

## Formulation and Evaluation of Nanosilver Loaded Silk Fibroin Topical Gel

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### ABSTRACT

The skin is providing barrier to the outside protecting the body from infection, radiation, and extremes of temperature. There are various types of wounds which can damage the skin including abrasions, lacerations, rupture injuries, punctures, and penetrating wounds.

Many of the wounds are superficial requiring local first aid including cleansing and dressing.

But some wounds are deeper and need medical attention to prevent infection and loss of function, due to damage to underlying structures like bone, muscle, tendon, arteries and nerves.

The purpose of medical care for wounds is to prevent complications and function. While important, cosmetic results are not the primary consideration for wound repair.

Wound healing, a common clinical entity contemporary to human beings, most often susceptible to microbial infection. The ultimate goal for wound healing is a speedy recovery with minimal scarring and maximal function.

Different approaches such as use of antimicrobials and cell growth promoting agents have been used for achieving the set goal. Silk fibroin, a cell growth promoter has been used as wound healing agent.

Out of the present antimicrobial agents silver nanoparticles exhibit broad spectrum of anti-microbial activity. The mono dispersed silver nanoparticles with narrow particle size distribution are more effective anti bacterial agents, because of high surface/volume fraction, so that large populations of silver atoms are in direct contact with the environment. Silver Nanoparticles are non toxic to human cell to the tune of 350µg/day.

### INTRODUCTION

#### 1.1 Wound

In medicine, a wound is a type of injury in which skin is torn, cut or punctured (an open wound), or in which blunt force trauma causes a contusion (a closed wound). In pathology, it refers to a sharp injury which damages the dermis of the skin<sup>1</sup>.

##### 1.1.1 Classification

The wounds can be classified into two types as open wounds and closed wounds.

##### a. Open Wounds

An open wound is a type of injury in which skin is torn, cut or punctured and surrounding tissues are exposed. Most commonly open wounds are susceptible to infection from pathogens all around and should be cared for well. Open wounds can be classified according to the object that caused the wound. The most common causative organisms associated with open wound infections include *Streptococcus pyogenes*, *Staphylococcus aureus*/MRSA, Enterococci and *Pseudomonas aeruginosa*<sup>1,2</sup>.

##### b. Closed Wounds

1. Closed wounds range from bruises or hematomas to crushing injuries. Different types of closed wounds have

one thing in common: the skin remains intact and is not broken. If the skin is not broken, the risk of infection is lower than it is for an open wound. Closed wounds typically are caused by a blunted impact, which ruptures a blood vessel or capillary under the skin. The blood oozes out, but typically stop bleeding within about 30 seconds after the injury occurs. As body reabsorbs the blood, the skin shall discolor<sup>1,2</sup>.

##### 1.1.2. Risk factors

The risk factors involved in the wounds can be summarized as follows:-

##### a. Cellulitis:

Cellulitis is a diffuse inflammation of connective tissue with severe inflammation of dermal and subcutaneous layers of skin. Cellulitis is caused by a type of bacteria entering the skin, usually by the way of a cut, abrasion or break in the skin. This break does not need to be visible. Group A Staphylococcus and *Streptococcus* are the most common of these bacteria, and which are part of the normal flora of the skin but cause no actual infection while on the skin's outer surface. The typical symptom of cellulitis is an area which is red, hot, and tender<sup>3</sup>.

##### b. Necrotizing subcutaneous Infection:

Necrotizing soft tissue infection is a rare but very severe

type of bacterial infection that can destroy the skin, muscles, and underlying tissue. Necrotizing refers to something that causes tissue death. A very severe and usually deadly form of necrotizing soft tissue infection is due to *Streptococcus pyogenes*, which is sometimes called "flesh-eating bacteria."

Necrotizing soft tissue infection develops when the bacteria enters the body, usually through a minor cut. The bacteria begins to grow and releases harmful substances (toxins) that break down materials in the tissue, that rapidly spreads the bacteria, leads to widespread effects such as shock.

The first sign of infection may be a small, reddish, painful spot or inflammation on the skin. This quickly changes to a very painful bronze- or purple-colored patch that grows rapidly. The center become black and die off. The skin may break open and fluid oozes out. The wound may quickly grow in less than an hour. Symptoms may include general ill feeling, sweating, nausea, dizziness, fever and finally shock. Without treatment, death may occur rapidly<sup>3,4</sup>.

#### **c. Gas Gangrene:**

Gas gangrene (also known as "Clostridial myonecrosis") is a bacterial infection that produces gas within tissues in gangrene. It is a lethal form of gangrene usually caused by *Clostridium perfringens* bacteria. It is a medical emergency. Gas gangrene is caused by exotoxin-producing Clostridial species (most often *Clostridium perfringens*, and *C. novyi* but less commonly *C. septicum* or *C. ramnosum*), which are mostly found in soil but also found as normal gut flora, and other anaerobes (e.g. *Bacteroides* and anaerobic streptococci)<sup>4</sup>. Symptoms include:

- Air under the skin (subcutaneous emphysema)
- Blisters filled with brown-red fluid
- Drainage from the tissues, foul-smelling brown-red or bloody fluid (serosanguineous discharge)
- Increased heart rate (tachycardia)
- Moderate to high fever
- Ordinary to severe pain around a skin injury
- Pale skin color, later becoming dusky and changing to dark red or purple
- Swelling around a skin injury
- Sweating
- Vesicle formation, combining into large blisters
- Yellow color to the skin (jaundice)

#### **1.1.3. Wound Healing**

Wound healing, or wound repair, is the body's natural process of regenerating dermal and epidermal tissue. When an individual is wounded by a set of events takes place in a predictable fashion to repairs the damage. These events overlap in time and must be artificially

categorized into separate steps: the inflammatory, proliferative, and remodeling phases (Some authors consider healing to take place in four stages, by splitting of different parts inflammation or proliferation into separate steps)<sup>5</sup>.

In the inflammatory phase, bacteria and debris are phagocytized and removed. The factors are released that cause the migration and division of cells involved in the proliferative phase<sup>5</sup>.

The proliferative phase is characterized by granulation tissue formation, collagen deposition, angiogenesis, epithelization, and wound contractions. In angiogenesis, new blood vessels grows from endothelial cells. In granulation tissue formation, fibroplasia, fibroblasts grow and forms a new, provisional extracellular matrix (ECM) by excreting collagen and fibronectin<sup>6</sup>.

In epithelization epithelial cells crawl across the wound bed to cover it. In contraction, the wound has been made smaller by the action of myofibroblasts, which establishes a grip on the wound edges and contract themselves using a mechanism similar to that in smooth muscle cells. When these cells roles are close to complete, unnecessary cells undergo apoptosis. In the remodelling phase and maturation, collagen has remodelled and realigned along tension lines and cells which are no longer needed are removed by apoptosis<sup>7,8</sup>.

In the remodelling phase and maturation, collagen is remodelled and realigned along tension lines and cells that are no longer needed are removed by apoptosis<sup>9</sup>.

#### **1.1.4. Approaches used in Wound Treatment**

There are mainly two approaches used in wound healing. One is the use of anti-microbial agents and antiseptics and the other is the use of tissue or cell growth factors. Out of these two approaches cell growth factors would be a better approach as it accelerates the natural wound healing process, while the antiseptics and antimicrobial only kills the microbes affecting the wounds<sup>9</sup>.

The advent and development of tissue engineering provides a new means of restoring tissues and organs. One of the challenges of tissue engineering is to establish a three-dimensional complex comprising cells and biomaterials. The three dimensional structure in which the cells acquire nutrition, exchange gas, and excrete waste is the physical foundation for the formation of new tissue and organs with shape and function. Such biomaterials often must be biodegradable. Therefore one of the prerequisite of practical tissue- engineering technology is to fabricate biodegradable material with superb biocompatibility. Silk fibroin is the material which is nontoxic, nonirritating, with good applicability. Moreover, it adheres well to fibroblast and epidermal

cells. Tsubouchi et al., has reported the wound healing activity of silk fibroin. Therefore, regenerated silk fibroin material is attractive for use as a biomaterial for wound healing. The silk fibres often have sericin present on them. Removal of sericin is an important step in isolation of silk fibroin because sericin has problems with biocompatibility and hypersensitivity to skin<sup>9,10</sup>.

## 1.2 Silk

Silks are generally defined as protein polymers that are spun into fibers by some Lepidoptera larvae such as silkworms, spiders, scorpions and flies. Silk proteins have been produced within specialized glands after biosynthesis in epithelial cells, followed by secretion into lumen of these glands where the proteins are stored prior to spinning into fibers. Silks differ widely in the composition, structures and properties depending on its specific source. The most extensively characterized silks are from the domesticated silkworm, *Bombyx mori*, and from spiders (*Nephila clavipes* and *Araneus diadematus*). Many of the more evolutionarily advanced spiders synthesize different types of silks. Each of these different silks has a different amino acid composition and exhibits mechanical properties adapted to their specific functions; reproduction as cocoon capsular structures, lining for prey capture, lifeline support (dragline), web construction and adhesion. Fibrous proteins present in silk render mechanical strength to the silk. Because of these impressive mechanical properties, this family of proteins provides an important set of material options in the fields of controlled release, biomaterials and scaffolds for tissue engineering. The relative environmental stability of these families of proteins, in comparison to globular proteins, in combination with their biocompatibility, unique mechanical properties, and options for genetic control to tailor sequence provides an important basis to exploit these natural proteins for biomedical applications<sup>10</sup>.

Waste silk is a raw material of the most important branch of silk industry. The raw materials of this industry consist of production of continuous filament thread and waste collected during the reeling of raw material and subsequent stages of its processing<sup>10</sup>. Waste silk can be classified as

- a) Waste from the cocoon
- b) Reeling waste
- c) Thread waste

Silk wastes, which are produced during the silk processing, can be reutilized. It can be used as a coarse yarn and as a spun silk, which can be incorporated in natural rubber to achieve physicochemical properties. Silk being a protein in nature, the researchers find its relevance in pharmaceutical

applications. To explore this, one needs to study the structural and chemical properties of silk.

### 1.2.1 Structure and Properties of Silk

Silk fibers are composed of two protein microfilaments (called as brins) embedded in a glue-like glycoprotein coating (Figure. 1.1A)<sup>10</sup>. The brins are fibroin filaments composed of bundles (of ca. 100nm in diameter) of nanofibrils (individually of ca. 5nm in diameter) which are preferentially aligned with the long axis of the fiber. The silk fibroin nanofibrils are composed of a complex of three proteinaceous components: a large protein, known as heavy chain (H-chain) fibroin (of ca.350k Da) that is linked to a second small protein, known as light chain (L-chain) fibroin (of ca.25 k Da) via disulfide bonds; and a third small glycoprotein, known as the P25 protein (of ca.30k Da) is associated via non-covalent hydrophobic interactions. The molar ratios of L-chain:H-chain:P25 are 6:6:1;

the H-chain is hydrophobic and contains blocks of (Gly-Ala-Gly-Ala-Gly-Ser)<sub>n</sub> that are known to form anisotropic  $\beta$ -sheet-rich nanocrystals, whereas the L-chain is very hydrophilic and relatively elastic in nature, and the P25 protein is believed to play a role in maintaining the integrity of the complex<sup>10</sup>.

Silkworm fibroin protein differs in composition depending upon source, extraction method and analysis. In general silks are insoluble in dilute acids and dilute alkalis, resistance to most proteolytic enzymes. It is hydrolyzed by conc. Sulfuric acid. Lithium bromide (LiBr) (9.3 M) solubilizes silk fiber, while specific mixture of acid solubilizes silk without decreasing molecular mass of the polymer. The mulberry silk, which is the domesticated Silk from *B. mori*, contain 72-81% fibroin, 19-28% sericin and traces of lipids, pigment and carbohydrates. The trace components of Mulberry *B.mori* cocoon silk comprises between 2.5-3.7 % of total material<sup>10,11</sup>.

Silk fibroin(SF) is composed of 18 kinds of amino acids, among them, the glycine (Gly, 44.60 % (mol percent)), the alanine (Ala,29.40%) and the serine (Ser, 12.10%) with simple side groups account for about 85%, that is about 4:3:1 in term of their molar ratios. They are arranged in their relatively ordered chain segment according to certain sequences, most of these chains locate in the crystal region of SF. A hexapeptide repeat Ser-Gly-Ala-Gly-Ala-Gly (SGAGAG) was first reported as structural element. On the other hand, the tyrosine (Tyr,5.17%(mol percent)), the phenylalanine (Phe, 0.63%) and the tryptophan (Try, 0.11%) with relatively large side-groups mostly locate in non-crystal region<sup>11,12</sup>.

### 1.2.2 Therapeutic Applications of Silk Fibroin

The therapeutic applications of silk fibroin includes:-

**a. Hypolipidemic Agent:**

The amino acid composition of silk fibroin is comparatively simple; it is rich in Glycine, Serine, Alanine and Tyrosine. Considering the physiological functions of these amino acids and recent research focused on cholesterol lowering ability of Glycine and Serine has and thus it was proved that silk fibroin has hypolipidemic activity<sup>12</sup>.

**b. Antidote in Alcohol Toxicity:**

Alanine promotes the metabolism of alcohol by liver thus useful in alcohol toxicity and fibroin is rich source of it<sup>13</sup>.

**c. Dementia:**

Tyrosine is effective in preventing dementia and fibroin is rich source of its content<sup>14</sup>.

**d. Anticoagulant / Antithrombogenic:**

The films of silk fibroin treated with 50% methanol for 15 min. and graft polymerized with ethyl methacrylate and styrene by methane plasma initiation. Their antithrombogenicities were investigated by blood clotting method and platelet adhesion tests. Activity was improved by the surface grafting and varied with the fraction of polar component of surface tension to the total surface tension of film<sup>14,15</sup>.

**e. Wound Healing**

Bioactive peptides with mol. wt. 300-5000 are obtained from silk fibroin hydrolysis. The peptides are said to have

immune stimulant activity, antioxidant and anticancer promoting effects<sup>15</sup>.

**1.3. Role of Silk- Fibroin in Wound healing**

Amino acids in silk fibroin play an important role in wound healing. Amino acid composition of different varieties of silk fibroin has been studied by Lucas et al. the main four varieties of Indian silks viz., Mulberry, Tusar, Eri, and Muga, have been investigated by Dhavalikar with reference to amino acid composition. In the case of Mulberry the composition due to glycine, alanine, and serine was found to be about 80%. The Amino acid composition in the case of Tasar, Eri, Muga revealed alanine as the major constituent with small quantities of glycine and serine. A portion of the amino acid sequence of silk fibroin produced by *Bombyx mori* has been identified<sup>16</sup>. The crystalline region of silk fibroin is composed mainly of the repetitive element Gly-Ala-Gly-Ala-Gly-Ser, and in amorphous domain of silk fibroin is found the amino-acid sequence Thr-Gly-Ser-Ser-Gly-Phe-Gly-Pro-Tyr-Val-Ala-Asp-Gly-Gly-Tyr-Ser-Arg-Arg-Glu-Gly-Tyr-Glu-Tyr-Ala-Trp-Ser-Ser-Lys-Ser-Asp- Phe-Glu- Thr. Alanine, Glycine, Serine are important amino acids for activating epidermal cell multiplication<sup>17,18</sup>. The silk fibroin acts as a natural wound healing agent at inflammatory phase, fibroplasia, and epithelization<sup>19</sup> as shown in the Figure1.2.

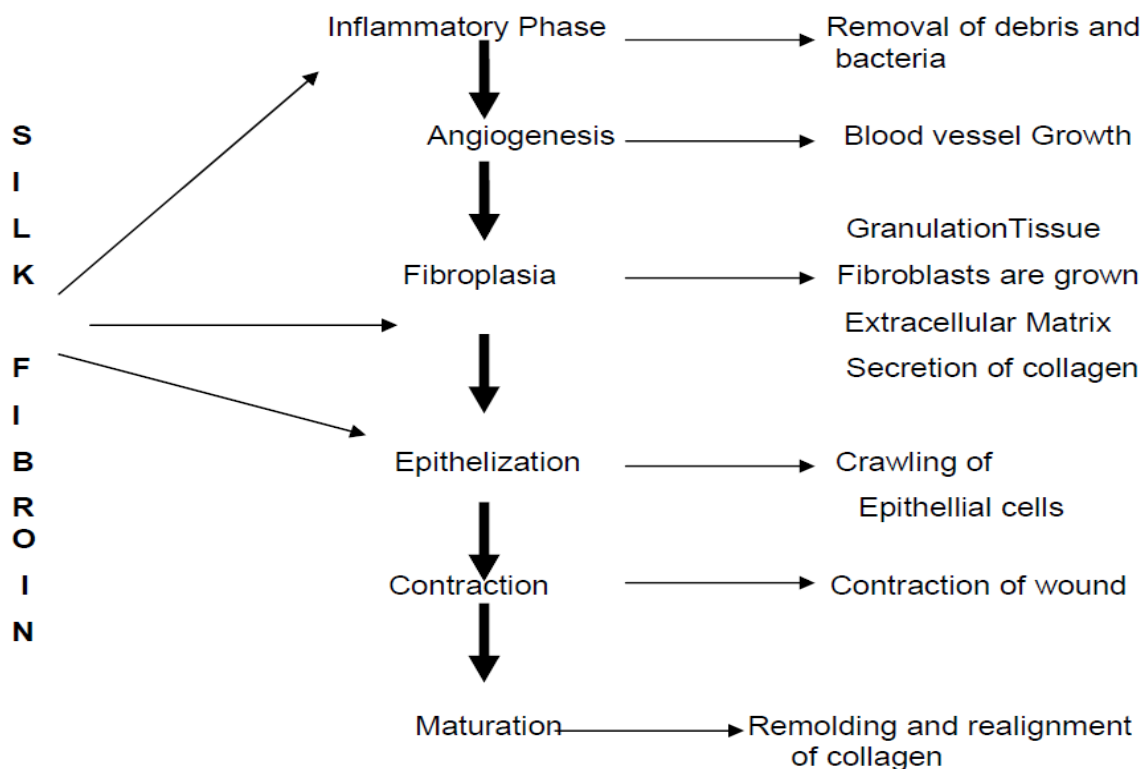


Figure1.2: Role of Silk Fibroin in natural wound healing process

### 1.3.1 Advantages of Silk- Fibroin in Wound healing

Silk fibroin as a wound healing material has several advantages over other synthetic materials such as

- Interact well with human cells in vitro are very well tolerated after implantation into animals in vivo.
- Do not evoke any degree of foreign body response and do not induce the local production of pro-inflammatory the implants.
- Favour the neo-angiogenic processes in a way cytokines.
- Do not cause fibrous scarring around or inside that the regenerated tissue is also well vascularised and thereby better able to survive and properly function.
- It is biocompatible, biodegradable, adherence to epidermal cells.
- It is attractive material for biomedical applications because it is a fibrous protein that has high permeability to oxygen and water, relatively low thrombogenicity. Therefore silk has been increasingly implemented in medical treatment and is preferred over other material for wound healing treatment<sup>20</sup>.

### 1.3.2 Limitations of Silk- Fibroin in Wound healing

Silk fibroin is a cell growth promoting agent and itself does not possess any antimicrobial action. Open wounds are the most subject to infection from pathogens all around us and should be cared for well. These pathogens may impair with the wound healing process induced by silk fibroin. Thus there is need to develop a formulation which have cell growth promoting activity along with anti-microbial activity<sup>21</sup>.

### 1.4 Role of Metal nanoparticles in health care

Upon reaching nanoscale, like other nanomaterials and primarily by virtue of immensely small size, silver particles exhibit remarkably unusual physicochemical properties and biological activities. Silver nanoparticles have shown bactericidal activity against various strains of bacteria including *Salmonella*, *Staphylococcus* and *Pseudomonas*<sup>30</sup>. Most importantly, silver attacks a broad range of targets in the microbes, so it is difficult for them to develop

resistance against silver, because they would require developing a host of mutations to protect themselves<sup>31</sup>. The antibacterial activity of silver appears to be due to disruption of the cell wall leading to increased cell wall permeability and depletion of ATP of cell affecting its viability. The monodispersed silver nanoparticles with narrow particle size distribution are more effective antibacterial agents, because of the high surface/volume fraction so that a large proportion of silver atoms are in

direct contact with their environment. Silver is known to be nontoxic to human cell to the tune of 350 µg/day<sup>32,33</sup>.

### 1.4.1 Characterization of Metal nanoparticles

The monodispersed metal nanoparticles can be characterized by following techniques:

- UV-visible spectroscopy
- X-ray diffractometry (XRD)
- Transmission Electron Microscopy (TEM)

UV-visible spectroscopy is one of the most widely used techniques for structural characterization of metal nanoparticles. Silver nanoparticles show band at about 420 nm whereas gold nanoparticles at about 510 nm in UV visible spectroscopy which is known as Surface Plasmon Resonance (SPR)<sup>34</sup>.

#### Physical basis of SPR

According to Mie's theory, only a single SPR band is expected in the absorption spectra of spherical nanoparticles, whereas anisotropic particles may give rise to two or more SPR bands depending on the shape of particles. The number of SPR peaks increases as there symmetry of the nanoparticles decreases. Thus, disks, spherical nanoparticles and triangular nanoplates of Ag shows one, two and more peaks, respectively<sup>37</sup>.

The X-ray diffraction patterns of silver shows characteristic peaks at  $2\theta = 38^\circ$ ,  $44^\circ$  and  $64^\circ$ , which corresponds to {111}, {200} and {220} crystal planes. Presence of these crystal planes indicates face central cubic crystalline structure of silver and also ensures that silver metal is in zero valent state. The XRD pattern of silver nanoparticles can also be used to determine their particle size. This can be done by mathematical analysis of the Bragg's peaks using the Scherrer formula<sup>38,39</sup>.

The TEM analysis helps to confirm the particle size of the nanoparticles. The electron diffraction pattern generated during TEM analysis gives an idea about the nature (e.g. crystalline or amorphous) of the particles. The crystalline particles shows presence of clearly split Debye-Scherrer rings. These rings represents {111}, {200} and {220} crystal planes of silver. Presence of these crystal planes indicates face central cubic crystalline structure of silver<sup>40,41,42</sup>.

### 1.4.2 Methods for stabilizing colloids

An important feature in the production of metal nanoparticles is their ability to remain dispersed in liquids without sign of agglomeration and coagulation, since their activity is governed by monodispersity and stability. Colloidal particles in a dispersion medium always show Brownian motion and hence collide with

each other frequently<sup>43,44</sup>. The stability of colloids is thus determined by the interaction between the particles during such a collision. There are two basic interactions: one being attractive and other repulsive. When attraction dominates, the particles will adhere with each other and finally the entire dispersion may coalesce. When repulsion dominates, the system will be stable and remain in a dispersed state<sup>44,45</sup>.

Because there are always strong, long-range attractive forces between similar colloidal particles, it is necessary to provide long range repulsion between the particles to impart stability<sup>46</sup>.

This repulsion should be at least as strong as the attractive force and comparable in range of the attractive interaction. Stability can be obtained by surrounding colloidal particles<sup>46,47</sup>.

- With an electrical double layer (electrostatic or charge stabilization).
- With chemically attached polymeric molecules (i.e. adsorbed) (steric stabilization)<sup>48</sup>.
- With free polymers into the dispersion medium (depletion stabilization).

### 1.5 Combination of Silk Fibroin with Silver nano particles

Considering the wound healing activity of silk fibroin and broad spectrum anti-microbial activity of silver nano particles, it is believed that the combination of Silk Fibroin with Silver nano particle may be highly beneficial in wound healing process since the combination could act on wounds by two ways:

- Cell growth promoting action of silk fibroin proteins
- Broad spectrum of anti-microbial action of Silver nano particles on the microbes affecting the wounds.

Therefore the current study focuses on preparation of silver nano loaded silk fibroin topical gel which may accelerate wound healing process<sup>66</sup>.

#### Aim and Objectives:

The primary aim of the present study is to formulate Nanosilver loaded silk fibroin for for these reasons:

- There is need to develop a formulation having dual role (i.e. cell growth promoting activity along with antimicrobial activity).
- Moreover, microbes are also unlikely to develop resistance against silver nanoparticles. Silver nanoparticles exert anti-microbial activity by increasing the bacterial wall permeability leading to loss of cell contents.
- The nanoparticles of Silver are more effective antibacterial agents, because of high surface area.
- Amino acid compositions of silk fibroin have wound healing activity as they promote the epithelization.

- The combination of silk fibroin and silver nanoparticles may accelerate the wound healing process.

#### Conclusion:

Nanosilver loaded silk fibroin gel was prepared successfully by *in situ* reduction of Ag<sup>+</sup> ions to zero valent silver atoms (silver nanoparticles) at room temperature using silk fibroin as the reduction agent. The prepared gel had more pronounced wound healing activity than marketed formulation which is believed to be due to the dual role played by NSF gel wherein silk fibroin acted as epidermal cell growth promoter and silver nanoparticles acted as antimicrobial agent leading to faster percent wound closure.

This investigation also proposes the need for such combinations of a cell growth promoting agent with anti-microbial agents in the treatment of wounds.

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