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# **Development of Biopolymer based Formulations for Haemostasis and Wound Healing under Civilian and Combat Operations**

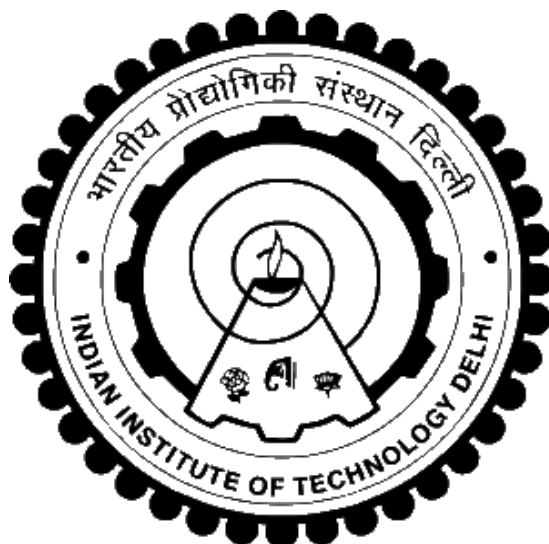
by

**Divya Tripathi**  
**Centre for Biomedical Engineering**

**Submitted**

**In fulfilment of the requirements of degree of Doctor of Philosophy**

**to the**



**INDIAN INSTITUTE OF TECHNOLOGY, DELHI**

**APRIL, 2022**

*Dedicated to my Family*

## CERTIFICATE

This is to certify that the thesis entitled “**Development of Biopolymer based Formulations for Hemostasis and Wound Healing under Civilian and Combat Operations**” being submitted by **Miss Divya Tripathi** to the **Indian Institute of Technology, Delhi** for the award of the degree of Doctor of Philosophy, in Biomedical Engineering is a record of the original bonafide research work carried out by her. **Miss Divya Tripathi** has worked under our guidance and supervision and has fulfilled the requirement for the submission of the thesis.

The results presented in this thesis are original and has not been submitted in partial or full, to any other university or institute for the award of any degree or diploma.

*Amit Kumar*  
2  
Dr. Amit Kumar, Scientist 'F'  
भारत सरकार, रक्षा मंत्रालय/ Govt. of India, Ministry of Defence  
Institute of Nuclear Medicine & Allied Sciences (DRDO)  
भारत सरकार, रक्षा मंत्रालय/ Govt. of India, Ministry of Defence  
S. K. Mazumdar Road, Delhi-110054

**Dr. Amit Kumar**  
Scientist F  
Institute of Nuclear Medicine and Allied Sciences,  
Defence Research and Development Organization,  
Delhi-110054

*H Singh*

**Prof. Harpal Singh**  
Professor  
Centre for Biomedical Engineering  
Indian Institute of Technology, Delhi  
Hauz khas, New Delhi-110016

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**Divya Tripathi**

## **ABSTRACT**

Haemorrhage is a serious medical emergency and one of the leading causes of disability and death of the military and civilian trauma. haemostatic materials have been extensively explored in the recent times for effective and rapid haemostasis, which includes alginate, chitosan, gelatine, collagen, zeolite, etc. These materials have limitations like poor adhesion, inefficient activation of wound healing factors, inflammation, and difficulty in removing the material from the wound site. Moreover, available formulations for haemostasis and wound healing are not stable in extreme environmental conditions like temperature and humidity therefore possess stability and storage issues. In case of visceral injuries, the lack of proper diagnosis and treatment remains one of the major challenges. Many formulations have demonstrated limited translational potential due to their toxic nature. Therefore, there is a need to develop improved formulations for visceral injuries. In the current research work, we have focused on the development of haemostatic biopolymer coated gauzes, bioactive composite scaffolds and treatment modalities for superficial and visceral organ injury management.

Primarily collagen and chitosan have haemostatic, tissue fix and wound healing properties, but the poor mechanical properties limit their application. Therefore, various concentrations of collagen (1–6%) / chitosan (1–2%) were used to develop biopolymer coated onto cotton gauzes, with and without glycerol as a plasticizer. Glycerol treated gauzes showed desired mechanical and adhesive property in comparison to chitosan/collagen coated gauzes alone. Developed gauzes were characterized using Differential Scanning Calorimetry (DSC), Thermal Gravimetric Analysis (TGA) and Fourier Transform Infrared Spectrophotometry (FTIR) to confirm the biopolymer coating and stability. Scanning electron microscopy (SEM) showed multilayer coating of the biopolymer on the gauzes. Surface Plasmon Resonance Assay confirmed that chitosan exhibited more binding affinity of 65 Response units (RU) in

comparison to collagen, which showed 55 RU with erythrocytes. Decrease in the value of plateletcrit and mean platelet volume confirmed platelet adhesion and aggregation over the surface of polymer coated dressings. Gamma Scintigraphy studies showed  $85 \pm 2\%$  formulation retention up to 12h at the wound site in comparison to  $40 \pm 3\%$  retention of the radiopharmaceutical alone. Collagen and chitosan coated gauze showed  $226 \pm 15$ s and  $179 \pm 12$ s haemostasis time, respectively, which was significantly less from  $506 \pm 15$ s in standard cotton gauze. Chitosan gauze showed faster wound healing in comparison to the collagen coated gauze.

In another approach, fabrication of three-dimensional composite scaffolds was carried out by lyophilization of variable concentrations of collagen and chitosan gel solutions. Fibrinogen and thrombin aerosol were deposited over the surface of scaffolds to enhance haemostasis and wound healing. Composite scaffolds were characterized using DSC, TGA, and FTIR to ascertain the aerosol deposition and stability. SEM showed multi-layered porosity with pore size of  $\sim 30 \mu\text{m}$  and mushroom like fibril growth of aerosol. A detailed investigation by Surface Plasmon Resonance confirmed higher binding affinity of collagen towards the human blood platelets and erythrocytes in comparison to chitosan and was found to increase with the increase in blood cell concentration from 480.8 to 886.4 RU for erythrocytes. Scaffolds showed higher binding response for platelets than erythrocytes, while fibrinogen and thrombin showed no or limited interaction. Highest blood sorption of  $83 \pm 4\%$  was observed in case of aerosol deposited scaffolds. Aerosol deposited scaffolds showed minimum clotting time of  $20 \pm 3$ s and bleeding time of  $38 \pm 4$ s, which was significantly lower compared to the scaffolds without aerosol treatment. Fibrinogen and thrombin aerosol deposited composite scaffolds with 2:1 concentration of chitosan/collagen showed complete wound contraction by day 14, while 50% was observed in case of the control group. *In vivo* studies revealed that chitosan had a crucial

role in the inflammatory phase, while collagen played an important role in the proliferation and maturation phase.

For the management of visceral injuries, we have validated the animal models for evaluation of developed formulation for acid induced and radiation induced injury generally involved in civilian/combat conditions. The first model was acid induced injury; evaluation of the injury was done using non-invasive method that is Fluorodeoxyglucose (*FDG*) uptake by Positron Emission Tomography Scan (PET). Group 1 animals (Control group) showed only  $20\pm 3\%$  of *FDG* accumulation which is due to minimal or no inflammation in the lungs. On the other hand, lungs of second group animals showed significantly increased  $\sim 69\pm 3\%$  *FDG* uptake which is probably due to severe inflammation and no significant change in the *FDG* uptake was observed till 21 days. In the third group of animals, *FDG* uptake was significantly decreased from  $\sim 69\pm 3\%$  to  $\sim 35\pm 3\%$  within 21 days. Decrease in the inflammation after the collagen peptide treatment confirms, that collagen peptide plays the vital role in reducing the inflammation and helps in healing of the pulmonary tissue. The second animal model was radiation induced lung injury which was developed in small animal model by controlled radiation exposure with specificity and accuracy. Overcoming all the restrictions, a device was designed for organ specific radiation induced injury with accuracy in small animal models. Novel features of the device include, organ specific radiation exposure, transparent design allows better visualization and control, uniformity in dose delivery from animal to animal. Comparison between the irradiated organs with the non-irradiated organs gave the conformation about the accuracy and efficiency of the device. Developed animal models were used for the analysis of therapeutic effect of collagen peptide at different time interval using various routes of administration. The present study demonstrates the radio therapeutic behaviour of collagen peptide when administered via different routes (oral and nebulizer), in the lungs of irradiated rodent model. To reveal the efficacy of our formulation in inhibiting

radiation induced pneumonitis and fibrosis, a variety of assays were performed. Biochemical parameters TNF- $\alpha$ , IL-6, CRP and ferric antioxidant levels showed the visceral wound healing effect of collagen peptide. Pre-treated Group-3(oral treatment) and Group-4(nebulizer treatment) showed declined concentration of IL-6 that was  $0.49 \pm 0.2$  ng/ml and  $0.38 \pm 0.3$  ng/ml respectively. The concentration of ferric antioxidants in the radiation control group (Group-5) was very high with the value of  $312.02 \pm 100$  u/M, lowest concentration of  $54.55 \pm 300$  u/M was observed in Group-4(treatment via nebulization). The concentration of C-reactive protein was highest in the Group-5 with the concentration of  $89.57 \pm 10$   $\mu$ /ml while lowest was observed in Group-4 with concentration of  $74.64 \pm 5$   $\mu$ /ml. On the other hand, histological studies reveal anti-inflammatory potential of collagen peptide. Present studies helped in both diagnoses and treatment of visceral injuries. The entire data generated during the current study collectively explained that collagen peptide has significant potential of minimizing radiation induced lung fibrosis and pneumonitis.

## सार

रक्तस्राव एक गंभीर चिकित्सा आपात स्थिति है और सैन्य और नागरिक आघात की विकलांगता और मृत्यु के प्रमुख कारणों में से एक है। हाल के दिनों में प्रभावी और तीव्र हेमोस्टेसिस के लिए हेमोस्टैटिक सामग्री का व्यापक रूप से पता लगाया गया है, जिसमें एलिगेट, चिटोसिन, जिलेटिन, कोलेजन, जिओलाइट आदि शामिल हैं। इन सामग्रियों में खराब आसंजन, घाव भरने वाले कारकों की अक्षम सक्रियता, सूजन, और घाव स्थल से सामग्री को हटाने में कठिनाई जैसी सीमाएं हैं। इसके अलावा, हेमोस्टेसिस और घाव भरने के लिए उपलब्ध फॉर्मूलेशन तापमान और आर्द्रता जैसी अत्यधिक पर्यावरणीय परिस्थितियों में स्थिर नहीं होते हैं इसलिए स्थिरता और भंडारण के मुद्दे होते हैं। आंत की चोटों के मामले में, उचित निदान और उपचार की कमी प्रमुख चुनौतियों में से एक बनी हुई है। कई योगों ने अपनी विषाक्त प्रकृति के कारण सीमित अनुवाद क्षमता का प्रदर्शन किया है। इसलिए, आंत की चोटों के लिए उपयुक्त फॉर्मूलेशन विकसित करने की भी आवश्यकता है। वर्तमान शोध कार्य में, हम हेमोस्टैटिक बायोपॉलिमर कोटेड गॉज, बायोएक्टिव कम्पोजिट स्कैफोल्ड्स और आंत के अंग चोट प्रबंधन के लिए उपचार के तौर-तरीकों के विकास पर ध्यान केंद्रित कर रहे हैं। मुख्य रूप से कोलेजन और चिटोसिन में हेमोस्टैटिक, ऊतक फिक्स और घाव भरने के गुण होते हैं, लेकिन खराब यांत्रिक गुण उनके आवेदन को सीमित करते हैं। इसलिए, प्लास्टिसाइजर के रूप में ग्लिसरॉल के साथ और बिना बायोपॉलिमर लेपित धुंध विकसित करने के लिए कोलेजन (1-6%) और चिटोसिन (1-2%) के विभिन्न सांद्रता का उपयोग किया गया था। ग्लिसरॉल से उपचारित गॉज ने अकेले पॉलीमर कोटेड गॉज की तुलना में वांछित यांत्रिक और चिपकने वाला गुण दिखाया। बायोपॉलिमर कोटिंग और स्थिरता की पुष्टि करने के लिए डिफरेंशियल स्कैनिंग कैलोरीमेट्री, थर्मल ग्रेविमेट्रिक एनालिसिस और फूरियर ट्रांसफॉर्म इन्फ्रारेड स्पेक्ट्रोफोटोमेट्री का उपयोग करके विकसित गॉज की विशेषता थी। स्कैनिंग इलेक्ट्रॉन माइक्रोस्कोपी ने धुंध पर बायोपॉलिमर के बहुपरत कोटिंग को दिखाया। सरफेस प्लास्मोन रेजोनेंस परख ने पुष्टि की कि चिटोसिन ने कोलेजन की तुलना में 65 रिस्पांस यूनिट्स (आरयू) की अधिक बाध्यकारी आत्मीयता प्रदर्शित की, जिसमें एरिथ्रोसाइट्स के साथ 55 आरयू दिखाया गया। प्लेटलेट क्रिट के मूल्य में कमी और बहुलक लेपित ड्रेसिंग की सतह पर प्लेटलेट आसंजन और एकत्रीकरण की पुष्टि की गई प्लेटलेट मात्रा का मतलब है गामा स्कैनिंग ग्राफी अध्ययनों ने अकेले रेडियोफार्मास्युटिकल के  $40 \pm 3\%$  प्रतिधारण की तुलना में घाव स्थल पर 12 घंटे तक  $85 \pm 2\%$  फॉर्मूलेशन प्रतिधारण दिखाया। कोलेजन और चिटोसिन लेपित धुंध ने क्रमशः  $226 \pm 15$  और  $179 \pm 12$  एस हेमोस्टेसिस समय दिखाया, जो मानक धुंध में  $506 \pm 15$  से काफी कम था। कोलेजन लेपित धुंध की तुलना में चिटोसिन धुंध ने तेजी से घाव भरने को दिखाया।

दूसरे, कोलेजन और चिटोसिन जेल समाधानों के चर सांद्रता के लियोफिलिजेशन द्वारा त्रि-आयामी समग्र मचानों का निर्माण किया गया था। हेमोस्टेसिस और घाव भरने को बढ़ाने के लिए स्कैफोल्ड की सतह पर फाइब्रिनोजेन और थ्रोम्बिन एरोसोल जमा किए गए थे। एरोसोल जमाव और स्थिरता का पता लगाने के लिए कम्पोजिट स्कैफोल्ड्स को डिफरेंशियल स्कैनिंग कैलोरीमेट्री, थर्मोग्रेविमेट्रिक एनालिसिस और फूरियर ट्रांसफॉर्म इन्फ्रारेड स्पेक्ट्रोफोटोमीटर का उपयोग करने की विशेषता थी। स्कैनिंग इलेक्ट्रॉन माइक्रोस्कोप ने  $\sim 30$  माइक्रोन के छिद्र आकार के साथ बहुस्तरीय संरचना और एरोसोल के फाइब्रिल जैसे मशरूम की वृद्धि दिखाई। सरफेस प्लास्मोन रेजोनेंस द्वारा की गई एक विस्तृत जांच ने चिटोसिन की तुलना में मानव रक्त प्लेटलेट्स और एरिथ्रोसाइट्स के प्रति कोलेजन की उच्च बाध्यकारी आत्मीयता की पुष्टि की और एरिथ्रोसाइट्स के लिए रक्त कोशिका एकाग्रता में 480.8 से 886.4 आरयू की वृद्धि के साथ वृद्धि पाई गई। स्कैफोल्ड्स ने एरिथ्रोसाइट्स की तुलना में प्लेटलेट्स के लिए उच्च बाध्यकारी प्रतिक्रिया दिखाई, जबकि फाइब्रिनोजेन और थ्रोम्बिन ने कोई या सीमित बातचीत नहीं दिखाई। एरोसोल जमा मचान के मामले में  $83 \pm 4\%$  का उच्चतम रक्त सोखना देखा गया था। एरोसोल जमा किए गए मचानों ने न्यूनतम थक्के समय  $20 \pm 3$  एस और रक्तस्राव का समय  $36 \pm 4$  एस दिखाया, जो एरोसोल उपचार के बिना मचानों की तुलना में काफी कम था। चिटोसिन/कोलेजन के 2:1 सांद्रण के साथ एरोसोल जमा मिश्रित मचानों

ने 14 दिन तक घाव का पूर्ण संकुचन दिखाया, जबकि नियंत्रण समूह के मामले में 50% देखा गया। विवो अध्ययनों से पता चला है कि भड़काऊ चरण में चिटोसिन की महत्वपूर्ण भूमिका थी, जबकि कोलेजन ने प्रसार और परिपक्वता चरण में महत्वपूर्ण भूमिका निभाई थी।

आंत की चोटों के प्रबंधन के लिए, हमने एसिड प्रेरित और विकिरण प्रेरित चोट के लिए विकसित फॉर्मूलेशन के मूल्यांकन के लिए पशु मॉडल को मान्य किया है। पहला मॉडल एसिड प्रेरित चोट था; चोट का मूल्यांकन गैर-आक्रामक विधि का उपयोग करके किया गया था जो कि पॉज़िट्रॉन एमिशन टोमोग्राफी स्कैन (पीईटी) द्वारा फ्लूरोडॉक्सीग्लुकोज (एफडीजी) है। समूह 1 के जानवरों (नियंत्रण समूह) ने एफडीजी संचय का केवल  $20 \pm 3\%$  दिखाया जो फेफड़ों में न्यूनतम या कोई सूजन नहीं होने के कारण होता है। दूसरी ओर, दूसरे समूह के जानवरों के फेफड़ों में काफी वृद्धि हुई  $\sim 69 \pm 3\%$  एफडीजी तेज जो शायद गंभीर सूजन के कारण है और एफडीजी तेज में 21 दिनों तक कोई महत्वपूर्ण परिवर्तन नहीं देखा गया था। जानवरों के तीसरे समूह में, 21 दिनों के भीतर एफ डीजी तेज  $\sim 69 \pm 3\%$  से  $\sim 34 \pm 3\%$  तक काफी कम हो गया था। कोलेजन पेप्टाइड उपचार की पुष्टि के बाद सूजन में कमी, कोलेजन पेप्टाइड सूजन को कम करने में महत्वपूर्ण भूमिका निभाता है और फुफ्फुसीय ऊतक के उपचार में मदद करता है। दूसरा पशु मॉडल विकिरण प्रेरित फेफड़ों की चोट था जिसे विशिष्टता और सटीकता के साथ नियंत्रित विकिरण जोखिम द्वारा छोटे पशु मॉडल में विकसित किया गया था। सभी प्रतिबंधों को पार करते हुए, छोटे जानवरों के मॉडल में सटीकता के साथ अंग विशिष्ट विकिरण प्रेरित चोट के लिए एक उपकरण तैयार किया गया था। डिवाइस की नई विशेषताओं में शामिल हैं, अंग विशिष्ट विकिरण जोखिम, पारदर्शी डिजाइन बेहतर दृश्य और नियंत्रण की अनुमति देता है, पशु से पशु तक खुराक वितरण में एकरूपता।

गैर-विकिरणित अंगों के साथ विकिरणित अंगों के बीच तुलना ने उपकरण की सटीकता और दक्षता के बारे में रचना दी। प्रशासन के विभिन्न मार्गों का उपयोग करते हुए अलग-अलग समय अंतराल पर कोलेजन पेप्टाइड के चिकित्सीय प्रभाव के विश्लेषण के लिए विकसित पशु मॉडल का उपयोग किया गया था। वर्तमान अध्ययन विकिरणित कृतक मॉडल के फेफड़ों में विभिन्न मार्गों (मौखिक और छिटकानेवाला) के माध्यम से प्रशासित होने पर कोलेजन पेप्टाइड के रेडियो चिकित्सीय व्यवहार को प्रदर्शित करता है। विकिरण प्रेरित न्यूमोनाइटिस और फाइब्रोसिस को रोकने में हमारे सूत्रीकरण की प्रभावकारिता को प्रकट करने के लिए, विभिन्न प्रकार के परीक्षण किए गए। जैव रासायनिक पैरामीटर TNF- $\alpha$ , IL-6, CRP और फेरिक एंटीऑक्सिडेंट स्तरों ने कोलेजन पेप्टाइड के आंत के घाव भरने के प्रभाव को दिखाया। पूर्व-उपचार समूह -3 (मौखिक उपचार) और समूह -4 (नेबुलाइजर उपचार) ने आईएल -6 की घटी हुई एकाग्रता को क्रमशः  $0.49 \pm 0.02\%$  एनजी/एमएल और  $0.38 \pm 0.02\%$  एनजी/एमएल दिखाया। विकिरण नियंत्रण समूह (समूह -5) में फेरिक एंटीऑक्सिडेंट की सांद्रता  $312.02 \pm 0.02\%$  यू/एम के मूल्य के साथ बहुत अधिक थी, समूह -4 (नेबुलाइजेशन के माध्यम से उपचार) में  $54.55 \pm 0.02\%$  यू/एम की सबसे कम एकाग्रता देखी गई थी। सी-रिएक्टिव प्रोटीन की सांद्रता  $19.96 \pm 0.01\%$  /एमएल की सांद्रता के साथ समूह 5 में सबसे अधिक थी, जबकि सबसे कम  $78.64 \pm 0.02\%$  /एमएल की एकाग्रता के साथ समूह 4 में देखी गई थी। दूसरी ओर, ऊतकीय अध्ययनों से कोलेजन पेप्टाइड की सूजन-रोधी क्षमता का पता चलता है। वर्तमान अध्ययनों ने आंत की चोटों के निदान और उपचार दोनों में मदद की। वर्तमान अध्ययन के दौरान उत्पन्न संपूर्ण डेटा ने सामूहिक रूप से समझाया कि कोलेजन पेप्टाइड में विकिरण प्रेरित फेफड़े के फाइब्रोसिस और न्यूमोनिटिस को कम करने की महत्वपूर्ण क्षमता है।

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## **LIST OF ABBREVIATIONS**

<b>TCCC</b>	Tactical combat causality care
<b>CCM</b>	Combat Casualty Care and Management
<b>EMS</b>	Emergency medical services
<b>ECM</b>	Extracellular matrix
<b>ALI</b>	Acute lung injury
<b>SPR</b>	Surface Plasmon Resonance
<b><math>^{99m}\text{TcO}_4^-</math></b>	Technetium 99m pertechnetate
<b>NHS</b>	N-hydroxy succinimide
<b>EDC</b>	N-ethyl-N-dimethylaminopropyl carbodiimide
<b>PBS</b>	Phosphate buffered saline
<b>HCL</b>	Hydrochloric acid
<b>CBC</b>	Complete blood count
<b>HGB</b>	Haemoglobin level
<b>PTL</b>	Platelets
<b>HCT</b>	Haematocrit
<b>MCV</b>	Mean corpuscular haemoglobin
<b>MCHC</b>	Mean corpuscular haemoglobin concentration
<b>RDW</b>	Red cell distribution width
<b>PCT</b>	Plateletcrit
<b>MPV</b>	Mean Platelet volume
<b>PDW</b>	Platelet distribution width
<b>EDTA</b>	Ethylene diamine tetra acetic acid
<b>FDG</b>	Fluorodeoxyglucose

<b>IL-6</b>	Interleukin -6
<b>TNF-<math>\alpha</math></b>	Tumour necrosis factor- $\alpha$
<b>CRP</b>	C-Reactive protein
<b>FRAP</b>	Ferric reducing antioxidant power
<b>MOF</b>	Multi organ failure
<b>ARDS</b>	Acute respiratory distress syndrome
<b>ATLS</b>	Advanced trauma life support
<b>MTF</b>	Medical treatment facility
<b>PDGF</b>	Platelet derived growth factor
<b>EGF</b>	Epidermal growth factor
<b>GAGs</b>	Glycosaminoglycans
<b>CPCSEA</b>	Committee for the purpose of control and supervision of experiments on animals