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Evaluation of Chitosan and Nano-chitosan Gel with and without 1% Silver Sulfadiazine as an Alternative for Burn Wound InfectionsTreatment in Albino Mice

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Abstract— Infected wounds are one of the most common health issues today, and there are numerous treatment options. These treatments, however, have drawbacks and are frequently expensive. We can produce a more effective, less expensive wound dressing for the treatment of infected wounds by employing chitosan or nano-chitosan alone or by combining each material with a pre-existing silver antibacterial like silver sulfadiazine. These findings demonstrate the combination of actives that inhibit and kill certain wound pathogens in a polymeric gel wound dressing after a series of antimicrobial efficacy tests *in vitro* and *in vivo*.

Keyword: Bcc, Chitosan, Nanochitosan, Burn infections.

Introduction

Burkholderiacepacia complex (Bcc) is a gram-negative bacterium group made up of at least nine species that is commonly isolated from the natural environment.These bacteria, on the other hand, can sometimes produce human diseases that are lethal who are weaker, for example, patients suffering from a foot ulcer. Since Bcc bacteria are not usually carried as commensal microbes, patient-to-patient transmission, hospital environments, including medical equipment and contaminated disinfectants, and the atmosphere are thought to be the key sources of infection. (Baldwin *et al*., 2007). *B. cepacia*is a Gram-negative bacilli, rod-shaped, non-sporeforming, motile, catalase-positive and lactose-non fermenting bacteria (Tavares*et al*., 2020).They're opportunistic pathogens that often affect people with cystic fibrosis, chronic granulomatous disease, or indwelling medical devices. *B. cepacia* complex clinical manifestations have been linked to occasional infections and outbreaks, particularly device-associated bloodstream infection in immunocompromised individuals, in hospitals and other health-care settings (Mann *et al*., 2010). Wound inflammation, peritonitis, septic arthritis, and ocular infections (UTI, neonatal meningitis, and brain abscess). The *B. cepacia* complex is a common cause of pneumonia, septicemia, and soft tissue abscesses in patients with cystic fibrosis, as well as pneumonia, septicemia, and soft tissue abscesses in people with chronic granulomatous disease (Porterand Goldberg, 2011).

Chitosan is a natural unbranchedhomopolymer made from chitin, a common by product of seafood processing, by removing the acetyl groups $COCH_3$ from the chitin original structure with alkali (Kurita, 2006). Chitosan is a non-toxic, high-molecular-weight polymer that resembles cellulose, a plant fiber. The only difference among chitosan and cellulose is that chitosan has an amine ($-NH_2$) group in position C-2 instead of cellulose's hydroxyl (-OH). Unlike plant fiber, however, chitosan has positive ionic charges, allowing it to chemically bind to negatively charged fats, lipids, cholesterol, metal ions, proteins, and macromolecules (Li *et al*., 1992). In this respect(Rout, 2001), due to their excellent properties, Chitin and chitosan have gained increasing commercial interest as potential resource materials, such as biocompatibility, biodegradability, adsorption, the ability to shape films, and the capacity to chelate metal ions.

Nano-chitosan is a natural substance of outstanding physical and chemical properties. It is bioactive and environmentally friendly. Physical crosslinking by ionic gelation between chitosan and unique negatively charged macromolecules such as pentasodiumtripolyphosphate has been used to make nano-chitosan (Calvo*et al*., 1997). Furthermore, chitosan and chitosan nanoparticle films and coatings can be employed as a carrier for natural or artificial antibacterial agents, antioxidants, enzymes, or functional compounds like plant extracts, probiotics, minerals, or vitamins (Ojagh*et al*., 2010).

The preparation of chitosan or nano-chitosan gel containing 1% silver sulfadiazine is described in this paper. In addition, the efficacy of chitosan or nano-chitosan gels (with and without silver sulfadiazine) on bacterial pathogen inhibition and burn wound healing in mice was compared to a commercially available 1% silver sulfadiazine cream *in vitro* and *in vivo*.

Methods

The study was conducted at Bacteriology and Molecular Laboratories in Biology Department, Sciences Faculty, Kufa University, Najaf/Iraq.

Patients and Clinical Specimens

During the study period from September 2020 to February 2021, 140 specimens were collected from patients suffering burns, incision wounds and diabetic foot ulcers infections to each sex: male (78 samples) and female (62 samples) with age group from 1-70 yearsfrom specialized burn center and middle Euphrates hospital in AL Najaf/Iraq.

Bacterial Isolates

The collected specimens were inoculated on three types of culture media which included blood agar and MacConkey agar,then use a sterile loop to spread on each dish. Dishes were incubated at 37˚C for 24 hrs.Following that, the dishes were checked for bacterial growth, and a single pure isolated colony was transferred to brain heart infusion agar for maintenance and morphological evaluation by Gram staining, as well as other biochemical assays(Colle*et al*., 1996) and vitek -2 compact system that confirmed the identification of isolates (Nakasone *et al*., 2007).

Susceptibility to Antibiotics Test (Disk Diffusion Method)

The antibiotic sensitivity report was performed according to Kirby-Bauer disc diffusion fashion on Mueller-Hinton agar (Bauer *et al.,* 1966).

Preparing the Gel of Chitosan, Nano Chitosan gel and Silver Sulfadiazine

1. Preparing of Silver Sulfadiazine (SSD) Salts

Ammoniacal solutions at equimolar concentrations (0.1 mol/L) were reacted in the dark with silver nitrate and the required anion to produce silver sulfadiazine and silver chloride (Wruble, 1943). The resulting white pastes were filtered and washed multiple times with Milli-Q water before being dried for 10 hrs at 60°C under reduced pressure. White and "fluffy" was the silver sulfadiazine solid, whereas white and "clumpy" was the silver chloride solid.

2. ChitosanGel

Chitosan (75.00% deacetylation) was dissolved in acetic acid at a concentration of 2% (v/v), resulting in a last condensation of 3% (w/v). Formaldehyde was applied to the chitosan solution at a rate of 0.002g per gram of chitosan in order to form the gel. Glycerin was also introduced at a 0.2g per gram of chitosan ratio. The gel was formulated after the mixture was totally mixed. 1% silver sulfadiazine

was added to a known quantity of the produced gel. This concentration was accomplished by incorporating 0.01g of silver sulfadiazine into each gram of chitosan gel. The gels were placed in closed containers at room temperature after homogenization(do Nascimento*et al.*, 2009).

3. Nano-chitosanGel

The preparation of nano-chitosan gel has been done by thedissolve 0.5g/L of chitosan using 1% acetic acid in a glass beaker (Pishbin*et al*.,2011; Arias *et al*., 2013).

Agar Well Diffusion Assay (Antibacterial activity experimental)

Bacterial suspensions were generated in accordance with the McFarland standard of 0.5., agar well diffusion method was used to determine the antibacterial activity of the following compounds:

A-Chitosan with silver sulfadiazine.

B-Nano-chitosan with silver sulfadiazine.

C-Nano-chitosan without silver sulfadiazine.

D-Silverdin (cream 1%) commercially available silver sulfadiazine.

Utilizing the micropipette,100 µl of *B. cepacea* grown in brain heart infusion broth (BHIB) 18-24hrs was spreadon an agar plate's surface of Muller Hinton agar (MHA). Utilizing a sterile cork borer, every culture plate had five holes drilled in it. One of the holes was fill up of 100 µl of chitosan with silver sulfadiazine gel; the second hole full up of 100 µl of nano chitosan with silver sulfadiazine gel; the third hole full up of 100 µl of nano-chitosan without silver sulfadiazine, fourth hole full up of 100µl of chitosan without silver sulfadiazine and fifth hole full up of 100µl commercially available silver sulfadiazine 1% cream. The culture plates were then incubated for 24 hrs at 37 ℃. The distinct inhibitory zone surrounding the gels compound was measured in millimeters (Murray *et al*., 1995; Olurinola, 1996).

Animals Models (*in vivo*)

Burn Wound Model

Twentyfemale mice albino weighing 18-24g and age 16weeks were used in the *in vivo* studies. The Institutional Animal Care Committee approved the protocol for this study, and it was carried out in compliance with the guidelines of the Animal College of Veterinary Medicine. The animals were kept in individual cages with unlimited access to water and food (rodent chow). Xylazine (10mg/kg) and ketamine (80mg/kg) intraperitoneal injections were used to anesthetize each mouse. The dorsum hair on the burned area was shaved by using electric hair clipper and then shaved with sharp blade. The shaved areas were cleansed with alcohol swab., and a 1.5 cm^2 aluminum square heated to 100°C was placed over the exposed skin for 5 seconds to cause the burn. A wheel weighing 200g was attached to the aluminum square before to infliction. As a result, all animals experienced the same strain on their skin (due to the weight of the wheel) (do Nascimento*et al.*, 2009).

Treatmentof burns

Five groups of animals were created. The wounds of group 1 mice (n=4) were treated with chitosan gel with silver sulfadiazine, group II mice (n=4) with nano chitosan gel with silver sulfadiazine, group III mice (n=4) with nano-chitosan gel without silver sulfadiazine, group IV mice (n=4) with commercially available silver sulfadiazine 1% cream , and group V mice (n=4) as a control (no treatment). Groups I, II, III and IV received gel applications every 48 hrs, while Group IV received

regular applications of silver sulfadiazine 1% creamevery 48 hrs. The wounds were washed with a sterile 0.9 % saline solution in between applications of gel or cream. Throughout the procedure, the wounds were left open (without bandages). The animals were observed every day in the morning, and the time it took to achieve full epithelialization was used to calculate healing time (in days) (do Nascimento*et al*., 2009).

Resultsand Discussion

During the study period from September 2020 to February 2021, 140 specimens were collected from patients suffering burns, incisional wounds and diabetic foot ulcers infections to each sex: male (78 samples) and female (62 samples) with age group from 1-70 years. The results of culture on blood agar showed 21/40 isolates belong to *Staphyllococcusaureus,* 12/40 isolates has been belong to coagulase negative staphylococciand 7/40 isolates belong to *Streptococcus spp., S. aureus* remains a leading cause of infections in burn centers (Chen *et al*., 2012). *S. aureus* is the most common bacteria causing both hospital and community associated infections, including bacteremia, pneumonia and burn infection (Luzzaro*et al*., 2011). In burn centers, the introduction and spread of methicillin-resistant *S. aureus* (MRSA) leads to poor outcomes such as prolonged hospitalization, bacteremia or sepsis, and even mortality, necessitating further prevention and treatment efforts (Issler-Fisher *et al*., 2015). Opportunistic and pathogenic Gram negative bacteria strains isolated from the patients suffering burns, incision wounds and diabetic foot ulcers infections are BCC, *E. coli*, *E. cloacae*, *P. mirabilis*, *P. aeruginosa*, *K. pneumonia*, *Acinetobacter* spp. *P. aeruginosa* and BCC were the most common bacteria, accounting for 50% of total isolates. *P. aeruginosa* is one of the most important pathogens causing different infections such as bacteremia and burn infections(Maraolo*et al*., 2018).This pathogen has adapted successfully to hospital surroundings due to the production of biofilms, which provide the pathogen with long-term survival advantages and efficiently prevent eradication by the host immune system or antimicrobial drug treatment (Groenewold*et al*., 2018). *P. aeruginosa*has become the leading cause of death in burn patients, accounting for more than 70% of all deaths(Roham*et al*., 2017).

Antibiotic Susceptibility

This test was conducted to all isolates byKirby-Bauer disk diffusion method was used to study routinely used antibacterial drugs (Bauer et al., 1966).All isolates test with fourteen antibiotics disc which included; carbapenem class (imipenem and meropenem);cephalosporins (ceftazidime); aminoglycoside (gentamycin); floroquniolone (ciproflaxcin,levofloxacin); tetracyclins (tetracyclin, doxycycline); ansamycin (rifampin); phenicols (chloramphenicol,pipracillin); folate pathway antagonists (trimethoprim sulfamethoxazole); macrolides (fusidic acid,erythromycin). The results were interpreted based on the inhibition zone diameter and compared to standard inhibition zones determined by CLSI, (2020).

The outcomes of this experiment revealed that *B.cepacia* and *P.aeurogenosa* have great resistance to most commonly antibiotics used in hospitals, *B.cepacia* and *P.aeurogenosa* showed susceptibility to the antibiotics utilized in this study differed.Fusidic acid exhibits the highest rate of resistance in both BCC and *P.aeurginosa* 100%, ceftazidim,rifampin 95% in BCC but high rate in *P. aeurginosa* 100%, chloramphenicol 85%,trimethoprimesulfamethoxazole 74%, erythromycin 70%, while gentamycin, tetracyclin, doxycycline and pipracillin with presentage 62%. This study show increase rate of resistance for meropenem proximally 50%, and imipenem 20% as show in table (1).

Table (1):Antimicrobials sensitivity test of*B.cepacia*and *P.aeurginosa*

Antibiotic type	*B.cepacia No. 46* (100%)			*P.aeruginosa* No.6 (100%)		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Chloramphinecol	6 (13)	2(4.3)	38(82.6)	2(33.3)	0(0.0)	4(66.6)
Ceftazidim	4 (8.6)	0(0.0)	42(91.3)	0(0.0)	0(0.0)	6(100)
Levofloxacin	18(39.1)	6(13)	22(47.8)	1(16.6)	0(0.0)	5(83.3)
Meropenem	20(43.4)	4(8.6)	22(47.8)	2(33.3)	0(0.0)	4(66.6)
Pipracillin	6 (13)	12(26)	28(60.8)	1(16.6)	2(33.3)	3(50)
Ciproflaxacin	18(39.1)	6(13)	22(447.8)	1(16.6)	0(0.0)	5(83.3)
Doxycyclin	16(34.7)	2(4.3)	28(60.2)	2(33.3)	0(0.0)	4(66.6)
Erythromycin	6 (13)	10(21.7)	30(65.2)	0(0.0)	1(16.6)	5(83.3)
Tetracyclin	12(26)	6(13)	28(60.8)	1(16.6)	0(0.0)	5(83.3))
Gentamycin	18 (39)	0(0.0)	28(60.8)	2(33.3)	0(0.0)	4(66.6)
Imipenem	38(82.6)	0(0.0)	8(17.3)	5(83.3)	0(0.0)	1(16.6)
Trimethoprim	10(21.7)	2(4.3)	34(73.9)	1(16.6)	0(0.0)	5(83.3)
Rifampin	2(4.3)	2(4.3)	42(91.3)	0(0.0)	0(0.0)	6(100)
Fusidic acid	0(0.0)	0(0.0)	46(100)	0(0.0)	0(0.0)	6(100)

Both species showed resistance to antibiotics under study (fusidic acid, chloramphenicol, ceftazidime, meropenem, imipenem, piperacllin, gentamycin, ciprofloxacine, tetracycllin, doxycycllin, rifampin, trimethoprim sulfamethoxazole, erythromycin and levofloxacine) approximately with the same percentages which may be due to that both species utilized similar mechanisms to resist these antibiotics, where *Bcc* has intrinsic resistance and is one of the most antimicrobial-resistant organisms encountered. Therefore, it needs to be correctly identified and differentiated from *P. aeruginosa* as *Bcc* antimicrobial drugs such as aminoglycosides, first and second generation cephalosporins, antipseudomonalpenicillins, and polymyxins have inherent differences. As a result, the correct identification of *B. cepacia* is extremely important (Omar *et al*., 2015). A study done *P. aeruginosa* isolates were found to be resistant to imipenem (13.4%) by Bashir *et al*., (2011).

P. aeruginosa strains showed 100% resistance to Ampicillin and Norfloxacin, 83.3 % resistance to piperacillin, ticarcillin, and tetracycline, 66.6% resistance to ceftazidime, imipenem, gentamicin, amikacin, tobramycin, andcotrimoxazole, and 50% resistance to cefoperazone, Cefotaxime was effective against 83.3% of *P. aeruginosa* strains, a study done according to Sivanmaliappan and Sevanan, (2011).

Several studies tested *B. cepacia* for antibiotic susceptibility where all agreed that this organism was highly resistant to multiple antibiotics. Gautam*et al.,*(2009) and Dizbay*et al.*, (2009) reported that

their *B. cepacia* isolates were most resistant to ceftazidime followed by ciprofloxacin each, then meropenem co-trimoxazole and piperacillin–tazobactam, comparing the results of our study to those two studies we observe that our isolates were more sensitive to meropenem.Because of its high transmissibility among hospitalized patients and numerous medication resistance, *B. cepacia* complex has been added to the list of nosocomial infections that cause major issues in clinical settings (Sousa *et al.,*2011).

Since the continuous increase of multidrug-resistant bacteria, the focus of research has shifted towards the antimicrobial activity of other agents such as plant-derived compounds (Khamenehe*t al*., 2019) nanoparticles (Savoia,2012 ; Sanchez-Lopez *et al*., 2020).

Microbiological Studies of Silver Sulfadiazine Loaded on Chitosan or on Nano-Chitosan Gel and Chitosan and Nano-Chitosan Gel without Silver Sulfadiazine

Wound infection is a major issue in burn therapy because it is the leading cause of death (D'Avignone*t al*., 2010 ; Merchant *et al*., 2015; Norbury*et al*., 2016). Pathogens have developed in response to antibiotic use over time (Wang *et al.,* 2018). With a scarcity of new antibiotics, breakthroughs that improve the efficacy of currently available antimicrobials are significant in reducing morbidity in burn patients (Kinche*t al*., 2014).Infection not only poses a major risk to the patient's life, but it also slows the healing process, resulting in a longer stay in the hospital and higher costs (Siafaka*et al*., 2016; Oryan*et al*., 2018).For a variety of reasons, burns are particularly vulnerable to infection. The loss of the epidermal barrier, along with protein and lipid denaturation, creates an ideal environment for microbial development (Sevgi*et al*., 2013).

From the results of the sterility studies of the chitosan/nano chitosan with SD-Ag (silver sulfadiazine), it was demonstrated that no growth of any of the inoculated microorganisms occurred after the incubation period. Moreover, it can be reported that the prepared chitosan and nano-chitosan gel showed an antibacterial effect on the selected isolates. Before dissolve of chitosan in acetic acid, the results of detection of concentration without inhibitory of bacteria isolate in three concentration (1%, 2% and 3%), while when dissolve of chitosan in acetic acid in three concentrations revealed that 3% conc. of acetic acid inhibit bacterial isolate (BCC and *P. aerogenosa*) as show in figure (1).

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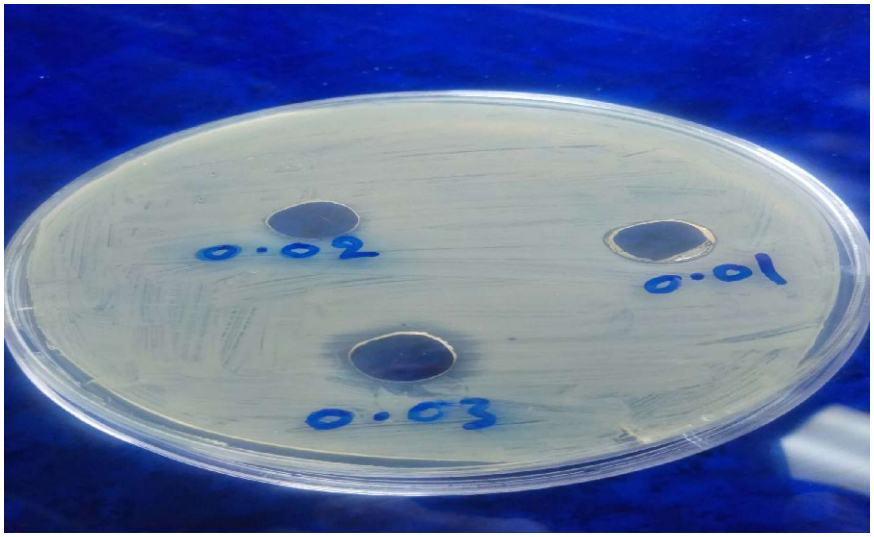

Figure (1): Antibacterial activity of concentrations of acetic acid in percentages (0.01, 0.02 and 0.03) on BCC isolate.

Antibacterial Activity of Chitosan and Nano-Chitosan Gel

Figure (2) shows antibacterial activity of commercial available silver sulfadiazine 1% cream (A), nano chitosan with SD-Ag (B), chitosan gel without SD-Ag(C) and chitosan gel with SD-Ag(D).

The inhibition zone of nano-chitosan with SD-Ag and chitosan with SD-Ag show high result compared with commercial available cream (silverdin1%). The activity of chitosan gel without SD-Ag was appear clear zone around well as show in well C.It is well known, that chitosan possesses antimicrobial activity against *P.aeruginosa* (Siafaka*et al*., 2016).

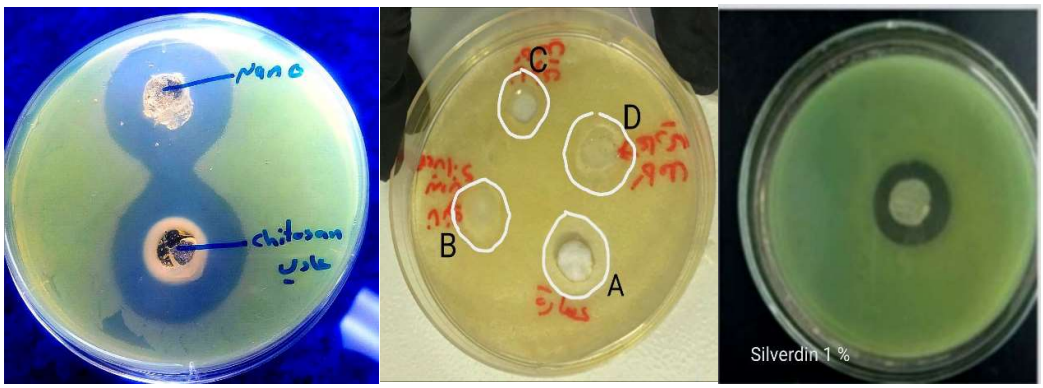

Figure (2): Zone inhibition diameters of chitosan gel with SD-Ag, Nano-chitosan with SD-Ag gel, chitosan without SD-Ag and commercial product of silver sulfadiazine (silverdin 1%).

Macroscopic Burn Healing *In vivo* Studies

The burn healing potential was assessed in this study throughout a 21-day experimental period, with the percentage of wound areas contracted being monitored on a regular basis. Experimental 2nd degree burns on the backs of mice were generated, and all lesions were treated for 21 days to see how well they healed.

1-Nano-chitosan with SD-Ag gel.
2-Chitosan with SD-Ag gel.
3- Chitosan/ Nano-chitosan without SD-Ag gel.
4-Silverdin cream (1% silver sulfadiazine).

According to the depth of tissue injury, a burn injury can be classed as first, second, third, or fourth degree burns. Second-degree burns have a white or yellow color, blistering, and a moist look, and can affect either the papillary layer (i.e. the top dermis) or the reticular layer (i.e. the deeper dermis) (MofazzalJahromi*et al*., 2018).
Mice's daily habits (such as food intake and activity) were observed to be normal. Figure (3) shows macroscopic photographs of the burn regions taken on days 0, 3, 7, 10, 14, 18, and 21. On the first day, all groups had necrosis and edema on the wound surface areas. Exudates were also visible, and the wound edges began to protrude. Edema was also visible on the wound's surface. After a few days, crust forms were visible on the skin. It was discovered that:

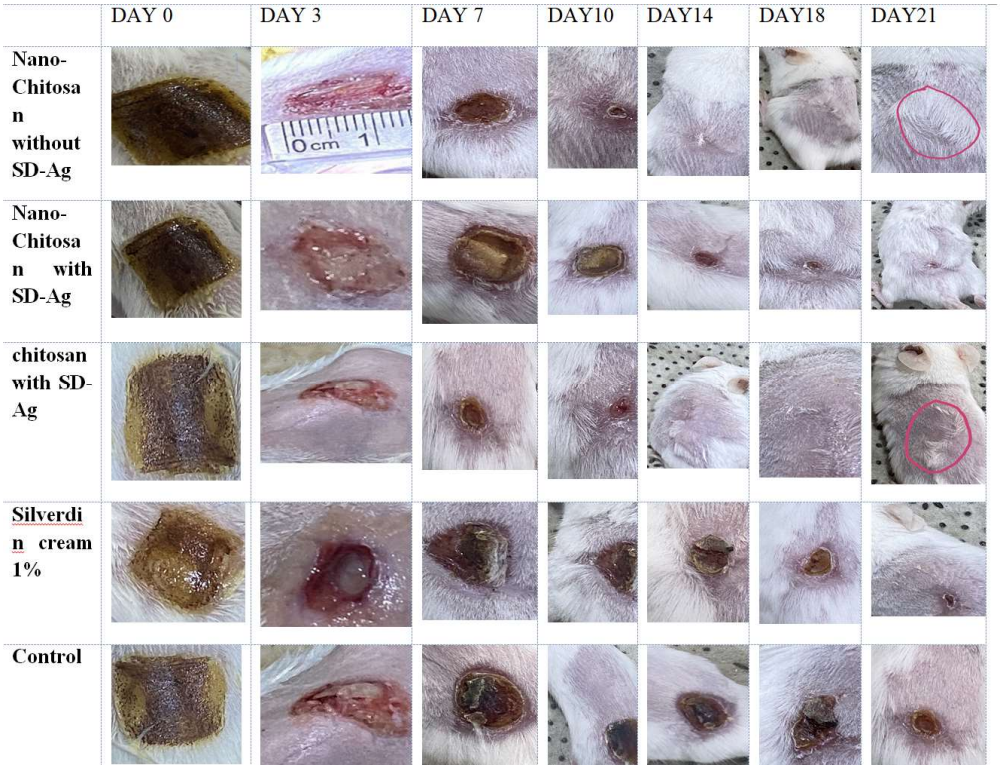

Figure (3): Effects of Nano-chitosan with silver sulfadiazine/ chitosan with silver sulfadiazine /Nano-chitosan without silver sulfadiazine and commercial product of silver sulfadiazine (silverdin 1%) on wound's evolution. five group were used as a control group (without

treatment). Macroscopic examples of wound healing with control, groups after excision on days 0, 3, 7, 10, 14, 18 and 21.

1- Nano-chitosan without SD-Ag gel show the best result for healing burn wounds after 12-13 days from initiation treatment and growth hair in the same area without leave any spot of burns area.

2-Nano-chitosan with SD-Ag hydrogel appeared decrease of healing of burn wounds.

3-Chitosan with SD-Ag hydrogel inhaling area appeared small and leave spot without growth of hair in same area.

4-Silverdein cream 1% show less active on burn wounds.

Although silver has extensive antibacterial efficacy, it has certain disadvantages, including damage to host cells (fibroblasts and keratinocytes) and the formation of bacterial resistant strains (e.g., *E. coli*) (Atiyeh*et al*., 2007).Topical antibiotic administration could be a viable alternative for treating burn wound infections. Several research have been conducted to overcome these limitations in the development of an ideal wound dressing that would include both skin regeneration and infection control, with an emphasis on silver antibacterial and biosensor properties (Liu *et al*., 2017).This particle optimization and characterization of physical characteristics, antibacterial efficiency, and fungicidal activity for the dressing with silver sulfadiazine (SSD) loaded chitosan nanoparticles (CSNPs) for the dressing with silver sulfadiazine (SSD) loaded chitosan nanoparticles (CSNPs). The multiplication of Gram negative and Gram positive bacteria, as well as Candida albicans, was inhibited as a result of the findings (El-Feky*et al*., 2017). The Ag-loaded scaffolds had a high bactericidal effect against the tested species, however cytotoxicity was seen in the fibroblasts (Biswas*et al*., 2018). In general, chitosan and its derivatives' antibacterial efficacy against Gram-positive and Gram-negative bacteria is debatable. According to certain published studies, unmodified chitosan is more effective against Gram-negative bacteria than against Gram-positive bacteria (No *et al*., 2002; Silva *et al*., 2010).

Conclusion

Chitosan and nano-chitosan are more efficient as a wound-healing accelerator than silver sulfadiazine cream. Notably, wounds covered with biopolymers, chitosan or nano-chitosan showed fast healing rate and scarless healing, which are similar the normal skin. Finally, it have decided that natural polymers have outperformed the commercial silver sulfadiazine cream, accelerate the healing rate and give strong inhibiting of bacterial pathogen for burn infections, which were lately found advanced therapeutic impact as wound dressings. The Chitosan and nano-chitosan were tested in *vivo* and *in vitro* for its potency against common strains of microbes *in* current study found in burn wounds were *Burkholderiacepacia* isolates.

Ethical approval

This study was ethically approved by the medical ethics committee in Al-Sadder Medical City, Najaf, Iraq. Moreover, adult patients and the parent of the children patients gave the informed consent before they gave the samples.

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