Hematological Changes after Caprine Demineralized Bone Matrix Implantation in Ulnar Critical Defect of Rabbit Model*

Alterações hematológicas após implante de matriz óssea desmineralizada caprina em modelo de defeito ulnar crítico em coelhos

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Abstract

Objective Several animal models have been used in fracture healing and bone graft studies, but hematological responses are seldom reported. Therefore, the present study reported the hematological changes observed in rabbits that underwent xenografting of caprine demineralized bone matrix (CDBM).

Method Twenty-four (24) male rabbits (2.5 ± 0.5kg) were acquired for the purpose of this study and were randomly assigned to three groups: autologous bone graft (ABG), unfilled (NC), and caprine demineralized bone matrix (CDBM). Blood samples were collected through cardiac puncture under xylazine-ketamine anesthesia on day 0 (baseline), and on days 28 and 56 postsurgery and were analyzed manually within 2 hours of collection. Statistical analysis was performed using a two-way analysis of variance (ANOVA) with repeated measures, and a p-value < 0.05 was considered significant.

Result There was an overall significant difference in the values of total white blood cell count ($p = 0.0043$), neutrophil count ($p < 0.0001$), monocyte count ($p = 0.0184$), red blood cell count ($p = 0.003$), hemoglobin concentration ($p < 0.0001$) and packed cell volume ($p < 0.0001$) across the days and the treatment groups. There was, however, no

Keywords
- bone grafting
- bone xenograft
- experimental animal model
- fracture healing
- hematology

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overall significant difference in lymphocyte count ($p = 0.4923$), basophil count ($p = 0.4183$), and eosinophil count (0.4806) within days.

Conclusion Response to CDBM grafting in rabbits could, therefore, be said to be characterized by marked leukocytosis with neutrophilia, lymphocytosis, and monocytosis by day 28 of postgrafting. This could form the basis with which hematology can be used to monitor body response of bone graft animal models.

Resumo

Objetivo Diversos modelos animais têm sido usados em estudos sobre enxertos ósseos e tratamento de fraturas, mas as respostas hematológicas são raramente relatadas. Este estudo descreveu as alterações hematológicas observadas em coelhos submetidos a xenoenxertos de matriz óssea desmineralizada caprina (MODC).

Métodos Vinte e quatro (24) coelhos machos (2,5 ± 0,5 kg) foram adquiridos para este estudo e divididos aleatoriamente em três grupos: enxerto ósseo autólogo (EOA); controle negativo sem preenchimento (SP) e matriz óssea desmineralizada caprina (MODC). Amostras de sangue foram coletadas por punção cardíaca sob anestesia com xilazina-quetamina no dia 0 (para estabelecimento dos valores basais) e aos dias 28 e 56 após a cirurgia; essas amostras foram submetidas à análise manual em até 2 horas após a coleta. A análise estatística foi composta por ANOVA de dois fatores com medidas repetidas e o valor de $p < 0,05$ foi considerado significativo.

Resultados Houve uma diferença geral significativa nos números de leucócitos totais ($p = 0,0043$), neutrófilos ($p < 0,0001$), monócitos ($p = 0,0184$) e hemácias ($p = 0,003$), na concentração de hemoglobina ($p < 0,0001$) e no hematócrito ($p < 0,0001$) ao longo dos dias e entre os grupos de tratamento. No entanto, não houve diferença global significativa no número de linfócitos ($p = 0,4923$), basófilos ($p = 0,4183$) e eosinófilos ($p = 0,4806$) entre os dias.

Conclusão A resposta ao enxerto de MODC em coelhos é, portanto, caracterizada por leucocitose intensa com neutrofilia, linfocitose e monocitose no 28° dia após o procedimento. Esses dados podem basear a utilização da hematologia no monitoramento da resposta corporal em modelos animais de enxerto ósseo.

Palavras-chave
- enxerto ósseo
- xenoenxerto ósseo
- modelo animal experimental
- consolidação da fratura
- hematologia

Introduction

Blood is a reflector of the status of any animal and has thus been used to know physiologic and pathologic states and for both diagnostic and prognostic evaluations of various conditions in animals.[1–4] Hematology is, therefore, performed to investigate metabolites in the body, responses to exposure to antigens, and the state of diseases in animals as different pathologic conditions or exposure to certain conditions affect some specific blood parameters.[1,5–7]

In the use of hemogram for diagnosis in rabbits, care must be taken as the hematological parameters are affected by so many factors. Age, gender, breed, ambient temperature, diurnal rhythm, and even mere transportation have been reported to cause derailment in the hemogram of rabbits.[8] For example, lymphocytopenia, leukocytosis and increased packed cell volume (PCV) have been reported in rabbits transported at 28°C for up to 3 hours, while cold stress was also reported to increase red blood cell (RBC) count. Furthermore, white blood cell count (WBC) varies due to diurnal fluctuations and variation, with total WBC being observed to be at its lowest in the later afternoon and evening when compared with the earlier hours of the day.[8] In addition, the erythrocytes also vary with gender, as the male rabbits have been observed to have a slightly higher erythrocyte count than females.[8] Also, infectious diseases do not typically cause leukocytosis in rabbits but present a shift from lymphocyte-predominant to neutrophil-predominant differential counts; furthermore, acute infections can be characterized by leukopenia with normal differential count.[8] Anemia, neutrophilia, leukocytosis, and monocytosis were reported in hepatic coccidiosis rabbit model contrasting with the eosinophilia known to accompany parasitic diseases in other animals.[8]

In fracture healing, monitoring of hematological parameters play a vital role as any deviation from the physiological reference range could be an indication of infection or graft response and, therefore, require urgent attention to avert the possibility of mal-union, delayed union, or non-union.[9] Hematology is, however, characterized by fluctuation within the normal physiological ranges, which may make its use in diagnosis difficult.[9] Decrease in total RBC is seen following trauma and surgery.[9] Stress, trauma, and surgery are known to cause leukocytosis, lymphocytopenia, and neutrophilia, which could also fluctuate within normal ranges after the initial

problem subsides. The increase or decrease of basophils, monocytes, and eosinophils could be subjective in fracture healing monitoring.[9] Due to the scarcity of information on hematological response of fracture healing in animal models, we, therefore, report in the present study, the observed hematological changes in rabbits that underwent xenografting of caprine demineralized bone matrix (CDBM).

Materials and Methods

Experimental Design

The present study was approved by the animal research ethics committee of the institution under the number (UDUS/FAREC/2019/AUP-R0–5). Twenty-four (24) male rabbits (2.5 ± 0.5kg) were randomly grouped into 3 groups, with 8 rabbits in each group. The groups were based on the treatment they received: autologous bone graft group (ABG), unfilled negative control group (NC), and CDBM group.

Preparation of CDBM and Critical Bone Defect

The preparation of the DBM, creation of the defect on the ulna, and bone grafting were performed with modifications following Arpağ et al.,[10] Monazzah et al.,[11] and Bigham-Sadegh and Oryan.[12]

Blood Sample Collection and Hematological Analysis

Blood samples were collected presurgery to serve as the baseline data for the hemogram, with specific interest in complete blood count (CBC) and leucocytic differential count. The blood samples were collected through cardiac puncture under xylazine-ketamine anesthesia. The samples were stored in a ethylenediaminetetraacetic acid (EDTA) sample bottle (JRZ Plastilab, Beirut, Lebanon) and processed under two hours of collection as adopted by Chineke et al.[7] The blood samples were subsequently collected on the 28th and on the 56th postoperative days for evaluation. This was with modification from Ajai et al.,[13] Bigham-Sadegh et al.[14] and Korkmaz et al.[15]

Data Analysis

The data generated was analyzed using a repeated measure mixed model approach with two-way analysis of variance (ANOVA) to detect differences in interaction of days and groups concurrently, and significance level was determined at $p < 0.05$ using InVivoStat 4.0.2 (Chelmsford, Essex, UK).

Results

The surgery and grafts implantation were performed successfully, and the animals recovered from anesthesia uneventfully. There was an overall significant leukocytosis ($p = 0.0043$) on days 28 and 56 compared with the baseline. On day 56, a significant difference was also observed in the neutrophil count ($p < 0.0001$), while on day 28, significant neutrophilia was observed in the CDBM group when compared with day 0; the number of neutrophils had reduced by day 56 though it was not statistically different from its baseline value. There was significant monocytosis in the ABG and CDBM groups on day 28, which returned to near baseline by day 56. Although there was no overall statistical difference in the lymphocyte count ($p = 0.4923$), on day 28, the lymphocyte counts of the ABG and CDBM groups were significantly higher from their baseline values. Significantly moderate eosinophilia was observed in the NC and CDBM groups on day 56. An overall significant difference was also observed for RBC ($p = 0.003$), hemoglobin concentration ($p < 0.0001$), and packed cell volume ($p < 0.0001$) across the days and the treatment groups. However, there was no overall significant difference in lymphocyte count ($p = 0.4923$), basophil count ($p = 0.4183$), and eosinophil count (0.4806). The result is presented in ►**Table 1** showing the significant differences across the days and among the groups.

Discussion

Since blood is a reflector of the health status of animals, responses to exposure to foreign bodies, and the state of disease in animals,[1–7] we used hematology to evaluate the responses of the experimental rabbits to the grafted caprine DBM, which is expected to initiate some antigenicity. Several reports have documented normal hematological parameters in rabbits in different geographical zones and conditions,[4,7,8,16] but the fluctuation within the normal physiological ranges make its use in diagnosis difficult,[9] especially in rabbits, in which gender, diurnal rhythm, breed, ambient temperature, age, and stress have been reported to affect their haematology.[7,8,16] It is, therefore, imperative to compare hematological responses with the baseline in any experimental condition, as seen in other studies.[1,17,18]

Leukocytosis, which is consistent with the stress of surgery, inflammation, and excruciating pain associated with fracture and fracture healing,[1,9,16,18,19] was observed in the three groups. The leukocytosis, which was exceptionally marked and significant in the CDBM group on day 28, could be as a result of the immune reaction to the implanted caprine DBM. The value of the leucocyte count had dropped by day 56 to near baseline value, which is indicative of reduced reaction to the implant. This was the same pattern of reaction that was observed for neutrophil and lymphocyte. This is in accordance with earlier reports,[16,20] which state that in conditions that triggers immunologic response, there is leukocytosis with marked lymphocytosis.

Many factors, such as time of the day of blood sampling and stress, are known to affect monocyte, eosinophil, and basophil counts.[16,20] Despite this, monocytosis has been reported to be consistent with chronic inflammation in rabbits,[16,20] and the same was observed for CDBM on day 28 when compared with its baseline. Eosinophilia and basophilia are markers of allergic and hypersensitive conditions. Mild eosinophilia here was not observed in this study until day 56 in CDBM group. This could be due to reduced immunogenicity of demineralized bone by the process of demineralization as reported.[21,22]

The reduced PCV observed on the 28th day in all the groups was as a result of blood loss to surgery. This is in accordance with the earlier reports[1,20] that anemia occurs when there is external hemorrhage. However, the animals in all the groups

Table 1 Hematological results (mean ± SEM) of experimental animals presurgery (day 0), on day 28 and on day 56 ($n = 8$)

	Day 0			Day 28			Day 56		
	ABG	NC	CDBM	ABG	NC	CDBM	ABG	NC	CDBM
WBC X 10^9 /L	3.71 ± 0.43	3.76 ± 0.36	4.53 ± 0.37	4.96 ± 0.36[a]	4.90 ± 0.72	6.75 ± 0.36*[a]	5.51 ± 0.34[b]	3.81 ± 0.19*	4.11 ± 0.24*
Neut[a] X 10^9 /L	0.76 ± 0.09	0.68 ± 0.08	0.81 ± 0.09	1.00 ± 0.07	0.84 ± 0.11	1.30 ± 0.11[a]	3.09 ± 0.27[a]	1.11 ± 0.13*[a]	1.19 ± 0.13*
Lym[c]b X 10^9 /L	2.90 ± 0.34	3.01 ± 0.28	3.63 ± 0.29	3.82 ± 0.29[a]	3.98 ± 0.61	5.24 ± 0.26*[a]	2.30 ± 0.09	2.55 ± 0.19	2.73 ± 0.14
Mono[c] X 10^9 /L	0.04 ± 0.01	0.07 ± 0.02	0.09 ± 0.02*	0.13 ± 0.02[a]	0.09 ± 0.01	0.21 ± 0.03[a]	0.07 ± 0.01	0.12 ± 0.02	0.12 ± 0.02
Eosin[d] X 10^9 /L	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01[a]	0.03 ± 0.01[a]
Baso[e] X 10^9 /L	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01
RBC X 10^{12} /L	3.47 ± 0.34	3.55 ± 0.12	3.58 ± 0.30	3.10 ± 0.18	3.71 ± 0.31[a]	4.51 ± 0.27*[a]	3.49 ± 0.27	4.97 ± 0.23*[a]	3.92 ± 0.21
Hg[f] (g/dL)	9.46 ± 0.46	14.09 ± 0.35*	11.23 ± 0.37*	8.37 ± 0.46	9.67 ± 0.47[a]	10.35 ± 0.80	10.43 ± 0.79	10.11 ± 0.39[b]	11.55 ± 0.41
PCV (%)	26.00 ± 1.34	35.58 ± 1.24*	31.50 ± 1.32*	24.63 ± 0.57	23.63 ± 0.96[a]	28.50 ± 0.87*	29.63 ± 0.94[a]	27.63 ± 0.71[b]	31.38 ± 1.19

Abbreviations: ABG, autologous bone graft; CDBM, caprine demineralized bone matrix; NC, negative control; PCV, packed cell volume; RBC, red blood cell count; SEM, standard error of the mean; WBC, white blood cell count.

Different alphabets indicate that the means differ significantly ($p < 0.05$) from day 0 within the same group, while the means bearing an asterisk differ significantly ($p < 0.05$) from ABG for their respective days.

[a]Neutrophils.

[b]Lymphocytes.

[c]Monocytes.

[d]Eosinophils.

[e]Basophils.

[f]Hemoglobin concentration.

have increased PCV values by day 56, though the values were not up to their corresponding baselines. This showed that there was regenerative response to the blood loss. In the same vein, the hemoglobin concentration also decreased on day 28 and had risen to almost above the baseline by day 56. On the contrary, the RBC count increased on days 28 and 56 for all the groups, except for the ABG on day 28, which was lower when compared with the baseline but not statistically significant. The different patterns observed for the RBC could be a result of the time of sampling and ambient temperature that affect the experimental animals.[16,20]

The inability to monitor the diurnal rhythm and environmental temperature in relation to the variation of hematological values obtained are the limitations of this study.

Conclusion

Despite several fluctuations that mar the interpretability of hematology in rabbits. It can be concluded from this study, the rabbits responded to the CDBM with marked leukocytosis with neutrophilia, lymphocytosis and monocytosis by day 28, which became near normal by day 56 post-grafting. This could therefore form a hematological basis with which the state of bone grafting animal models could be monitored. It is however strongly recommended that baseline is taken in every study involving hematology in rabbits, putting diurnal rhythm and environmental temperature into consideration.

Conflict of Interests

The authors have no conflict of interests to declare.

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References

1 Alimi OA, Abdulwahab WF, Amid SA, et al. Hematological prediction study of peritonitis following laparotomy in goats. J Vet Med Sci 2020;82(05):531–535
2 Tambuwal FM, Agaie BM, Bangana A. Haematological and Biochemical Values of Apparently Healthy Red Sokoto Goats. In: Proceeding of 27th Annual Conference. FUTA, Akure, Nigeria. Nigerian Society of Animal production (NSAP)2002:50–53
3 Akinrinmade JF, Akinrinde AS. Hematological and serum biochemical indices of West African dwarf goats with foreign body rumen impaction. Niger J Physiol Sci 2012;27(01):83–87
4 Etim NN, Williams ME, Akpabio U, Offiong EEA. Haematological Parameters and Factors Affecting Their Values. Agric Sci 2014;2 (01):37–47
5 Aderemi FA. Effects of replacement of wheat bran with cassava root sieviatesupplemented or unsupplemented with enzyme on the haematology and serum biochemistry of pullet chicks. Trop J Anim Sci 2004;7(01):147–153
6 Doyle D. William Hewson (1739-74): the father of haematology. Br J Haematol 2006;133(04):375–381
7 Chineke C, Ologun A, Ikeobi CO. Haematological Parameters in Rabbit Breeds and Crosses in Humid Tropics. Pak J Biol Sci 2006;9 (11):2102–2106
8 Moore DM, Zimmerman K, Smith SA. Hematological Assessment in Pet Rabbits: Blood Sample Collection and Blood Cell Identification. Clin Lab Med 2015;35(03):617–627
9 Kumar D, Bhargava MK, Singh R, et al. Haematological Changes during Fracture Healing in Goats. IOSR J Agric Vet Sci 2016;9(09):1–3
10 Arpağ OF, Damlar I, Altan A, Tatli U, Günay A. To what extent does hyaluronic acid affect healing of xenografts? A histomorphometric study in a rabbit model. J Appl Oral Sci 2018;26(00):e20170004
11 Monazzah S, Oryan A, Bigham-Sadegh A, Meimandi-Parizi A. Application of bovine bone versus bovine DBM graft on bone healing of radial defect in rat. Comp Clin Pathol 2017;26(06): 1293–1298
12 Bigham-Sadegh A, Oryan A. Selection of animal models for preclinical strategies in evaluating the fracture healing, bone graft substitutes and bone tissue regeneration and engineering. Connect Tissue Res 2015;56(03):175–194
13 Ajai S, Sabir A, Mahdi AA, Srivastava RN. Evaluation of Serum Alkaline Phosphatase as a Biomarker of Healing Process Progression of Simple Diaphyseal Fractures in Adult Patients. Int Res J Biol Sci Int Res J Biol Sci 2013;2(02):2278–3202
14 Bigham-Sadegh A, Mirshokraei P, Karimi I, Oryan A, Aparviz A, Shafiei-Sarvestani Z. Effects of adipose tissue stem cell concurrent with greater omentum on experimental long-bone healing in dog. Connect Tissue Res 2012;53(04):334–342
15 Korkmaz M, Oztürk H, Bulut O, Unsaldi T, Kaloğlu C. [The effect of definitive continuous distraction employed with the Ilizarov type external fixation system on fracture healing: an experimental rabbit model]. Acta Orthop Traumatol Turc 2005;39(03):247–257
16 Washington IM, Van Hoosier G. Clinical Biochemistry and Hematology. The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents 2012:57–116
17 Toth C, Klarik Z, Kiss F, Toth E, Hargitai Z, Nemeth N. Early postoperative changes in hematological, erythrocyte aggregation and blood coagulation parameters after unilateral implantation of polytetrafluoroethylene vascular graft in the femoral artery of beagle dogs. Acta Cir Bras 2014;29(05):320–327
18 Grover RK, Sobti VK. Clinical, haematological and radiological evaluation of fragmented autogenous cortical bone grafting of radius in dogs. Zentralbl Veterinarmed A 1998;45(05):303–308
19 Arens D, Wilke M, Calabro L, et al. A rabbit humerus model of plating and nailing osteosynthesis with and without Staphylococcus aureus osteomyelitis. Eur Cell Mater 2015;30:148–161, discussion 161–162
20 Melillo A. Rabbit Clinical Pathology. J Exot Pet Med 2007;16(03): 135–145
21 Guizzardi S, Di Silvestre M, Scandroglio R, Ruggeri A, Savini R. Implants of heterologous demineralized bone matrix for induction of posterior spinal fusion in rats. Spine 1992;17(06):701–707
22 Bigham AS, Shadkhast M, Bigham Sadegh A, Shafiei Z, Lakzian A, Khalegi MR. Evaluation of osteoinduction properties of the demineralized bovine foetal growth plate powder as a new xenogenic biomaterial in rat. Res Vet Sci 2011;91(02):306–310