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Immunohistochemical Analysis of Vascular Endothelial Growth Factor (VEGF) Expression in Rat Wound Tissue Treated with Silk Biomaterials

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Abstract

Wound healing is a complex and intricate process that involves interaction of various growth factors and cytokines to promote optimal tissue regeneration. Healthy wound healing relies on the rate of formation of extracellular matrix (ECM) and angiogenesis process.VEGF is a critical signaling molecule that promote the growth of blood vessels influence the synthesis and organization of ECM components by acting as a chemotactic agent. This study focuses on inducing wounds in rat models, testing the healing efficiency of silk biomaterials by analyzing the VEGF biomarker using immunohistochemistry (IHC) techniques. The IHC data from captured images were analyzed using Olympic software. These biomarkers serve as characteristic features, objectively measured to gauge the progress of the biological processes in Immunohistochemistry (IHC) studies.The findings of this research shed light on the potential of Silver Nanoparticles loaded Silk Fibroin Gel (AgNp-SF Gel) enhances wound healing processes, particularly through the modulation of VEGF expression, offering valuable insights for the development of advanced wound care strategies.

Key Words: Biomaterial, Silk Fibrion, VEGF, Wound healing, Rat Animal study, Immunohistochemistry.

1. INTRODUCTION

Skin forms a self- renewing and self-repairing interface between the body and the environment. It provides an effective barrier against the microbial invasion and has properties that can protect against mechanical, chemical, osmotic, thermal and photo damage. Dermal wound healing is a complex process that requires interaction between cells, extra cellular matrix components, cytokines and growth factors (Shaw T.J and Martin P., 2009). Silk Fibroin (SF) is natural polymer extracted by Silkworm cocoons known for its wound healing efficiency (Zhang W., 2017) (Mazurek Ł., 2022). The healthy wound healing requires formation of granulation tissue that is made up of collagen and other proteins in the extracellular matrix (ECM) of the tissue. The Angiogenesis provide nutrients to the newly forming granulation stage and one of important growth factor involved is vascular endothelial growth factor (VEGF),a chemotactic agent attracting the immune cells to carry out healing process (Philip Bao et al., 2009). The present study focuses on the wound creation in the rat animal and checking the wound healing efficiency of Silk Fibroin Film (SF Film), Silver Nanoparticle added Silk Fibroin Film (AgNp SF-Film), Silk Fibroin Gel (SF Gel), Silver Nanoparticles added Silk Fibroin Gel (AgNp SF-Gel). The usage of silver nanoparticles in wound healing has received considerable attention due to emergence of antibiotic-resistant strains and low tendency to develop resistance (Beyth N et al ., 2015) (Paladini F et al., 2019).The efficiency of healing is estimated by the expression level of VEGF biomarker in the wound tissue using the Immunohistochemistry technique (IHC). The IHC data of the captured image analyzed in Olympic software. These Biomarkers are characteristic features and it is objectively measured as an indicator of biological process in IHC studies.In IHC immune staining method assists to identify the proteins (Antigens) in the tissue based on the principle of Antigen-antibody binding.

2. MATERIALS AND METHODS

2.1 Extraction and Purification of Silk Fibrion (SF)

High-quality Silk cocoons were selected and carefully cut into cube-shaped fragments. 5 grams of the cut cocoon pieces were subjected to a degumming process. This involved boiling them in a solution of 0.05% (w/v) Na_2CO_3 for 30 minutes followed by rinsing in pure distilled water thrice. Magnetic stirrer removes any residual sericin. The fibers were then air-dried overnight at 37°C. The silk fibers are dissolved in a 9M lithium bromide solution maintaining temperatures between 70°C to 80°C for 1 hour The solution dialysed against distilled water with MWCO of 3500 over a for 3 days to remove salts. To further purify the solution, centrifugation was carried out twice at 10000 rpm for 25 minutes. The resultant supernatant solution was devoid of aggregates, and preserved at a temperature of 4°C. The pure SF solution was convereted into SF film, AgNp SF-Film, SF Gel, AgNp SF-Gel following the established protocol (Sufia et al., 2023).

2.2 Rat Model for Wound Phase Analysis

The wound healing rate for the Silk fibroin (SF) were analysedusing the rat wound model. For this study thirty (30) male Wistar rats were used with 5 rats in 6 groups.The male Wister rats (250-300 gm) were acclimatized to new environment for a week under SPF (specific pathogen-free animal) facility with a 12:12 h light–dark cycle. All surgical procedures were conducted under the supervision of veterinary surgeons. The rats are randomly assigned into 6 groups with two time points:

7thday and 14th day. The grouping are: Negative control (D.H2o), Positive control (Betnovate ointment), SF film, AgNp SF-Film, SF Gel, AgNp SF-Gel. Prior to this the animal are treated with anesthetic drug, a cocktail of Ketamine and Xylene. After anaesthetization the hair on the back was shaved using new razor blade disinfected with 70% ethanol. Using the marker, the size of the rat skin to be cut is marked accordingly. The 1x1 cm full thickness wound incised in the area of the dorsal region (5cm from ear) of all the experimental animals. Negative control Group 1 (G1) wounds were kept open without any wound dressing material or without application of any ointment. While the Positive control Group 2 (G2) wounds were covered with commercially available Betnovate ointment. Group 3 (G3) wounds were covered with SF film. The Group (G4) wounds were covered with SF film with silver nanoparticles (AgNps-SF film). Sterile conditions were maintained throughout the treatment. The Group 5 (G5) wounds were treated with SF Gel. Group (G6) wounds were treated with SF gel with silver nanoparticles (AgNps-SF-gel). The wounds were covered with cotton gauze cloth and fixed by bandage to prevent dislodging of the dressing materials. The animals were kept in separate cages in an air-conditioned room (temperature, 23 ± 2°C; relative humidity, 50 ± 10 %) for 12 hr light- dark cycle and were fed with commercial rat feed and water until they were sacrificed. The Animals were sacrificed by CO_2 asphyxiation on the 7th day and 14th day from the creation of wound. The wound tissues aimed to perform Immunohistochemistry (IHC) were stored in 10% formalin for histological observation. The grouping of rat for testing the efficiency of blends made from silk biomaterial are listed in table 1.

Table 1: Grouping of Wister rat for Silk Biomaterial treatment

S.NO	TREATMENT	GROUPING
Group 1	No treatment	Negative Control
Group 2	Betnovate ointment	Positive Control
Group 3	SF Film	G3 group
Group 4	AgNps-SF film	G4 group
Group 5	SF Gel	G5 group
Group 6	AgNps-SF Gel	G6 group

2.3 Immunodetection of VEGF

For the immunodetection of VEGF, endogenous peroxidase in the deparaffinized (in hot air oven) and alcohol subjected rehydrated tissue sections were inactivated by incubating for 10 min in 3% H_2O_2. After antigen recovery, the tissue sections were then blocked with 3% bovine serum albumin in phosphate-buffered saline for duration of 1 h at 37 ^{0}C. The excess liquid is drained out followed by subjecting the sections with anti-VEGF polyclonal rabbit antibody (1:200 in 3% bovine serum albumin, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4^{o}C overnight. Then the slides were incubated with biotinylated secondary antibody (1: 400 in bovine serum albumin, Santa Cruz Biotechnology Inc.) for 2 hrs. For visualization of the antibody binding site were carried out by incubation with 5% diaminobenzidine in phosphate-buffered saline. The distribution and staining of positive cells for the VEGF marker done with Image-Pro Plus 6.0 software (Media Cybernetics Inc., Rockville, MD, USA) to analyze the integrated optical density (IOD) in quantity. There is direct relation between integrated optical density and intensity of VEGF marker, thus the higher the value of the IOD the higher the content of VEGF.

3. RESULTS

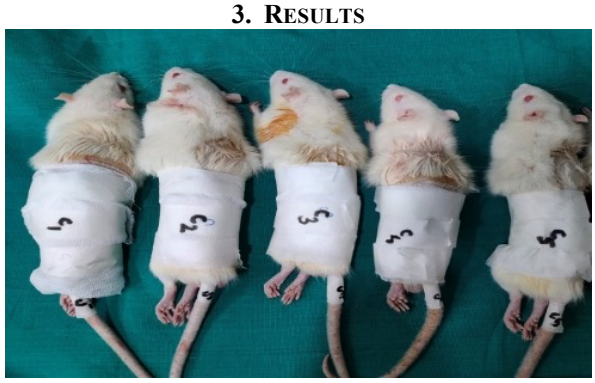


Figure 1: Batch of wound dressed Wistar rats

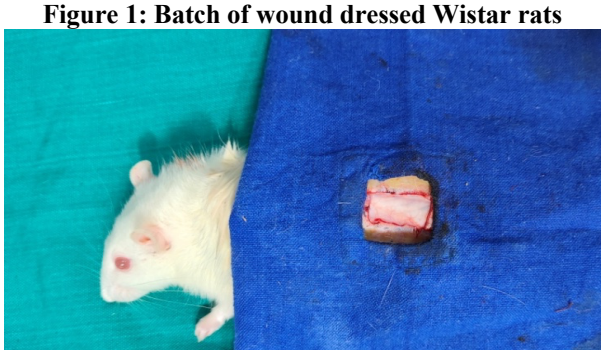


Figure 2: Creation of skin wound on the back of Wister rat

Figure 3: The healing efficiency observed in Ointment treated wound tissue (G2)

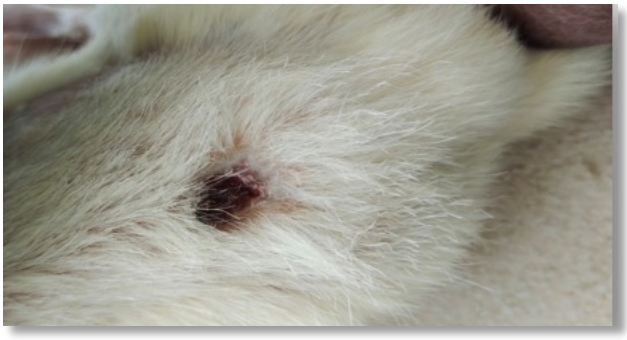


Figure 4 : The healing efficiency observed in SF-Film treated wound tissue (G3)

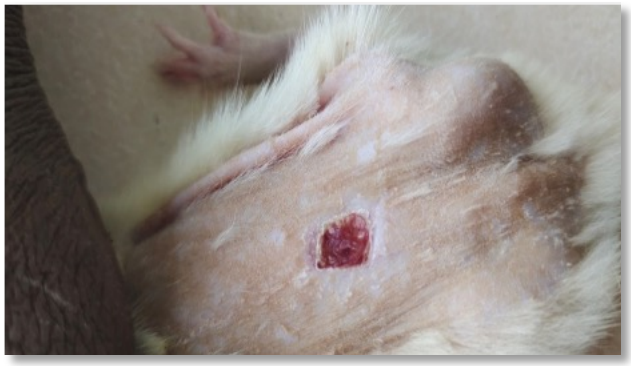


Figure 5: The healing observed in AgNp- SF Film treated wound tissue (G4)

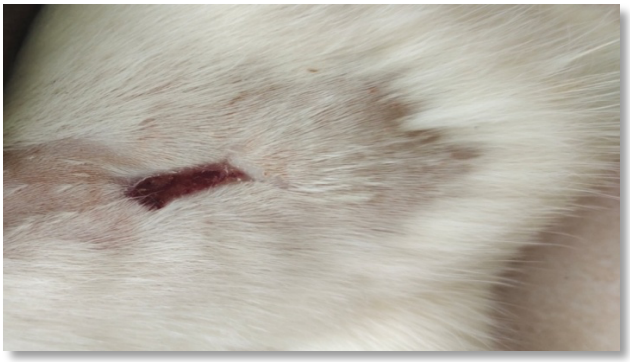


Figure 6: The healing observed in SF Gel treated wound tissue (G5)

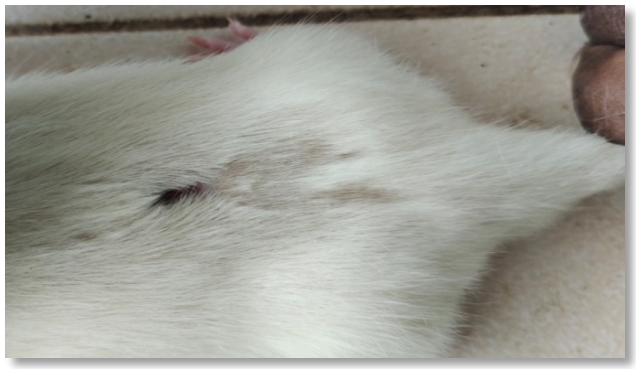
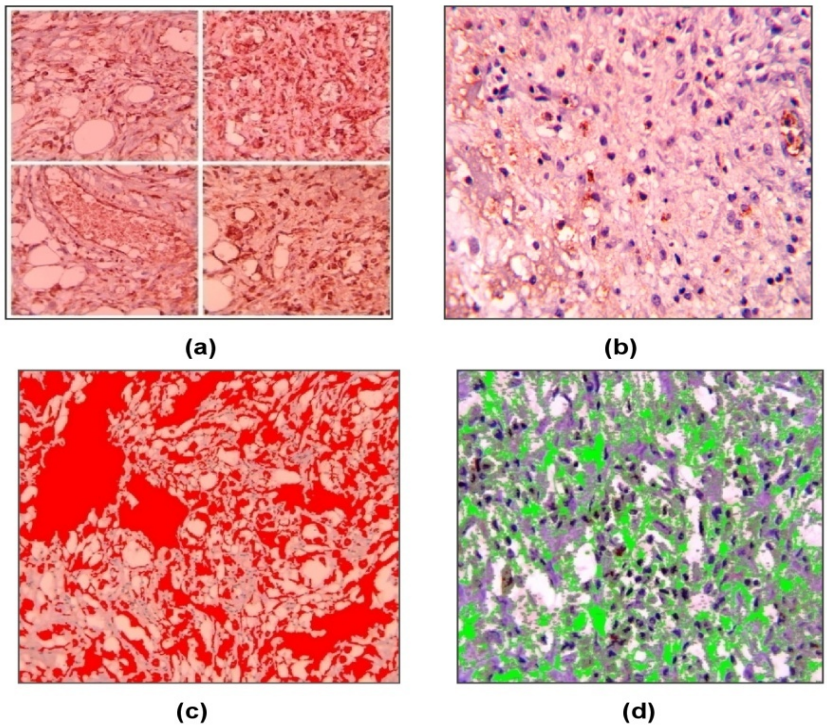
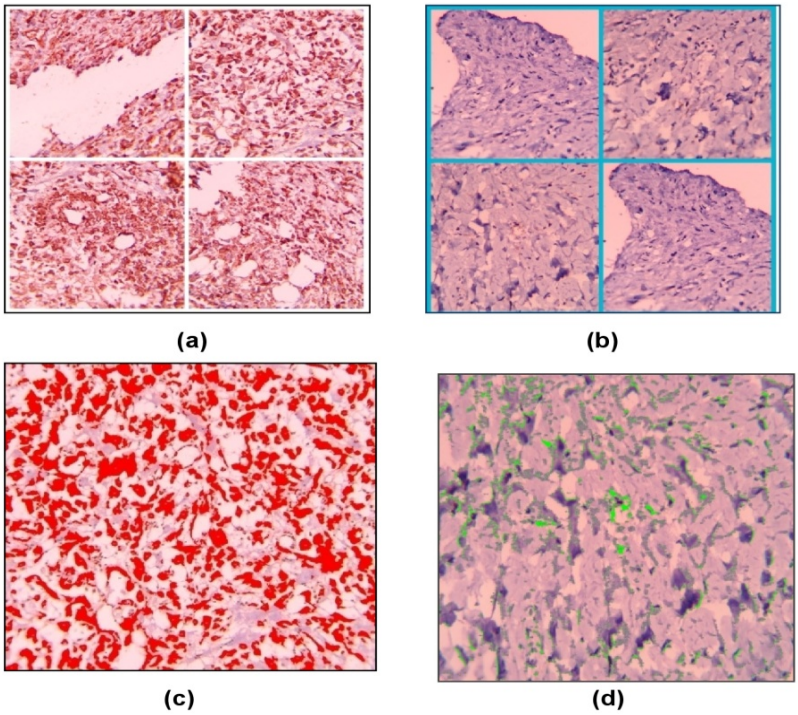


Figure 7: The healing efficiency observed in AgNps- SF Gel treated wound tissue (G6)

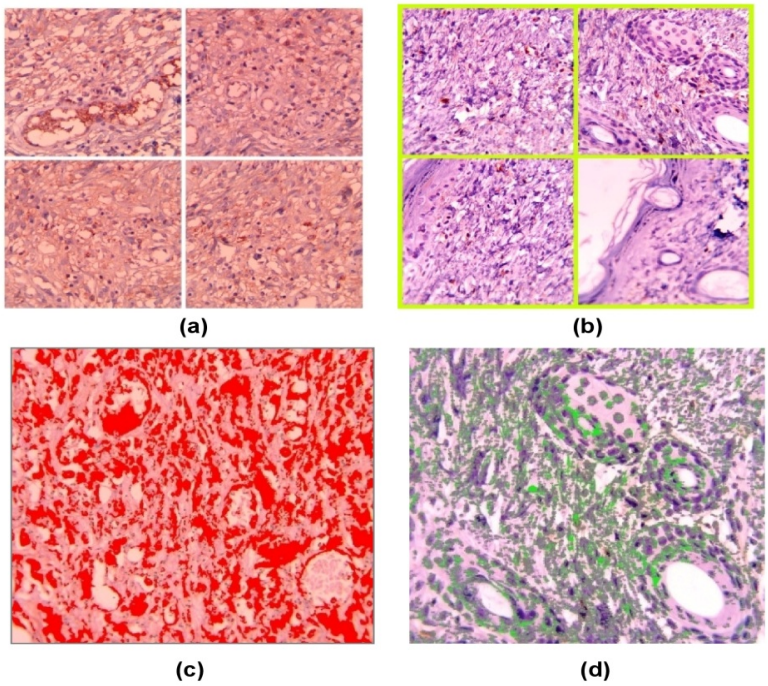


Picture 1 : Untreated wound tissue on 7th and 14th day of post operation.
(a)G1 negative control on 7th day
(b) G1 negative control on 14th day
(c) Analyzing of IoD on Image-Pro software on 7th day
(d) Analyzing of IoD on Image-Pro software on 14th day

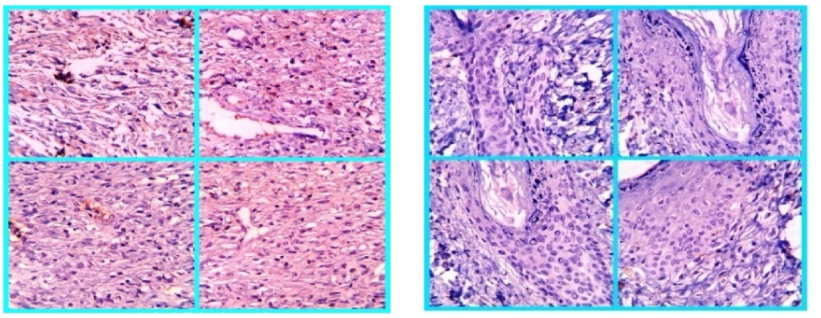
4. The Immunohistochemistry (IHC) Studies:



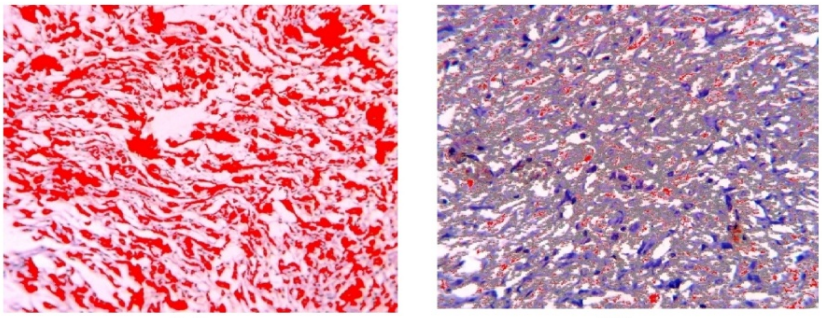
Picture. 2 : Ointment treated wound tissue on 7th and 14th day of post operation.
(a)G2 +ve control on 7th day
(b) G2 +ve control on 14th day
(c) Integrated optical density (IoD) using Image-Pro analysis on 7th day
(d) Integrated optical density (IoD) using Image-Pro analysis on 14th day



Picture 4 :AgNp-SF Film treated wound tissue on 7th&14th day of post operation.
(a)G4 treated with AgNp-SF Film on 7th day
(b) G4 treated with AgNp-SF Film on 14th day
(c) Analyzing of IoD on Image-Pro software on 7th day
(d) Analyzing of IoD on Image-Pro software on 14th day

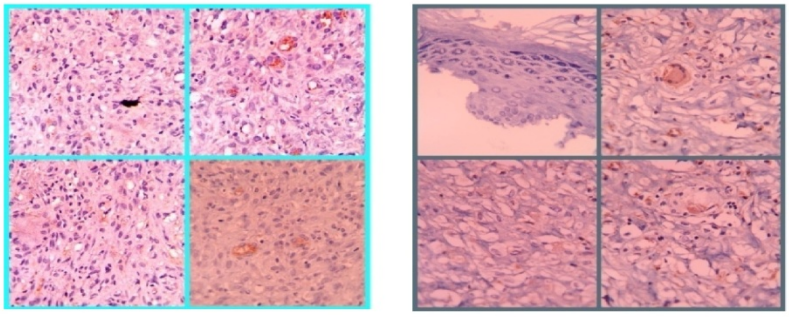


(a) (b)

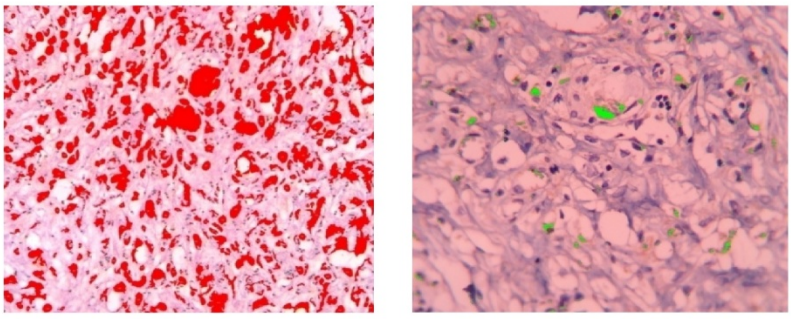


(c) (d)

Picture 5: SF Gel treated wound tissue on 7th and 14th day of post-operation.
(a)G5 treated with SF Gel on 7th day
(b) G5 treated with SF Gel on 14th day
(c) Analyzing of IoD on Image-Pro software on 7th day
(d) Analyzing of IoD on Image-Pro software on 14th day

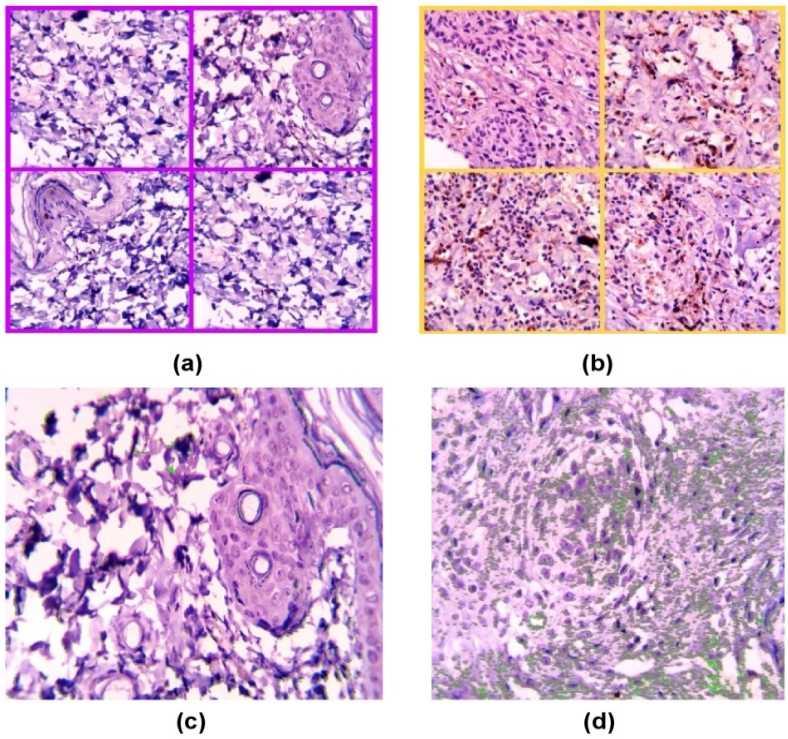


(a) (b)



(c) (d)

Picture 3: SFFilm treated wound tissue on 7th and 14th day of post operation.
(a)G3 treated with SF Film on 7th day
(b) G3 treated with SF Film on 14th day
(c) Analyzing of IoD on Image-Pro software on 7th day
(d) Analyzing of IoD on Image-Pro software on 14th day



Picture 6: AgNp-SF Gel treated wound tissue on 7th and 14th day of post operation.
(a)G6 treated with AgNp-SF Gel on 7th day
(b) G5 treated with AgNp-SF Gel on 14th day
(c) Analyzing of IoD on Image-Pro software on 7th day
(d) Analyzing of IoD on Image-Pro software on 14th day

Table 2 A: Area wise VEGF - IHC data per micro meter square scale for the SF biomaterials treated wound tissue on the 7th day.

Quantitative Analysis of Immunohistochemistry (7th Day - VEGF)			
Sno	**Name of Group**		**Area IHC (Micro meter square)**
1	Negative	G1	4120.919
2	Ointment	G2	1826
3	SF Film	G3	1122
4	AgNp-SF Film	G4	1501
5	SF Gel	G5	811
6	AgNp-SF Gel	G6	50.16

Table 2 B: Graphical representation for the Quantitative Analysis of Immunohistochemistry (IHC) for SF biomaterials treated wounds on 7th day.

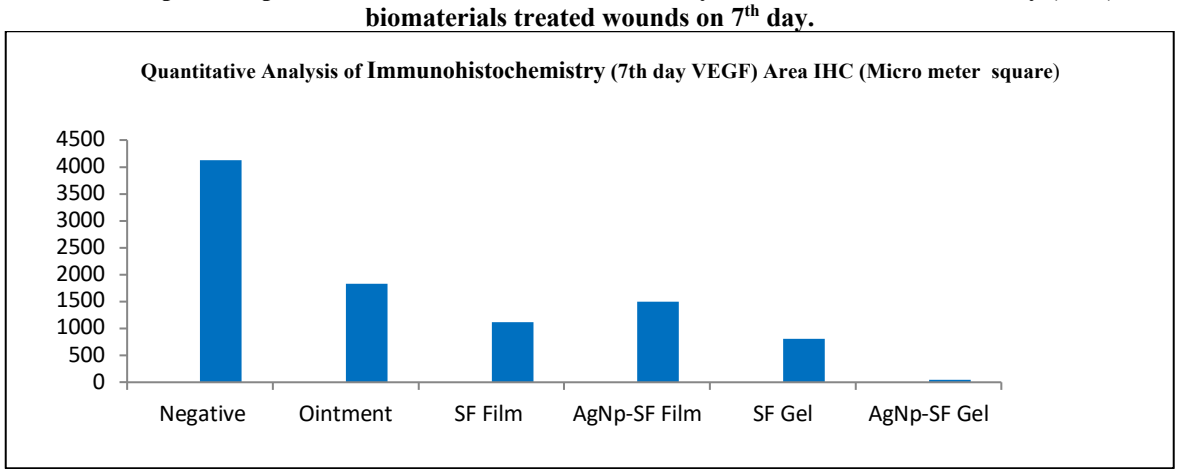
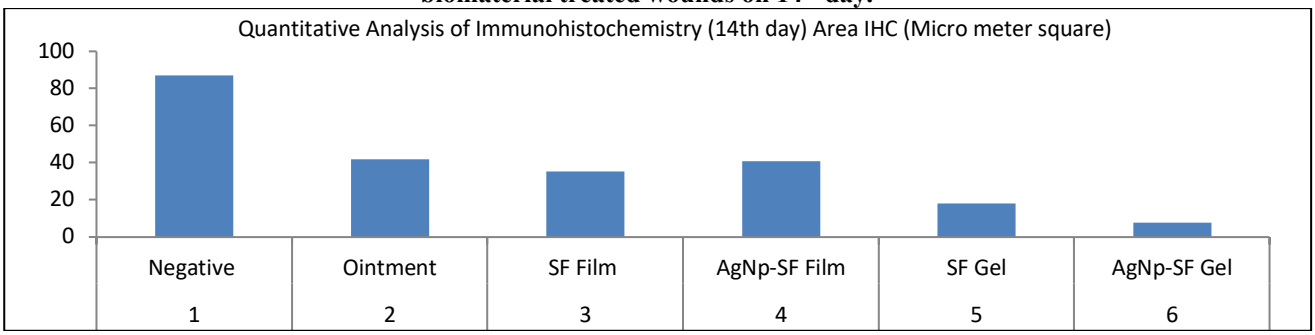


Table 3 A: Area-wise VEGF – Immunohistochemistry data per micro meter square scale for the SF biomaterial treated wound tissue on the 14th day.

Quantitative Analysis of Immunohistochemistry (14th Day - VEGF)			
S.No	**Name of Group**	**Group**	**Area IHC (Micro meter square)**
1	Negative	G1	86.9242
2	Ointment	G2	41.88
3	SF Film	G3	35.45
4	AgNp-SF Film	G4	40.88
5	SF Gel	G5	18.02
6	AgNp-SF Gel	G6	7.84

Table 3 B: Graphical representation for the quantitative analysis of Immunohistochemistry (IHC) for the SF biomaterial treated wounds on 14th day.



5. Discussions

The wound healing is a complex with overlapping stages starts from hemostasis angiogenesis and ends to scar formation (Gonzalez AC et al., 2016). Although there are numerous growth factors and cytokines released in response to injury. One among the most important pro-angiogenic growth factor involved in angiogenesis is vascular endothelial growth factor (VEGF). Probability of delay in wound healing is connected with the progression of angiogenesis which depends on the concentration of VEGF in the blood. Owing to the importance of VEGF, the IHC has been done on the rat tissue treated with SF film, AgNp SF-Film, SF Gel, AgNp SF-Gel as Silk Fibroin is well known for its healing efficiency (Sultan, M.Tet al., 2018). As per the results and observation of the 7th day wound tissues, the Ointment treated wound samples are at granulation stage with minimum to moderate number of blood capillaries (Picture 2), while untreated wound tissue or negative control are still proceeding from vascularisation to granulation tissue with matrix of capillaries surrounded by endothelial cells or VEGF positive (Picture 1a). The SF Film and AgNp-SF Film, both were present in contraction stage with minimum vasculature with moderate VEGF staining (Picture 3a& 4a). The SF- Gel and AgNp-SF Gel expressed mild to very low VEGF respectively (Picture 5a& 6a). The AgNp-SF Gel treated wounds are at granulation stage and proceeding to epithelization stage or remodelling stage with very low VEGF staining (Picture 6a). Thus overall performance of the SF AgNps-Gel treated wounds on 7th day was quite appreciable with its entrance in the remodelling or epithelization stage. Both the SF Gel based scaffold treated wounds showed mild to very low VEGF expression that marks the completion of neovascularisation and proceeding to remodelling phase.

With regard to the observations on the 14th day, Ointment treated wound tissue shown granulation stage and with overlapping epithelization phase (picture 1b). Mostly the 14th day wounds were preceeding to their epithelization stages with different intensities. While the SF Film treated wound found with connective tissue composed of fibrous tissue and remodeling can be appreciatedwith keratinization (Picture 3 b), while epithelization was going on in AgNp SF-Film wound (Picture 4 b). The SF Gel and AgNp SF-Gel both the treated wounds were at remodelling stage with dense connective tissue formation with epithelization but with different intensities in their respective performances (Picture 5 b &6 b).The graphical representation at Table 2 (a &b) and3(a & b) represent the area wise VEGF distribution per micro metre square scale for the 7th day and 14th day SF biomaterials treated wounds respectively. The distribution of VEGF in the AgNp SF-Gel wounds were comparatively very low with 50.16 μm^2 and 7.84 μm^2 emerging as best SF biomaterial over SF Films.While the AgNp SF-Film performance was not as expected. This data was analysed using the Olympic software. AgNp-SF Gel showed faster healing rate while AgNp-SF Film has shown less appreciable performance than its counterpart SF Film. The addition of AgNPs to SF film has increased the brittleness which might have hindered the release rate causing low healing performance (Fengchao Sun et al., 2023). Therefore, from the above results it is once again reiterated that the healing progression has been found in the order of AgNPs - SF Gel, SF Gel, SF Film and AgNPs-SF film. From the overall performance of the SF biomaterial, the Gel based treatments are appreciable in their performance showing faster healing rate in comparison with the SF Film or sheet form.

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