peak (KJELGAARD-HANSEN & JACOBSEN, 2011; OOHASHI et al., 2019). In this study, a significant increase in PFA was found two months before the animals showed clinical signs (MARTINEZ-SUBIELA et al., 2014). Additionally, the analysis of PFA has yielded promising results in autoimmune disorders in calves (BEZUIDENHOUT & PRETORIUS, 2020), infectious diseases in cats and dogs (PALTRINIERI et al., 2007; KOCATURK et al., 2010), parasitic diseases in rabbits and dogs (FREITAS, 2011; MYLONAKIS et al., 2012; CÉRON et al. 2018) and tumoral diseases in cats (SELTING et al., 2000). However, the correlation between these proteins and trauma is not yet fully understood (CHRISTENSEN et al., 2015).

Recently, GOVAERT et al. (2020) reported that there are a small number of studies that correlate accurate biomarkers with the diagnosis of bone repair complications, emphasizing the need for validation as to the type and value of the analyzed parameters.

Thus, this study aimed to measure the serum concentration of albumin, ceruloplasmin, haptoglobin, c-reactive protein, and transferrin, and to verify the applicability of these PFA as laboratory parameters (biomarkers) for monitoring inflammation associated with bone repair in rabbits.

## MATERIALS AND METHODS

Twenty-two healthy New Zealand White breed male rabbits (*Oryctolagus cuniculus*), aged 160 to 180 days, weighing 3.8Kg ( $\pm 0.3$ ), were used. The leporids were kept in individual cages, dewormed with 15.0 milligrams per kilo (mg/Kg) oral solution of toltrazuril, and fed with commercial feed, Tifton hay (*Cynodon* spp.), and water *ad libitum*. This research was approved by the ethics committee on the use of animals of the Faculty of Agricultural and Veterinary Sciences, UNESP Jaboticabal campus, Brazil, under protocol number 019155/17.

For the surgical procedure, rabbits were pre-medicated with meperidine at a dose of 5.0mg/Kg intramuscularly (IM) and induced with 3V% isoflurane using a face mask and subsequent orotracheal intubation. Animals were positioned in the right lateral decubitus position and had the right thoracic limb prepared for aseptic surgery (chlorhexidine 2% and 0.5%). A 2.5cm skin incision was made in the craniomedial region of the radius and division of the adjacent musculature, to enable visualization of the proximal, middle shaft of the right radius. A partial radius osteotomy 1 cm long was performed using an oscillatory saw. The fragment was removed along with its periosteum, forming bone failure. Soft tissue was sutured in a simple pattern separated with nylon thread (4-0). Postoperatively (PO), the rabbits were administered enrofloxacin (5.0mg/Kg subcutaneous, once a day per 7 days), morphine (2.0mg/Kg, IM, after 4 hours PO), and tramadol hydrochloride (5.0 mg/kg subcutaneous, twice a day by 3 days and once a day for 2 days).

The right thoracic limb of the animals was evaluated radiologically in craniocaudal and mediolateral positions immediately after surgery and at 15-day intervals (M15d, M30d, and M45d). The radiographic characteristics were quantified from 1 to 4 in relation to the percentage of bone callus formation (adapted from FRANCO, 2017) by two blinded evaluators. Score 1, 2, 3 and 4 were characterized by 0 to 25%, 5% to 50%, 50 to 75%, and 75 to 100%, of bone failure filled with bone callus, respectively.

Three milliliters (mL) of blood were collected from the external jugular vein, in sterile tubes, with and without ethylenediaminetetraacetic acid (EDTA), at predetermined time points (M): preoperative (M0), and 6, 24, and 36 h (M6h, M24h, and M36h), as well as 7, 15, 21, 30, and 45 days (M7d, M15d, M21d, M30d, and M45d) postoperative. Blood counts were processed using a veterinary hematology analyzer, and the differential leukocyte count was

analyzed under optical microscopy. The serum was obtained by centrifuging tubes without EDTA at 5,000 revolutions per minute (rpm) for five min. The total protein was measured with a semiautomatic spectrophotometer and the serum proteinogram by the polyacrylamide gel electrophoresis method.

The hemogram and proteinogram were analyzed using the Tukey test. The radiographic analysis was analyzed using the Kruskal-Wallis test. A p-value of <0.05 was considered to be statistically significant. These analyzes were performed by the computer program R CORE TEAM.

## RESULTS AND DISCUSSION

Fifteen days postoperatively (M15d), only 28% of the animals had evolved to score 2 on the radiographic evaluation, which was not statistically significant compared to their initial score (M0). This is consistent with the inflammatory phase and the beginning of the formation of cartilaginous callus (RAHN, 2002). At 30 days (M30d), 54% of the rabbits had a score of 2, 36% had a score of 3, and 10% had a score of 4. These results were significantly different ( $P \leq 0.05$ ) between M0 and M15d. Finally, at 45 days, a higher percentage of scores 3 and 4 were observed. However, there was no statistical difference ( $P \leq 0.05$ ) with M30d, despite differences ( $P \leq 0.05$ ) between the M0 and M15d. At M45d, bone nonunion was clinically established in 80% of the animals, characterizing the complication of fracture management (METSEMAKERS et al., 2018).

The albumin averages (Table 1) displayed slight variations at each time period, with a numerical decrease between M24h and M36h. However, this was not statistically significant. TOTHOVA et al. (2019b) obtained similar results in an experimental procedure performed with a failure in the articular cartilage and trochlear portion of the femur in sheep, where the lowest numerical concentration was 15 days postoperatively. Having kept the different inflammatory stimuli, BOZUKLUHAN et al. (2018) also reported no statistical difference between healthy cows with different degrees of endometritis. MYLONAKIS et al. (2012) obtained stable albumin values, while C-reactive protein (pCR) and haptoglobin were altered in dogs with inflammatory complications triggered by an *E. canis* infection. Even so, HÜBNER et al. (2016) reported a drop in albumin in abdominal procedures in humans in the immediate postoperative period. Therefore, we believed that our results showed that there were no significant inflammatory complications to alter the concentrations of albumin, in agreement with MYLONAKIS et al. (2012), BOZUKLUHAN et al. (2018), and TOTHOVA et al. (2019b).

A peak of haptoglobin was observed at M24h (Table 1), which was up to 2 times greater and statistically different ( $P \leq 0.05$ ) from M0. CÉRON et al. (2017) reported that proteins that increased 2 to 4 times after a stimulus were moderate, as observed in this study. These findings are similar to those by TOTHOVA et al. (2019a) in pigs and TOTHOVA et al. (2019b) in sheep, where an increase

Table 1 - Mean values followed by the standard deviation of the mean ( $\pm$ ) of the acute phase proteins: C-reactive protein ( $\text{mg/dL} \times 10^{-1}$ ), ceruloplasmin ( $\text{mg/dL} \times 10^{-1}$ ), haptoglobin ( $\text{mg/dL}$ ), albumin ( $\text{mg/dL}$ ), and transferrin ( $\text{mg/dL}$ ) measured in the serum of rabbits submitted to a partial ostectomy of the right radial bone, at the following time points (M): preoperative (M0), and 6, 24, and 36 h (M6h, M24h and M36h), as well as 7, 15, 21, 30, and 45 days postoperative (M7d, M15d, M21d, M30d, and M45d).

M	C-reactive protein	Ceruloplasmin	Albumin	Haptoglobin	Transferrin
M0	0.018 $\pm$ 0.007ab	0.175 $\pm$ 0.06a	3.635 $\pm$ 0.08ab	0.168 $\pm$ 0.13bcde	0.372 $\pm$ 0.09a
M6h	0.009 $\pm$ 0.003c	0.154 $\pm$ 0.06ab	3.733 $\pm$ 0.04ab	0.165 $\pm$ 0.04bcde	0.342 $\pm$ 0.02ab
M24h	0.009 $\pm$ 0.002c	0.186 $\pm$ 0.05a	3.445 $\pm$ 0.04bc	0.375 $\pm$ 0.09a	0.332 $\pm$ 0.03abc
M36h	0.009 $\pm$ 0.003c	0.163 $\pm$ 0.05ab	3.549 $\pm$ 0.03abc	0.210 $\pm$ 0.08abcd	0.297 $\pm$ 0.03bc
M7d	0.013 $\pm$ 0.005abc	0.127 $\pm$ 0.03ab	4.070 $\pm$ 0.04a	0.138 $\pm$ 0.05cde	0.346 $\pm$ 0.10ab
M15d	0.012 $\pm$ 0.004bc	0.100 $\pm$ 0.03b	3.770 $\pm$ 0.04ab	0.324 $\pm$ 0.22ab	0.337 $\pm$ 0.05ab
M21d	0.010 $\pm$ 0.004c	0.095 $\pm$ 0.03b	3.659 $\pm$ 0.05ab	0.279 $\pm$ 0.25abc	0.321 $\pm$ 0.05ab
M30d	0.011 $\pm$ 0.004c	0.170 $\pm$ 0.07ab	3.032 $\pm$ 0.06cd	0.090 $\pm$ 0.10de	0.252 $\pm$ 0.04cd
M45d	0.019 $\pm$ 0.005a	0.131 $\pm$ 0.03ab	2.750 $\pm$ 0.04d	0.070 $\pm$ 0.01e	0.213 $\pm$ 0.07d

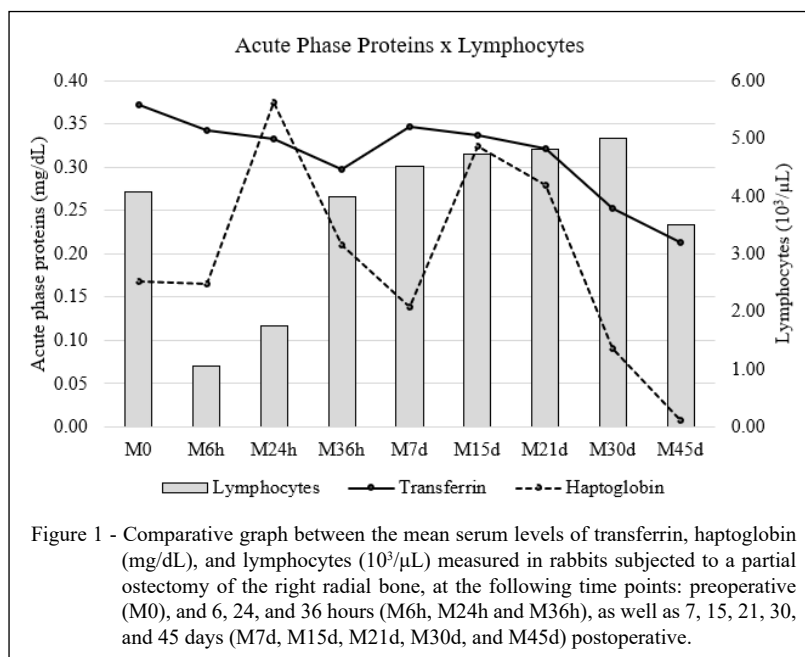
Averages followed by different letters in the columns differ from each other.

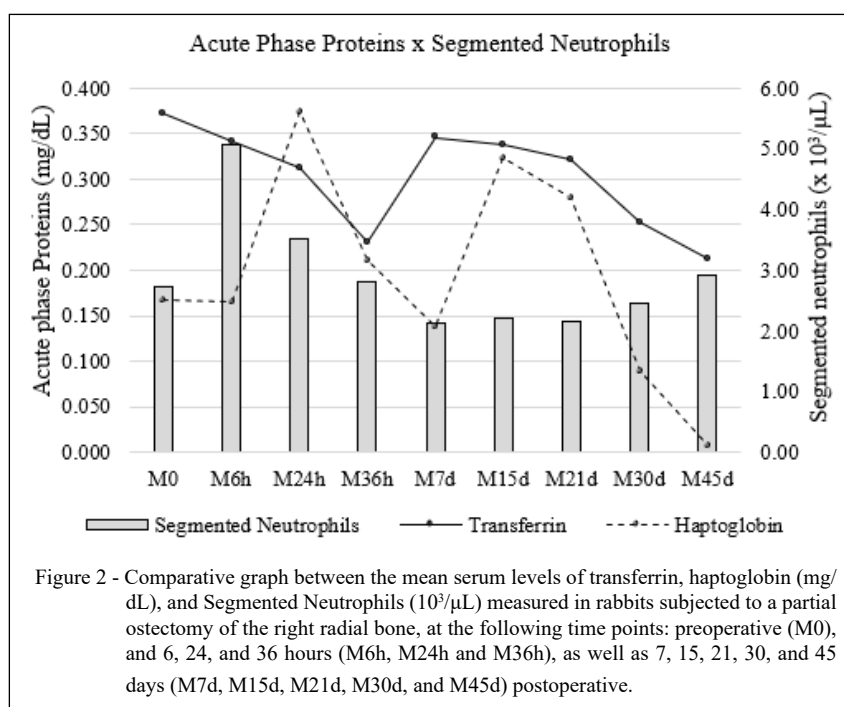
in haptoglobin concentration was observed 7 days after an experimental failure in the articular cartilage and portion of the femoral trochlea. This restored to baseline values after 14 days and was lower after 30 days. In addition, the inflammatory process directly related to surgery, anesthesia, and animal handling can be stressful, leading to an increase in cortisol (MURATA et al., 2004; TRALL, 2007) and, consequently, haptoglobin (CALDIN et al., 2009; BUSK et al., 2010). These data may justify minor but recurring peaks in M15d and M21d, in addition to those observed in the immediate post-surgical period (M24h). In M45d, it is possible to suggest that the animals were more adapted to handling since the values of this PFA were lower.

According to MEYES (1995) and BUSH (2004), it is possible to observe changes in the leukogram that suggest stressful episodes such as lymphopenia, which was observed at 6h and M24h, is related to the maximum haptoglobin peak in M24h (Figure 1). Interestingly, the peak at M24hrs preceded the increase in the concentration of neutrophils, suggesting that this PFA can potentially anticipate an inflammatory process. The same was suggested by GEORGIEV et al. (2009) and CÉRON et al. (2014). Even though the lymphocytes do not suggest any type of stress linked to the second haptoglobin peak (M15d) it is possible to observe in figure 2 that the segmented neutrophils count had a small numerical increase.

Table 1 shows higher pCR ( $P \leq 0.05$ ) at M0 than any subsequent time periods up to M30d. A study in dogs (CHRISTENSEN et al., 2015; SCHMIDT et al., 2018) and rabbits (OOHASHI et al., 2019) observed an increase in the serum concentration of pCR related to morbid processes, with a peak between 24 and 48 hours. These values returned close to baseline after 7 days, which leads us to believe that the pCR peak could have occurred between M36h and M7d, but was not identified.

CERÓN et al. (2018) described a dose-dependent connection of pCR concentration to complex inflammatory processes, such as septic or autoimmune processes, where low values are observed in small intestinal, dermal inflammation, or diseases of viral origin and, to a greater extent, in sepsis, metastasis, and autoimmune diseases. FRANSSON et al. (2007), for example, observed lower concentrations of pCR in dogs with an aseptic uterine inflammation (mucometra) than in those with pyometra. Once again, such findings confirmed that the pCR values were not elevated in the present study due to the lack of significant post-surgical complications. In addition, CÉRON et al. (2014) correlated low pCR values and high haptoglobin to situations that cause an increase in serum cortisol, as occurs in stressful events. This could also justify the lower pCR values at M6h and M36h compared to baseline.





Higher concentrations of ceruloplasmin were identified at M24h. However, the mean values were not significantly different ( $P \leq 0.05$ ) from M0. At M15d and M21d, the values were smaller and statistically different from M0 ( $P \leq 0.05$ ) (Table 1). Following the same response pattern discussed earlier, there is an increase in this PFA in more severe inflammatory clinical conditions, followed by a reduction after 7 days (KAYA et al., 2016; BOZUKLUHAN et al., 2018). FREITAS et al. (2011) identified a gradual increase in ceruloplasmin in rabbits infected with *Eimeria stiedai*. The values obtained were dose-dependent on the evolution of the inflammatory process. GEORGIEVA et al. (2009) observed a subtle increase in ceruloplasmin in rabbits that were experimentally inoculated with *E. coli* compared to the haptoglobin values, which were up to 10 times higher. It was deduced that the magnitude of the inflammatory process generated by the surgical procedure, as well as the complications related to bone nonunion, were not sufficient to determine the response pattern of serum ceruloplasmin in the species used.

Transferrin decreased from the first time point evaluated, reaching a negative peak at M36h, which was significantly different ( $P \leq 0.05$ ) from M0. At M36h, a significant negative transferrin peak ( $P \leq 0.05$ ) was observed. In addition, hemoglobin values at M36h and M7d were significantly ( $P \leq 0.05$ ) lower

than those at M6h. ULUTAS et al. (2005) described an association between anemia and a decrease in haptoglobin and transferrin. This suggested these blood collections in a short time between M0 and M36h may have influenced the hemoglobin values and, consequently, the PFA mentioned above. Even so, a haptoglobin peak at M24h followed by a leukocyte increase (Figure 2) demonstrates that there was already an inflammatory process responsible for the serum alteration of PFA and that it could have been greater had there not been a significant drop in hemoglobin. A comparative analysis of the inflammatory profile from a leukometric point of view and PFA in figure 2 reveals that transferrin is responsive to inflammation as early as M6h. At this time, NS are sequestered to the inflammatory focus and display a decrease in serum concentration as described by SWENSON and REECE (1996). These findings are consistent with the statements by MURATA et al. (2004), PALTRINIERI (2007), and CÉRON et al. (2017) that PFA can anticipate the diagnosis of inflammatory conditions even when they are still subclinical.

## CONCLUSION

The measurement of acute-phase proteins proved to be viable, considering the conditions in

which the present study was carried out. Among all variables studied, haptoglobin and transferrin showed best correlation with the inflammatory process of bone healing. Additional studies are needed to determine the sensitivity of acute-phase proteins as predictors of complications in fractures treatment.

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## ETHICS AND BIOSAFETY COMMITTEE

Protocol 019155/17.

## DECLARATION OF CONFLICTS OF INTERESTS

The authors declare no conflicts of interest. The founding sponsors had no role in the development of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript and in the decision to publish the results.

## AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

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