RESEARCH ARTICLE
Open Access

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2023.54.1.91-97

Evaluation of keeping quality of canine platelet rich plasma under different storage conditions

Deny Jennes[1*], Soumya Ramankutty[2], S. Anoop,[3]
N. Madhavan Unny[4], S. Sudheesh Nair[5] and K.D. John Martin[6]
Department of Veterinary Surgery and Radiology,
College of Veterinary and Animal Sciences, Mannuthy, Thrissur- 680 651,
Kerala Veterinary and Animal Sciences University, Kerala, India.

Citation: Jennes,D., Ramankutty,S., Anoop,S., Madhavan Unny,N., Sudheesh,S.N. and Martin, J.K.D. 2023. Evaluation of keeping quality of canine platelet rich plasma under different storage conditions. *J. Vet. Anim. Sci.* **54**(1):91-97
DOI: https://doi.org/10.51966/jvas.2023.54.1.91-97

Received: 14.09.2022 *Accepted: 01.11.2022* *Published: 31.03.2023*

Abstract

Platelet rich plasma (PRP) therapy is an integral part of regenerative medicine as the platelets possess a good healing capacity owing to the presence of a wide variety of growth factors in the platelet granules found in the cytoplasm of the platelet. Autologous PRP was prepared from the blood of the patient itself, without any preservatives. Storage of PRP was one of the main hurdles of the treatment modality. During storage, the platelet counts may get reduced, undergo activation or get contaminated with bacteria as no preservatives are used in the preparation of autologous PRP. Cytological changes and microbial quality of the PRP during storage at 4°C and -20°C for seven days were analysed in this study. Reduction in platelet count and the chance of microbial contamination were less when autologous PRP was stored at -20°C compared to 4°C.

Keywords: *Canine, autologous platelet rich plasma, PRP, double centrifugation method, storage changes*

Platelet rich plasma (PRP) is defined as the plasma fraction of the blood which contains platelet concentration above that of the normal baseline (Arnoczky and Shebani-Rad, 2013). It was obtained through the centrifugation of blood mixed with anticoagulants (Lee *et al*., 2016). PRP has a proven effect on wound healing and tissue regeneration as it provides an array of growth

#Part of MVSc thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala

1. *MVSc Scholar*
2 and 5. *Assistant Professor*
3. *Professor and Head, Department of Veterinary Surgery and Radiology, College of Veterinary and Animal Sciences, Pookode*
4. *Professor and Head, Department of Clinical Medicine, Ethics and Jurisprudence, College of Veterinary and Animal Sciences, Pookode*
5. *Professor and Head*

**Corresponding author: denyjennis@gmail.com., Ph: 9495960160*

factors. Apart from the growth factors, platelets also proved to contain some peptides which possess antimicrobial properties (Drago *et al*., 2013). Platelet rich plasma in storage is often found to undergo cytological changes and early activation of growth factors. Moreover, as the autologous PRP is prepared without the addition of any preservatives, susceptibility to microbial contamination is also postulated. The present study evaluated the platelet count, cytological changes and microbiological quality of autologous PRP in storage under two different temperatures.

Materials and methods

The study was conducted with venous blood samples collected from six dogs selected irrespective of age, breed and sex. For the preparation of autologous canine PRP, 10mL venous blood was collected either from the cephalic or jugular vein of the animal by using a 20 G scalp vein set following aseptic precautions into vials containing Ethylenediaminetetraacetic acid (EDTA). The fresh blood was subjected to a complete cell count examination using an automatic haematology analyser to make a baseline count for comparison.

The collected 10 mL of blood was subjected to two-stage centrifugations. The modified syringe method (Laiju, 2022) was followed for the preparation of PRP. First-stage centrifugation was performed at a speed of 200 g for five minutes at 20°C. The first centrifugation separated the whole blood into three different layers viz, the bottom layer consisted of red blood cells, the middle layer contained white blood cells and the topmost layer rich in platelets. The middle and top layers were transferred into another sterile centrifugation tube and subjected to further centrifugation at 500g for 20 minutes at 20°C. After the second centrifugation platelets along with a small number of erythrocytes and leucocytes were concentrated in the bottom as a pellet while the platelet poor plasma (PPP) formed a layer above it. Two-thirds of the PPP was discarded and the platelet concentrated at the bottom was mixed in the remaining PPP to prepare the PRP solution. The freshly prepared PRP was subjected to cytological and microbial quality assessment. The cell counts were measured using an automatic haematology analyser. Cell morphology in PRP was studied by making the smear of PRP, stained with Field's stain and observed under the oil immersion objective of a light microscope. The microbial quality of the PRP was assessed through bacterial culture studies on blood agar.

To find out the changes after storage in PRP, the prepared PRP was divided into two aliquots – among which one was stored at 4°C in the refrigerator and the other stored at -20°C for seven days. On the seventh day of storage, the PRP was thawed to room temperature and subjected to cytological and microbial assessment as mentioned before.

Results and discussion

Total erythrocyte count (RBC)

The mean (n = 6) values of total erythrocyte count ($\times 10^6/\mu L$) in blood and freshly

Table 1. Comparison of the values in fresh blood and in PRP

Cases	Erythrocyte count ($\times 10^6/\mu L$)		Leucocyte count ($\times 10^3/\mu L$)		Haemoglobin concentration (g/dL)		Volume of packed red cells (%) (VPRC)		Platelet count ($\times 10^3/\mu L$)	
	CBC	Fresh PRP	CBC	Fresh PRP	CBC	Fresh PRP	CBC	Fresh PRP	CBC	Fresh PRP
1.	5.79	0.16	16.40	7.20	12.60	0.00	37.00	1.10	190.00	1254.00
2.	6.74	0.13	10.30	7.50	13.90	0.00	45.50	0.90	436.00	1783.00
3.	5.39	0.35	7.20	6.60	14.10	0.00	37.80	0.00	282.00	1071.00
4.	7.60	0.36	12.10	11.00	15.80	0.50	53.20	2.80	625.00	1006.00
5.	4.97	1.21	16.00	10.30	11.60	1.70	33.00	7.20	491.00	1791.00
6.	5.26	0.59	13.60	14.20	12.10	0.60	33.20	0.00	236.00	961.00
Mean ± SE	5.96 ± 0.41	0.47 ± 0.16	12.60 ± 1.43	9.47 ± 1.19	13.35 ± 0.63	0.47 ± 0.27	39.95 ± 3.23	2.00 ± 1.12	376. 67 ± 68.76	1311.00 ± 155.94

prepared PRP were 5.96 ± 0.41 and 0.47 ± 0.16 respectively. A significant reduction in the erythrocyte count ($p < 0.001$) was noticed which was indicative of the efficiency of centrifugation. The results obtained were similar to the findings of Shin *et al.* (2017) in canine PRP and Laiju (2022) in bovine PRP.

The mean value of erythrocyte counts of PRP stored under 4°C and -20°C, after storage for seven days, were 0.67 ± 0.23 and 0.57 ± 0.20 respectively. No significant difference in erythrocyte count was noted between freshly prepared PRP and the PRP stored at 4°C and -20°C.

Total leucocyte count (WBC)

The mean values of leucocyte count ($\times 10^3/\mu L$) in whole blood and freshly prepared PRP were 12.60 ± 1.43 and 9.47 ± 1.19 respectively. A decrease in the leucocyte count in PRP was observed compared to whole blood but the reduction was not statistically significant ($p > 0.01$). Shin *et al.* (2017) and Laiju (2022) observed a significant reduction ($p < 0.05$) in the leucocyte count in canine and bovine PRP respectively. According to Franklin *et al*. (2015), the ideal properties of the PRP were always uncertain and each animal's PRP may vary in composition. The method of PRP preparation may also affect cell composition.

A significant reduction ($p = 0.002$) in the mean value of leucocyte count was noticed following storage under -20°C (6.97 ± 0.88) when compared to storage at 4 °C (13.33 ± 2.03). Leucocyte count was significantly decreased ($p=0.002$) at -20 °C compared to freshly prepared PRP and no statistically significant reduction was observed by storage at 4 °C. Decreased count of leucocytes observed was due to the degeneration of cells under low temperature (Hussain *et al.*, 2017).

Haemoglobin concentration (Hb)

The mean values of haemoglobin concentration (g/dL) in whole blood and freshly prepared PRP were 13.35 ± 0.63 and 0.47 ± 0.27 respectively. A significant reduction ($p < 0.001$) in the haemoglobin concentration was noted in the PRP compared to whole blood, indicating an effective first-stage centrifugation.

A statistically significant reduction was not evident in the haemoglobin concentration of fresh PRP and stored PRP. Storage of PRP under 4°C and -20°C had not shown any significant variation in haemoglobin concentration. The mean values were 0.33 ± 0.3 and 0.32 ± 0.28 respectively.

Volume of packed red cells (VPRC)

Volume of packed red cells (%) in whole blood and PRP were 39.95 ± 3.23 and 2.00 ± 1.12 respectively. A significant reduction ($p < 0.001$) of VPRC concentration was noted in the PRP compared to whole blood. The findings were similar to the observation of Schnabel *et al.* (2007) in which a significant reduction ($p < 0.0001$) of VPRC in PRP and PPP were observed compared to whole blood.

A significant increase ($p < 0.001$) in VPRC was observed between fresh PRP and PRP stored at 4°C, but the difference was not significant when stored at -20°C. The mean values of VPRC in PRP after seven days of storage were 4.10 ± 1.32 at 4°C and 2.0 ± 0.99 at -20°C. A significant reduction ($p < 0.001$) was noted at -20°C compared to 4°C storage. According to Lee and Kang (2016), at 4ºC hypoxia causes degeneration of RBCs which leads to erythrocyte swelling and consequently increases the haematocrit values. Fluctuations in the VPRC in PRP during storage at different temperatures are poorly understood due to the availability of limited literature.

Total platelet count

A significant increase ($p < 0.001$) in the mean value of platelet count ($\times 10^3/\mu L$) was noticed in the freshly prepared PRP compared to whole blood. The values are 1311.00 ± 155.94 in PRP and 376.67 ± 68.76 in whole blood. The observations were in accordance with the results of Schnabel *et al.* (2007), Shin *et al.* (2017) and Laiju (2022). The double centrifugation method adopted for the preparation of PRP was effective in concentrating the platelets.

Platelet count was significantly

reduced ($p < 0.001$) when PRP was stored for seven days at 4°C and -20°C than fresh PRP, but more reduction was noticed at 4°C than at -20°C. The mean value of platelet counts under storage after seven days were 612.83 ± 112.89 at 4°C and 1230.00 ± 148.58 at -20°C. A significant reduction ($p < 0.001$) in platelet count was observed in PRP stored at 4°C compared to storage at -20°C. Storage of platelets leads to biochemical, structural and functional changes and these were caused by the multifactorial process. It included energy consumption, pH decrease, platelet activation and apoptosis (Mittal and Kaur, 2015). Johnson *et al*. (2016) stated that the refrigerated (2–6°C) storage caused morphologic and metabolic changes in platelets. Platelet aggregation was induced by low temperatures and it was more in chilled conditions (Kattlove and Alexander, 1971). Andia *et al.* (2020) opined that the freeze-drying of PRP preserved the platelet function, concentration of cytokines and functionality.

Microbial quality

Aseptic precautions during the blood collection procedure would prevent microbial growth in stored PRP. There was no microbial growth in any of the samples of PRP on the day of preparation, but microbial growth was noticed in three out of six samples following storage under 4°C. The organisms isolated from each sample were coagulase-negative *Staphylococcus spp*., *Pseudomonas aeruginosa* (Fig. 5) and *Chromobacterium violaceum.* The finding was similar to the observation of Wu *et al.* (2014) in which, - *Stenotrophomonas maltophilia* was isolated from human PRP stored at 4°C. Among samples kept at -20°C, no microbial growth was detected on the seventh day (Table 5). According to Bielecki *et al.* (2007), PRP possessed bacteriostatic property and the antimicrobial property was mainly against *Staphylococcus aureus* and *Escherichia coli.*

Cytology and platelet morphology

Microscopical evaluation of PRP smear with Field's stain revealed purplish blue-coloured platelets with a small number of erythrocytes and leucocytes. The platelets were distributed uniformly in the smear of fresh PRP, without any clumping (Fig. 1). The above findings were in accordance with the findings of Laiju (2022) regarding bovine PRP smear. The smear of PRP stored at 4°C revealed an apparent reduction of platelet number in all microscopic fields, changes in the platelet morphology like change in shape and increased number of fragments, clumping of the platelets, lysis of erythrocytes and a smaller number of leucocytes (Fig. 2). The smear of PRP maintained at -20°C revealed comparatively less reduction of platelet count in the microscopical fields, fewer alterations in the morphology of platelets, a smaller number of leucocytes, and lysis of erythrocytes without any clumping of platelets (Fig.3).

Blood-derived therapeutic products are generally safe and effective in the clinical application (Marx *et al*., 1998). Platelets are observed to support wound healing on a large scale. During the time of injury, platelets will immediately come and adhere to the damaged tissue and initiate the healing mechanism through the release of cytokines and growth factors (Lee *et al*., 2016).

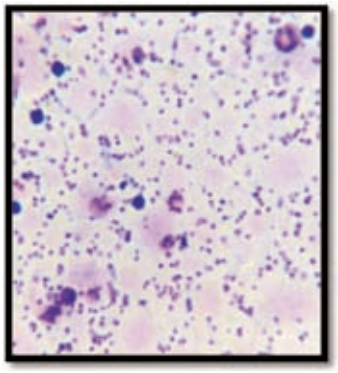

Fig. 1. PRP on day 0

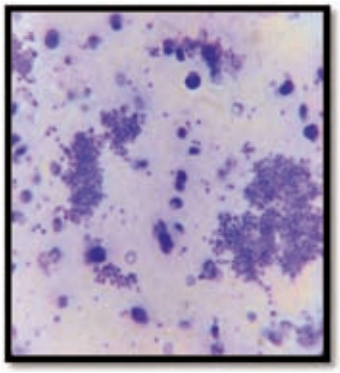

Fig. 2. Clumping of platelets 4°C

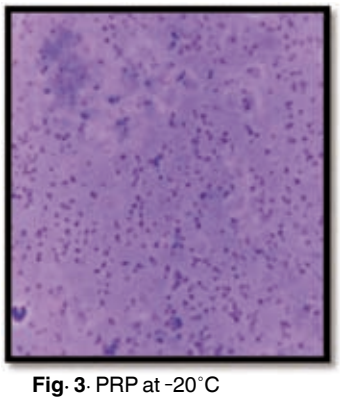

Fig. 3. PRP at -20°C

Lee *et al.* (2016) studied human PRP eye drops stored at -20° C for long-term usage and at 4°C for daily application. Simona *et al.* (2017) evaluated the storage changes of PRP after 48 and 72 hours of storage at 4°C and at -20° C. The platelet morphology was altered greatly with an increased number of fragments, indicating that the platelets had undergone activation in samples kept at 4°C. But PRP under -20° C did not show any alteration in platelet morphology indicating that the platelets were in an inactive state under deep freezing. According to the study of Wen *et al.* (2018), the storage of human PRP at 22° C for seven days with constant agitation in the platelet incubator of the blood bank revealed that the platelet count and growth factors concentration were sustained compared to the first day indicating that using stored PRP can be used for injections over a period of time during a course of treatment.

The basic principle of the PRP preparation during the double centrifugation

Table 2. Cell counts after storage of PRP

Cases	Day 7									
	4°C					-20°C				
	RBC count ($\times10^6$/µL)	WBC count ($\times10^3$ /µL)	Hb (g/dL)	VPRC (%)	Platelet count ($\times10^3$ /µL)	RBC count ($\times10^6$/µL)	WBC count ($\times10^3$ / µL)	Hb (g/dL)	VPRC (%)	Platelet count ($\times10^3$ /µL)
1.	0.33	13.60	0.00	2.50	462.00	0.22	5.80	0.00	0.90	1166.00
2.	0.34	6.80	0.00	1.40	991.00	0.17	6.30	0.00	0.00	1668.00
3.	0.36	10.80	0.20	2.30	605.00	0.44	5.20	0.00	1.80	966.00
4.	0.47	11.00	0.00	3.50	311.00	0.38	5.90	0.10	0.00	977.00
5.	1.78	20.80	1.80	10.30	901.00	1.47	7.50	1.70	6.40	1703.00
6.	0.71	17.00	0.00	4.60	407.00	0.71	11.10	0.10	2.90	900.00
Mean ± SE	0.67 ± 0.23	13.33 ± 2.03	0.33 ± 0.30	4.10 ± 1.32	612.83 ± 112. 89	0.57 ± 0.20	6.97 ± 0.88	0.32 ± 0.28	2.00 ± 0.99	1230.00 ± 148.58

Table 3. Comparative analysis of PRP variables before and after storage

	RBC ($\times10^6$/µL)	WBC ($\times10^3$ /µL)	RBC ($\times10^6$/µL)	Hb (g/dL)	VPRC (%)	Platelet ($\times10^3$ /µL)
Whole blood	5.96 ± 0.41^a	12.6 ± 1.43^a	5.96 ± 0.41^a	13.35 ± 0.63^a	39.95 ± 3.23^a	376.67 ± 68.76^c
Fresh PRP	0.47 ± 0.16^b	9.47 ± 1.19^a	0.47 ± 0.16^b	0.47 ± 0.27^b	2.00 ± 1.12^c	1311.00 ± 155.94^a
PRP after 7 days storage at 4°C	0.67 ± 0.23^b	13.33 ± 2.03^a	0.67 ± 0.23^b	0.33 ± 0.3^b	4.10 ± 1.32^b	612.83 ± 112.89^c
PRP after 7 days storage at -20°C	0.57 ± 0.20^b	6.97 ± 0.88^b	0.57 ± 0.20^b	0.32 ± 0.28^b	2.00 ± 0.99^c	1230.00 ± 148.58^b
F-value (P-value)	95.476** (<0.001)	8.234** (0.002)	95.476** (<0.001)	248.98** (<0.001)	90.289** (<0.001)	37.88** (<0.001)

*** Significant at 0.01 level; ns non-significant Means having different letter as superscript differ significantly within a column*

Table 4. Results of bacterial culture of PRP before and after storage

Cases	Fresh PRP	After 7 days storage	
		4°C	-20°C
1.	no growth	Coagulase -ve *Staphylococcus spp.*	no growth
2.	no growth	*Pseudomonas aeruginosa*	no growth
3.	no growth	*Chromobacterium violaceum*	no growth
4.	no growth	no growth	no growth
5.	no growth	no growth	no growth
6.	no growth	no growth	no growth

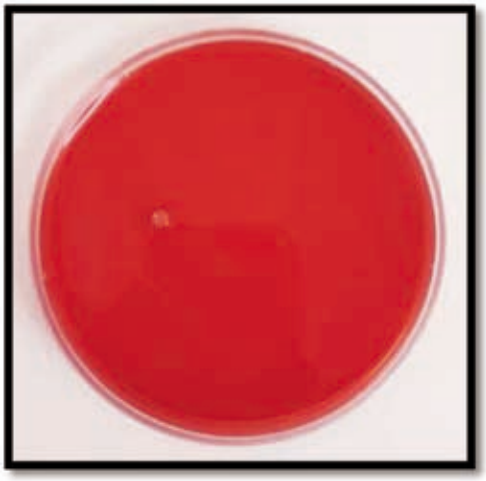

Fig. 4. Blood agar

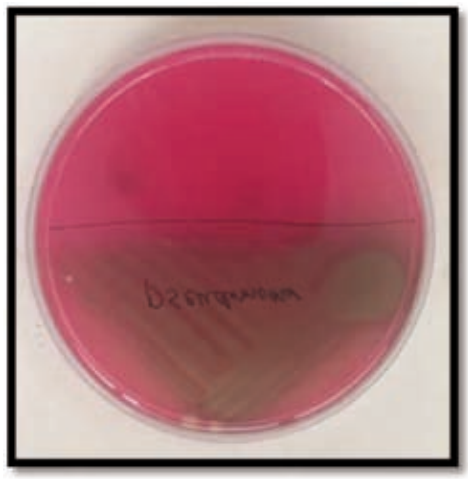

Fig. 5. *Pseudomonas spp* growth in blood agar

method was the density gradient separation of the blood cellular elements (Araki *et al.,* 2012). According to Bausset *et al*. (2012) to the centrifugation speed of 1000g or more has reduced the platelet reactivity and induced spontaneous auto-aggregation of platelets *in vitro*. The higher centrifugation force also altered the morphology of the platelets during the preparation of PRP. The two-stage centrifugation with 200 g for five minutes and at 500g for 20 minutes at 20°present study was found to preserve the PRP and platelet morphology. The results on the storage of PRP showed that -20°C was the suitable temperature for the preservation and to maintain the quality of PRP than 4 °C.

Conclusion

Due to the preservative-free preparation nature of autologous PRP, it was susceptible to microbial contamination when stored under refrigeration. The platelet count was also reduced and morphology altered during the storage period when PRP was stored at 4°C than at -20°C. For long-term storage of platelets -20°C was suitable compared to 4°C.

Acknowledgements

The authors are thankful to the Dean, College of Veterinary and Animal Sciences, Mannuthy, Central instrumentation laboratory and to the Professor and Head, TVCC, Mannuthy for the help and support in performing the research.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Alio, J.L., Abad, M., Artola, A., Rodriguez-Prats, J.L., Pastor, S. and Ruiz-Colecha, J. 2007. Use of autologous platelet rich plasma in the treatment of dormant corneal ulcers. *Ophthalmol*. **114**: 1286-1293.

Andia, I., Perez-Valle, A., Amo, C.D. and Maffulli, N. 2020. Freeze-Drying of Platelet-Rich Plasma: The Quest for Standardization. *Int. J. Mol. Sci.* **21**: 1-18.

Araki, J., Jona, M., Eto, H., Aoi, N., Kato, H., Suga, H., Doi, K., Yatomi, Y. and Yoshimura, K. 2012. Optimized preparation method of platelet-concentrated plasma and noncoagulating platelet-derived factor concentrates: maximization of platelet concentration and removal of fibrinogen. *Tissue Eng. Part C Methods*. **18**: 176-185.

Arnoczky, S.P. and Shebani-Rad, S. 2013. The basic science of platelet rich plasma (PRP): what clinicians need to know. *Sports Med. Arthrosc*. **21**: 180-185.

Bausset, O., Giraudo, L., Veran, J., Magalon, J., Coudreuse, J., Magalon, G., Dubois,

C., Serratrice, N., Dignat-George, F.O., and Sabatier, F. 2012. Formulation and Storage of Platelet-Rich Plasma Homemade Product. *Biores. Open Access*. **1**: 115-123.

Bielecki, T.M., Gazdzik, T.S., Arendt, J., Szczepanski, T., Krol, W. and Wielkoszynski, T. 2007. Antibacterial effect of autologous platelet gel enriched with growth factors and other active substances: an in vitro study. *J. Bone Joint Surg. Br.* **89:** 417-420.

Drago, L., Bortolin, M., Vassena, C., Taschieri, S. and DelFabbro, M. 2013. Antimicrobial activity of pure platelet rich plasma against microorganisms isolated from oral cavity. *BMC Microbiol*. **13**: 1-5.

Franklin, S.P., Garner, B.C. and Cook, J.L. 2015. Characteristics of canine platelet-rich plasma prepared with five commercially available systems. *Am. J. Vet. Res*. **76:** 822-827.

Hussain, K., Awakan, V. and Assad, A. 2017. Storage-induced changes in haematologic parameters of blood. Int. J. Med. Lab. Res. 2: 1-6.

Johnson, L., Tan, S., Wood, B., Davis, A. and Marks, D.C. 2016. Refrigeration and cryopreservation of platelets differentially affect platelet metabolism and function: a comparison with conventional platelet storage conditions. *Transfusion*. **56**: 1807-1818.

Kattlove, H.E. and Alexander, B. 1971. The effect of cold on platelets: cold induced platelet aggregation. *Blood*. **38**: 39-48.

Laiju, M.P. 2022. Autologous platelet rich plasma for treatment of sole lesions and expression profile of associated biomarkers in dairy cattle. PhD thesis, Kerala Veterinary and Animal Sciences University, Pookode, 248p.

Lee, J.H., Kim, M.J., Ha, S.W. and Kim, H.K. 2016. Autologous platelet-rich plasma eye drops in the treatment of recurrent corneal erosions. *Korean J. Ophthalmol*. **30**:101-107.

Lee, J.M. and Kang, J.S. 2016. Changes of haematological references depends on storage period and temperature conditions in rats and dogs. *Lab. Anim. Res.* **32:** 241-248.

Marx, R.E., Carlson, E.R., Eichstaedt, R.M., Schimmele, S.R., Strauss, J.E. and Georgeff, K.R. 1998. Platelet-rich plasma: growth factor enhancement for bone grafts. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod*. **85**: 638-646.

Mittal, K. and Kaur, R. 2015. Platelet storage lesion: an update. *Asian J. Transfus. Sci*. **9:** 1-3.

Schnabel, L.V., Mohammed, H.O., Miller, B.J., McDermott, W.G., Jacobson, M.S., Santangelo, K.S. and Fortier, L.A. 2007. Platelet rich plasma (PRP) enhances anabolic gene expression patterns in flexor digitorum superficialis tendons. *J. Orthop. Res*. **25:** 230-240.

Shin, H.S., Woo, H.M. and Kang, B. 2017. Optimisation of a double centrifugation method for preparation of canine platelet rich plasma. *BMC Vet. Res*. **13**: 1-8.

Simona, D.P., Chiara, C., Francesca, A., Rosario, P., Giusi, V. and Elisabetta, G. 2017. Platelet rich plasma eye drops: preparation, storage and clinical use in dogs and cats. Preliminary results. *Arch. Vet. Sci. Technol*. **104**: 1-4.

Wen, Y.H., Lin, W.Y., Lin, C.J., Sun, Y.C., Chang, P.Y., Wang, H.Y., Lu, J.J., Yeh, W.L., Chiueh, T.S. 2018. Sustained or higher levels of growth factors in platelet-rich plasma during 7- day storage. *Clin. Chim. Acta.* **483**: 89–93.

Wu, T.E., Chen, C.J., Hu, C.C. and Cheng, C.K. 2014. Three case reports: Easy to prepare autologous platelet rich plasma in the treatment of refractory corneal ulcers. *Taiwan J. Ophthalmol*. **2014**: 1-4. ■