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Short Communication

HAEMATO-BIOCHEMICAL ALTERATIONS ASSOCIATED WITH THE USE OF EGGSHELL MEMBRANE AS A DRESSING MATERIAL FOR FULL-THICKNESS WOUNDS IN A RABBIT MODEL

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ABSTRACT: Eggshell membrane (ESM) is a traditional but least explored natural wound dressing agent. Hematobiochemical parameters are important in assessing the deleterious effect of materials used in the wound study. The present study investigated the hematological alterations associated with using ESM as a wound dressing material. Various parameters such as hemoglobin (g/dl), differential leukocyte count (DLC) (%), glucose (mg/dl), and total protein (g/dl) were analyzed at different intervals. The values of heterophile gradually increased up to day 14, then started to decrease in all the groups and returned to normal by day 28. There was a significant decrease in lymphocytes in all the groups as there was an increase in heterophile, reaching the base value by day 28. A significant increase in TP was recorded from day 7 to day 21 in all the groups compared to the day 0 values. There was no significant variation in any of the other parameters evaluated. Overall, using ESM as a wound dressing agent has the least interference with hematological parameters. However, the relative decrease in the number of lymphocytes in DLC may be due to increased heterophils.

Key words: Eggshell membrane, Bone marrow-derived mesenchymal stem cells, Wound healing, Wound dressing, Rabbit.

In recent years, there has been extensive development in the field of wound dressings, leading to significant advancements. In ancient times, traditional natural products were directly used as dressing materials. However, over time, there have been notable changes, and newer dressing and healing materials with specific functions have been developed (Zahedi *et al.* 2010, Suvaneeth *et al.* 2021, Mridha and Ghosh 2022). Studies have shown that simple gauze dressings are not suitable for all types of wounds because they absorb moisture and exudates, which can dry out the wound environment and cause further damage during dressing changes (Kucharzewski *et al.* 2019). Instead, there are now various synthetic and natural dressing materials available that yield satisfactory results. Natural dressing options include polysaccharides, proteoglycans, and proteins (Mogoºanu and Grumezescu 2014). One such natural dressing material is the eggshell membrane (ESM), which consists of a protein network containing collagen I, V, and X, as well as other important proteins that contribute to enhanced tissue regeneration (Guha Ray *et al.* 2018). ESM has the potential to positively impact the quality and speed of wound healing. It serves as an ideal dressing material because it protects wounds, provides pain relief, and accelerates the healing process.

Additionally, ESM is readily available and cost-effective (Yang *et al.* 2003). Guarderas *et al.* (2016) demonstrated the potential of ESM in promoting accelerated wound healing in the early stages of cutaneous wounds. However, it is important to consider the possibility of hematological changes when using an external dressing agent on a wound bed. These changes in blood parameters serve as valuable initial indicators to evaluate the effects of agents used for wound treatment (Yakubu *et al.* 2007). Rabbit

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is selected as the study animal, as, it serves the purposes related to testing of various biomedical procedures (Nas *et al.* 2022). The objective of the present study is to investigate the hematological alterations associated with the use of ESM as a dressing agent in conjunction with bone marrow-derived mesenchymal stem cells (BM-MSCs) for treating full-thickness skin defects in rabbits.

The study

Experimental animals

A total of twenty-five New Zealand White rabbits, which were in good health condition, were utilized for this study. These rabbits were given a standard diet and unrestricted access to water, and they were kept under consistent management conditions throughout the entire study duration (Sharun *et al.* 2021a). To ensure the rabbits' comfort and ease of handling, a two-week acclimatization period was provided prior to the study. During this period, the animals were familiarized with the researchers' approach and handling techniques.

Experimental design

This study aimed to assess the hematobiochemical changes associated with the application of BM-MSCs (bone marrow-derived mesenchymal stem cells), ESM (eggshell membrane), and their combination in rabbits with full-thickness skin defects. To induce anesthesia in the animals, a combination of xylazine hydrochloride (6 mg/kg body weight) and ketamine hydrochloride (60 mg/kg body weight) was administered, with a 10-minute interval between the injections of the two drugs (Sharun *et al.* 2021b). A full-thickness excision wound measuring 2x2 cm square was then created in the dorso-caudal region of each rabbit (Sivanarayanan 2012). The rabbits were randomly divided into five groups, each consisting of five animals. Group A received no treatment (Fig. 1a), group B was treated with only fibrin glue (FG) (Fig. 1b), group C received FG and ESM as a dressing (Fig. 1c), group D received FG and BM-MSCs (Fig. 1d), and group E received a combination of FG, ESM, and BM-MSCs (Fig. 1e).

Ethical statement

The methods and experimental protocols were conducted in compliance with the approved guidelines of the Institute Animal Ethics Committee (IAEC) at the Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Bareilly, Uttar Pradesh, as stated in order No. 26-3/2020-21/JD(R).

Collection and processing of blood

Blood samples were obtained from the animals on days 0, 7, 14, 21, and 28 for further analysis. A cardiac puncture method was used to collect 2 ml of blood from each animal. Within two hours of collection, 20 µl of blood was used to determine the hemoglobin levels and prepare blood smears for enumerating the differential leukocyte count. The remaining blood sample was subjected to centrifugation at 3000 rpm for 10 minutes to separate the plasma. The obtained plasma was stored at -20°C to facilitate biochemical tests. Methanol-fixed Giemsa-stained blood smears were examined to assess the differential leukocyte count, hemoglobin levels were measured using Sahli's haemoglobinometer, and plasma glucose and total protein were also evaluated.

Statistical analysis

The parametric data were subjected to repeated measures of Analysis of Variance (ANOVA) for comparison. Furthermore, the non-parametric data were compared using Kruskal-Wallis one-way ANOVA.

Analysis of the result

Previous studies (Osigwe *et al.* 2017, Olamilosoye *et al.* 2018) have observed changes in various hematological parameters when using different agents for wound treatment. Hence, evaluating hematological parameters can serve as a valuable means to assess the potential

Table 1. Monocyte in rabbits belonging to different treatment groups (Mean ± SE).

Groups	Day 0	Day 7	Day 14	Day 21	Day 28
A	4±1.58	3.8±1.3	6.6±1.67*	5±1.58	5±1.58
B	4.8±1.92	4.6±1.95	6.4±1.14	3.6±2.41	3±1.58
C	4.6±2.3	4.2±1.64	8±2**	3.8±1.92	4.4±1.52
D	5.4±1.14	4.8±1.1	5.6±1.52	4±1.87	2.6±1.82
E	4.2±1.92	4.2±0.84	7±1.58*	4.6±1.52	3.6±2.41

*Significantly different from day 0 values of the respective group ($p < 0.05$).
**Highly significantly different from day 0 values of the respective group ($p < 0.01$).

Table 2. Total protein (g/dl) in rabbits belonging to different treatment groups (Mean ± SE).

Groups	Day 0	Day 7	Day 14	Day 21	Day 28
A	6.51±0.88	8.01±0.5*	10.79±0.61**	8.48±0.59*	6.6±0.72*
B	6.58±0.73	8.75±1.12**	11.4±0.65**	8.33±1.49	6.8±0.96
C	6.1±0.61	8.01±0.73**	11.33±0.92**	8.32±1.73*	6.62±0.54
D	6.4±1	7.96±1.04*	11.01±1.16**	8.97±0.79**	6.83±0.68
E	6.39±0.68	8.5±1.21**	11.62±0.51**	8.29±0.55*	6.7±0.66

*Significantly different from day 0 values of the respective group ($p < 0.05$).
**Highly significantly different from day 0 values of the respective group ($p < 0.01$).

adverse effects of diverse wound dressing agents.

The concentration of hemoglobin (Hb) in the blood should ideally fall between 10 and 15 g/dl. Throughout the study period, the Hb levels (g/dl) in all groups of animals remained within the normal range, indicating minimal interference from the treatment and wound healing on hematocrit values (Fig. 2a). Heterophils in rabbits, equivalent to neutrophils in other mammals, constitute the second most commonly encountered leukocyte (20-75%).

During the wound-healing process, various white blood cells (WBCs) are recruited to aid in the repair process (Almadani *et al.* 2021). A significant increase in heterophils was observed from day 7 to day 21 in all groups compared to day 0 values. However, following a gradual increase until day 14, heterophil levels started to decline in all groups and returned to normal by day 28 (Fig. 2b), indicating the normal progression of repair. Lymphocytes typically account for around 30% of the total cell count in healthy rabbits. Diseased rabbits often exhibit reduced lymphocyte levels, which can also decrease during periods of stress (Flecknell 2000). In our study, a decrease in lymphocytes was observed in all groups as heterophil levels increased (Fig. 3a). However, lymphocyte levels reached baseline values by day 28. This relative decrease in lymphocyte count in the differential leukocyte count (DLC) may be attributed to increased heterophils, but the values remained within physiological limits. Other parameters such as eosinophils, monocytes (Table 1), and basophils do not play a significant role in the normal wound healing process, and their variations were negligible (Sivanarayanan 2012, Madhu *et al.* 2013).

Normal glucose levels in rabbits range from 5.5 to 8.2 mmol/l, and hyperglycemia is common in rabbits under stressful conditions, including transportation, handling, and heat (Flecknell 2000). In our study, there were no significant variations in glucose levels, which can be attributed to favorable environmental conditions, suitable

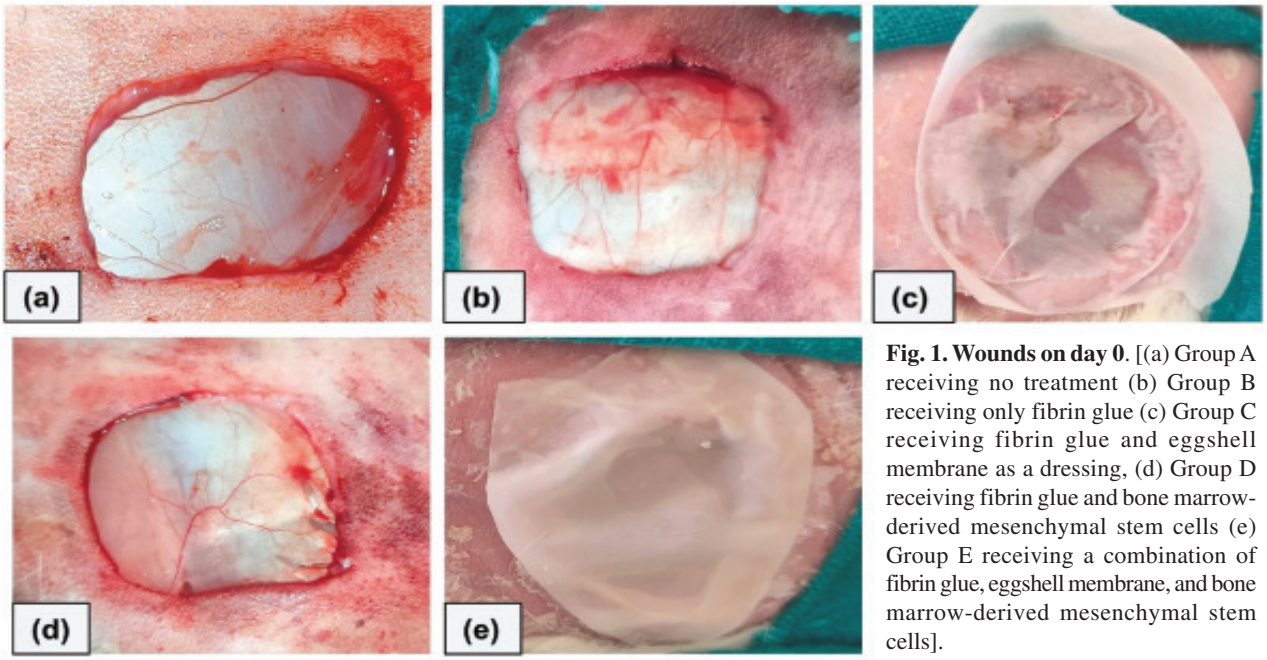

Fig. 1. Wounds on day 0. [(a) Group A receiving no treatment (b) Group B receiving only fibrin glue (c) Group C receiving fibrin glue and eggshell membrane as a dressing, (d) Group D receiving fibrin glue and bone marrow-derived mesenchymal stem cells (e) Group E receiving a combination of fibrin glue, eggshell membrane, and bone marrow-derived mesenchymal stem cells].

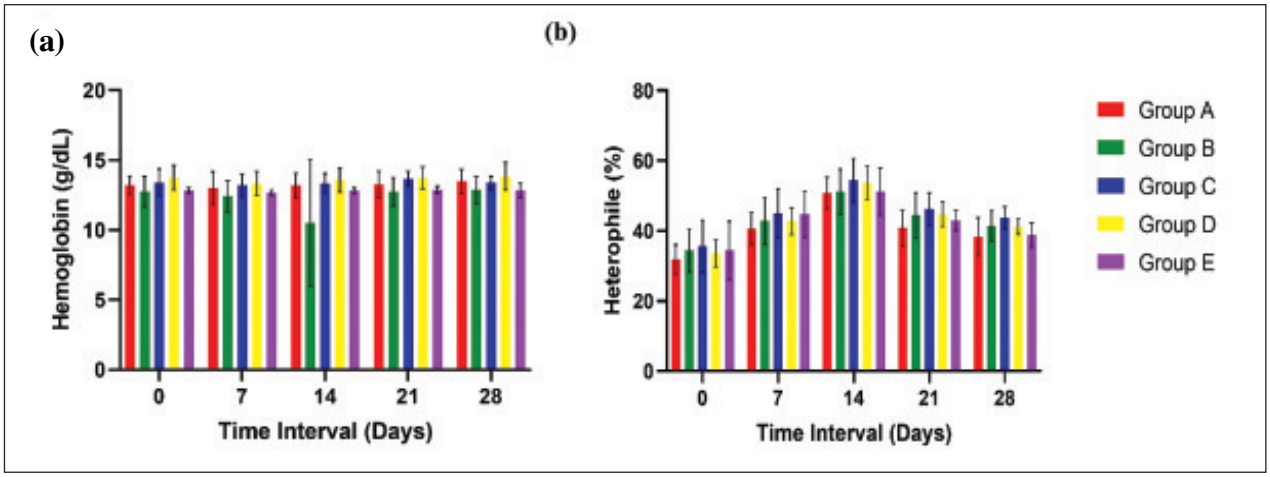

Fig. 2. (a) Values of haemoglobin (g/dL) in rabbits from different treatment groups. (b) Values of heterophile (%) in rabbits from different treatment groups [Mean ± SE].

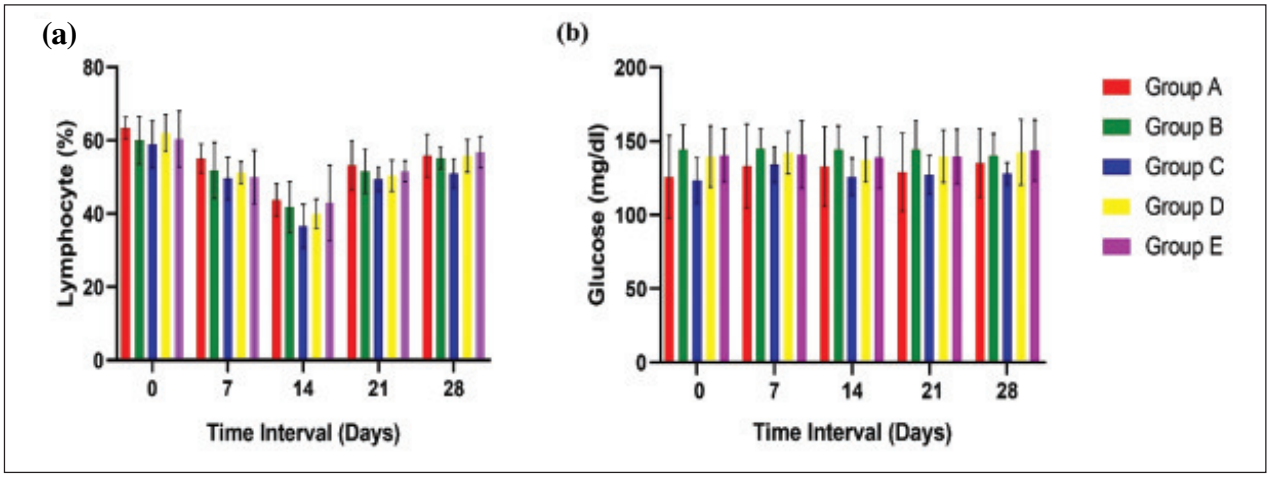

Fig. 3. (a) Values of lymphocyte (%) in rabbits from different treatment groups. (b) Values of glucose (mg/dL) in rabbits from different treatment groups [Mean ± SE].

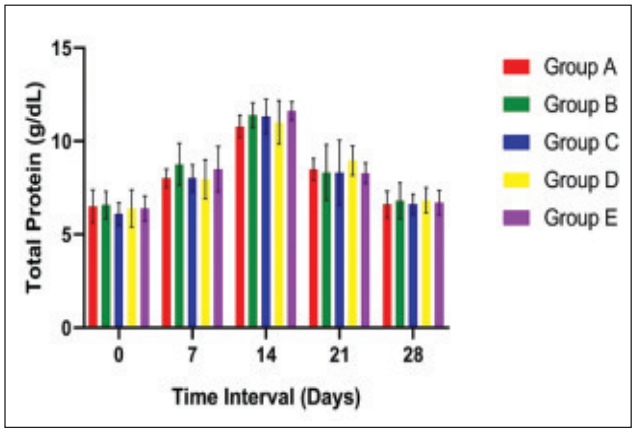

Fig. 4. Values of total protein (g/dL) in rabbits from different treatment groups [Mean ± SE].

animal housing conditions, and minimal effects of anesthesia (Fig 3b). Normal total protein (TP) values range from 4.9 to 7.1 g/dl, with an albumin-to-globulin (A: G) ratio of 0.7-1.89. Excessive compression during venipuncture can lead to falsely high TP readings (Flecknell 2000). A significant increase in TP was observed from day 7 to day 21 in all groups compared to day 0 values (Fig. 4) (Table 2). However, TP values started to decline in all groups after a gradual increase until day 14, eventually returning to normal levels by day 28. The gradual increase in TP during the initial phase of healing is likely linked to the body's physiological response to the healing process (Yazarlu *et al.* 2021).

The findings of this study shed light on the hematological changes associated with the use of ESM, FG, and BM-MSCs for full-thickness skin defects in rabbits. However,

it is important to note that this study focused solely on preliminary parameters. Future research should explore the evaluation of additional parameters such as stress/ antistress markers. Furthermore, further investigation is required to examine the immunological and toxicological effects associated with using ESM as a dressing agent.

Conclusion

There were no notable variations in clinical and hemato-biochemical parameters among the treatment groups, and the observed values remained within acceptable physiological ranges. The findings of this study indicate minimal interference between the treatment and wound healing in relation to hematocrit values. However, it is important to note that this study serves as a preliminary investigation, and further research is needed to fully explore the potential of ESM in promoting wound healing.

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