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Evaluation of Hemostatic Effectiveness in a Standard Swine Hemorrhage Model of Severe Bleeding: A Comparative Study of Chitosan Gauze and Kaolin Gauze

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ABSTRACT

Hemorrhage remains a critical factor contributing to avoidable fatalities in combat scenarios and civilian emergencies. Significant advancements have been made in managing extremity hemorrhage through tourniquet application, although not all injuries can be effectively treated with this method. In the United States, the Committee on Tactical Combat Casualty Care (CoTCCC) has emphasized the importance of employing hemostatic dressings during initial trauma care as an alternative when tourniquet application is not feasible. Similar recommendations have been adopted globally, highlighting the pivotal role of topical hemostats in managing such critical situations. Among the recommended topical hemostats, hemostatic gauze, composed of biomaterials including chitosan and kaolin, has demonstrated superior efficacy in rapidly achieving hemostasis. The objective of this study was to evaluate the hemostatic efficacy of axiostat gauze (AG) in an extremity arterial hemorrhage model in swine and compare it with QuikClot Combat Gauze (QCG). The femoral artery was selected to create a model of severe arterial hemorrhage. This model is widely accepted as a standardized combat injury model by military laboratories, including those of the US Department of Defense. The findings indicate that AG has a superior safety profile, evidenced by the absence of particle release, and offers advantages in terms of faster hemostasis, reduced overall blood loss, and greater fluid absorbency compared to QCG. Although AG achieved quicker hemostasis, both AG and QCG ultimately provided effective eventual hemostasis in all animals, demonstrating comparable overall efficacy. In addition to its hemostatic performance, AG also possesses antimicrobial properties and is biocompatible. These attributes make AG a highly effective option for managing severe traumatic bleeding. Thus, AG can be recommended for use in both military and civilian pre-hospital settings as a strategic solution for critical bleeding situations.

Keywords: Chitosan, Kaolin, Axiostat gauze, Quikclot combat gauze, Swine hemorrhage model, hemostasis.

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INTRODUCTION

Uncontrolled bleeding is the leading cause of preventable deaths on the battlefield, often determining whether soldiers survive long enough to receive advanced medical care. Rapid and effective intervention to stop hemorrhaging is crucial in such critical situations. An analysis of conflicts in Afghanistan and Iraq from 2001 to 2011 revealed that a staggering 90% of combat-related fatalities occurred before the patient could reach surgical facilities. Alarmingly, about 90% of these potentially preventable deaths were due to hemorrhage.(1)

In remote or conflict-ridden areas, access to advanced medical care may be severely limited or delayed. The absence of well-equipped medical facilities and trained personnel can hinder the timely administration of life-saving interventions, exacerbating the risks associated with severe bleeding. The critical nature of trauma and battlefield bleeding demands rapid interventions. However, the chaotic and unpredictable nature of these scenarios can create logistical challenges in delivering timely medical assistance. Efficient coordination, transportation, and communication are crucial to ensure that

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patients receive appropriate care as quickly as possible.[2-4] Unaddressed severe trauma and battlefield bleeding have far-reaching implications. Rapid blood loss can lead to hemorrhagic shock, organ failure, and ultimately death.[5-7] Furthermore, prolonged surgeries, increased blood transfusion requirements, and extended hospital stays place substantial burdens on healthcare systems, military resources, and patient recovery. Fatalities resulting from hemorrhage commonly transpire within the initial hours following an injury. Thus, the development and deployment of hemostatic agents accessible to patients or first responders before hospitalization assume paramount importance. Notably, advancements in hemostatic agents, surgical adjuncts, and blood products have substantially enhanced the pre-hospital management of critical hemorrhage.[8] An ideal hemostatic agent for the battlefield and pre-hospital hemorrhage control should have characteristics like quick and effective, easy administration, easy removal, low cost, prolonged stability, and good biocompatibility with no adverse effects.[2,9]

Numerous absorbable hemostats and sealants are available on the market; nevertheless, their widespread utilization is hindered by their inability to effectively manage profuse bleeding, lack of antimicrobial properties and, elevated costs and limited absorption capabilities. As a result of these factors, hemostatic agents have frequently been substituted with traditional laparotomy sponges and cotton gauze swabs during surgical procedures for fluid absorption. However, these conventional gauze dressings prove inadequate in staunching bleeding, leading to extended durations in the operating theater and necessitating blood transfusions, thereby escalating surgical expenses. Consequently, a demand arises for novel hemostatic solutions that are both efficacious and cost-effective.

The Committee on Tactical Combat Casualty Care (CoTCCC), introduced by the US Special Operations Command in 2002, serves as the established benchmark for pre-hospital care on the battlefield. Today, it operates under the control of the Department of Defense (DoD) and the Joint Trauma System (JTS).[10] CoTCCC endorses the use of a tourniquet as the recommended method to manage critical external bleeding that can be controlled anatomically by tourniquet application, including traumatic amputation scenarios. However, for severe bleeding that cannot be controlled with a limb tourniquet, CoTCCC has recommended several hemostatic dressings to control such bleeding. CoTCCC guidelines suggested applying hemostatic dressings with at least 3 minutes of direct pressure.[11] Topical hemostatic agents and dressings have significantly contributed to the successful management of extremity and compressible junctional hemorrhage, with ongoing advancements in product efficacy. Since the addition of QCG to CoTCCC Guidelines in 2008, chitosan-based hemostatic gauze dressings have shown comparable efficacy in severe hemorrhage based on animal studies.[12]

Chitosan has emerged as a promising candidate with remarkable hemostatic properties. It aids in hemostasis through various mechanisms, particularly electrostatic interactions. The positively charged amino groups of chitosan interact with the negatively charged surfaces of blood cells and tissues, enhancing adhesion and promoting clot formation. Additionally, chitosan helps activate platelets, leading to the formation of a fibrin-supportive matrix at the bleeding site, ensuring effective hemostasis. Recent evidence highlights that in military and battlefield environments, chitosan's rapid hemostatic action is invaluable, providing immediate interventions that can save lives. Combat-related injuries often involve profuse bleeding, making chitosan-based hemostats indispensable for controlling hemorrhage until comprehensive medical care can be administered.[13,14] Apart from hemostatic properties, the antimicrobial, biocompatibility and biodegradability of chitosan have been extensively studied.[15] Recent reports from civilian and military settings highlight successful outcomes and a favorable safety profile over many years of use. As a result, chitosan-based hemostats are widely adopted by US military Special Operations Forces, NATO militaries, and emergency medical services, underscoring their effectiveness and safety in clinical practice.[15]

The widely used FDA-approved hemostatic dressings include QuikClot Combat Gauze (kaolin-coated gauze), Celox Gauze, HemCon Chito Gauze, and Axiostat Patch. While Celox Gauze and ChitoGauze are chitosan-coated gauze with a potential risk of chitosan particle leaching during application, Axiostat is a 100% chitosan patch, making it more suitable for topical application as a safe hemostat. Additionally, the FDA-approved Axiostat Gauze (AG) is a 100% non-woven flexible gauze ideal for controlling severe bleeding for critical trauma applications.

The present study is conducted to compare the hemostasis efficacy and safety of the Axiostat gauze (AG) with Quikclot combat gauze (QCG) in a swine femoral arterial hemorrhagic model. QCG is well established and widely used by US Army, thus making an ideal comparator for this study. In the swine study, parameters such as time to hemostasis, blood loss reduction, survival rate and survival time were analyzed to evaluate the hemostasis efficacy and safety. AG utilizes proprietary Protonated Bioadhesive Technology (PBT®) embedded within its 100% chitosan non-woven fibers. This technology significantly enhances clotting efficiency by facilitating charge-based interactions with blood components, activating platelets via Toll-like receptors (TLR), initiating the clotting cascade, and promoting fibrin plug formation. Our previous studies have demonstrated that chitosan can activate platelets via Toll-like receptor 2 (TLR2), facilitating hemostasis in individuals on antiplatelet medications.[16] Kheirabadi *et al*. developed a swine model for hemorrhage to measure the efficacy of various hemostatic agents. The study model is based on the swine femoral artery injury model. This model for evaluation of hemostatic efficacy has gained acceptance as a standardized combat injury model from most of the investigators of military laboratories, including US Department of Defense.[17,18] Based on this model, several hemostatic dressings, including Celox Trauma Gauze, HemCon

ChitoGauze and BloodSTOP Ix Battle Matrix dressing, evaluate the hemostatic efficacy and safety.[2,19]

MATERIALS AND METHODS

Materials

Axiostat gauze (AG) samples were taken from Axio Biosolutions Pvt. Ltd., Quikclot combat gauze(QCG) samples and other experimental reagents and study accessories were purchased from online e-commerce stores.

Physical Characterization:

Appearance studies

The microstructure and overall surface morphology of both the dressing, including AG and QCG, were visualized using a scanning electron microscope (SEM) (JEOL Ltd., JSM-7900F) at an accelerating voltage of 3 kV.

Fluid absorbency

Both AG and QCG dressings underwent absorbency testing according to the BS EN 13726 standard. This test provided a measure of each dressing's ability to absorb fluid under standardized conditions. Each 5 × 5 cm dressing was initially weighed when dry (Wd). It was then immersed in 50 ml of Solution A for one hour and subsequently placed vertically to drain excess liquid. Solution A, designed to mimic the ionic composition of human serum or wound exudate, contains 142 mmol/L of sodium ions and 2.5 mmol/L of calcium ions from chloride salts. The dressing's weight after absorbing Solution A (Ww) was then recorded. Absorbency was calculated using the formula:

$$\text{Absorbency} = W_d/W_w$$

Apart from that, 5 cm diameter samples of each dressing were fully saturated in saline for 24 hours to evaluate how effectively the AG and QCG dressings retain absorbed fluid under pressure. After removal from the saline, the samples were initially weighed (w1), placed on a flat, dry, and clean surface, and subjected to an approximately 1000 gm weight for 3 minutes. Following the removal of the weight, the samples were reweighed (w2).

The fluid retention capacity was then calculated using the following formula:

$$\text{Percentage retention} = \frac{W2}{W1} \times 100$$

Particulate release from the topical hemostats

Based on ISO 10993-12, the sample extraction is prepared in ultrapure water free from particles and the extract is analyzed for particles by turbidimetry and microscopic methods. 5 × 5 cm of AG and CG were placed in a clean, particle-free glass container. The weight of the samples was noted prior to the extraction. The required amount of ultrapure water was added to the container as per ISO 10993-12 and extraction is continued at 50°C for 72 hours. Then, the extract is tested for the particulate matter by two different methods, i.e., turbidimetry and microscopic examination. For turbidity, the sample extract was analyzed at different time points (15 minutes, 24, 72 hours). A 1.5 mL extract from each of the samples at different time points was taken and the absorbance at 660 nm in a UV-visible spectrophotometer, considering ultrapure water as blank. The absorbance is then converted into turbidity by the following equation.

$$Turbidity\ (NTU) = 2.3\ \frac{Absorbance\ (A)}{Optical\ path\ length\ in\ meter\ (L)}$$

The observed particles were counted using the microscopic image of the extracts. The ultrapure water used for extract is used as control and imaged using microscope.

Antimicrobial Efficacy

Barrier to bacteria property

In this study, 5 x 5 cm of AG was used as the test sample, while similarly sized sterile cotton gauze served as the control. Both samples were challenged with approximately 10^6 CFU/mL of selected bacterial strains under aseptic conditions. The study included three gram-positive strains: *Staphylococcus aureus* (ATCC 29737), *Staphylococcus epidermidis* (ATCC 12228), *Micrococcus luteus* (ATCC 9341), and three gram-negative strains: *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 14169), and *Salmonella abony (ATCC 6017).* Nutrient Agar served as the growth medium for all bacterial strains.

Following inoculation, the bacterial interaction with both test and control dressings was allowed for 24 hours at room temperature to facilitate microbial migration through the material. Subsequently, the dressing materials were removed from the petri plates, which were then incubated at 37°C for 48 hours. Qualitative evaluation of bacterial growth was performed on all test and control plates after the 48-hour incubation period through the visualization method. The experimental setup and data analysis were appropriately designed to assess the barrier properties of the AG against bacterial infiltration.

Antimicrobial log reduction

The antimicrobial efficacy of AG dressing was evaluated against 11 most common wound pathogenic bacteria and fungus. The test organisms including *S. aureus* (ATCC 29737), *Staphylococcus epidermidis* (ATCC 12228), *Micrococcus luteus* (ATCC 9341), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 14169), *Aspergillus niger* (ATCC 16404), *Candida albicans* (ATCC 10231) etc.

The study methods are designed from ASTM E2315, AATCC 100 and AATCC 30 antifungal test protocol guidelines. Dressing samples, cut into 5 x 5 cm squares, were inoculated with approximately 10^5 to 10^6 CFU/mL of microorganisms and incubated at 37°C for 24 hours. After incubation, dressings were vortex-mixed in sterile PBS to eliminate remaining organisms and neutralize residual antimicrobial activity. Extracts were then serially diluted and plated on tryptone soya agar (TSA) or Sabouraud dextrose agar

(SDA) to enumerate bacterial and fungal colonies, respectively, after appropriate incubation periods.

In-vitro hemostatic efficacy

In-vitro blood clotting test involves quantification of non-coagulated erythrocytes after treatment with hemostatic materials. These free erythrocytes undergo hemolysis after dilution with water and hemoglobin is quantified using UV-spectroscopy. Blood from three healthy volunteers was collected into BD-citrated vacutainer blood collection tubes. The test is performed on fresh blood within 4 hours of collection. The test sample weighed 30 mg and was placed into a glass test tube. Then 0.25 mL of citrated blood was added directly onto the surface of the test sample, followed by 20 µL of $CaCl_2$ solution (0.2 M). The test tubes were incubated at 37°C for 10 minutes. After 10 minutes, 10 mL of DI water was added slowly into the test tubes. The red blood cells that had not been trapped in the blood clot were lysed and the free hemoglobin was dispersed in water. The concentration of this free hemoglobin was determined by measuring the absorbance of the supernatant at 545 nm using a UV-spectrophotometer. A solution of 0.25 mL of citrated blood with 20 µL of $CaCl_2$ solution (0.2 M) is used as a control. The blood clotting index of the test sample was calculated as a percentage of the control using the following formula.

$$Blood\ clotting\ index\ (BCI) = \frac{Test\ sample\ absorbance}{Control\ absorbance}\ X\ 100$$

In-vivo Hemostatic Efficacy

Ethical approval for swine arterial hemorrhage model

The study protocol (I/2018-39/IAEC/CVSc-Hyd/16/07/18) was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, New Delhi, India) and conducted at Dr. P.V. Narsimha Rao Telangana Veterinary University, Hyderabad, India. All animals received care and were used in strict compliance with the CPCSEA Guidelines.

Study design

The study design consisted of two study groups, AG and QCG, with 3 animals in each group. QCG, a product of Z-Medica Co. (Wallingford, CT), is a non-woven medical gauze impregnated with kaolin. The AG, a product of Axio Biosolutions Pvt. Ltd. (Ahmedabad, India), is a non-woven chitosan gauze (Table 1).

Animals

Yorkshire cross-bred male pigs weighing 38 to 45 kg were used in this study. The swine extremity hemorrhage model employed in the study was previously reported in the literature for the study of hemostasis.[22] The study was conducted in male pigs to avoid any possible differences in coagulation between the female pigs due to the estrus cycle.

Table 1: Description of the test items

Test item details	*AG*	*QCG*
Composition	100% Chitosan gauze	Kaolin-coated gauze
Manufacturer	Axio Biosolutions Pvt. Ltd.	Z-Medica Co.

Anesthesia and pre-surgery preparations

Before the surgery date, venous blood samples were collected for the complete blood count and standard clotting tests were performed to ensure that these parameters were within the normal range and met the inclusion criteria. After at least 1-week of acclimation, pigs were fasted for a minimum 12 hours before the surgery with free access to water.

Surgical procedure

The right carotid artery was cannulated for withdrawal of the blood and was used for continuous recording of blood pressure and heart rate throughout the experiment. The right jugular vein was catheterized (8.5/9 Fr catheter) for administering resuscitation fluid during hemorrhage and before achieving hemostasis. A midline laparotomy was performed, and the internal organs were grossly examined for normal appearance. The abdomen was closed by suturing with Vicryl (size 1) sutures, and the skin was stapled.

An incision of 10 cm was made on the skin in the groin area parallel and close to the femoral artery, the femoral artery was isolated by excising the muscles that directly covered the artery. A 5 cm section of the artery was dissected free from the surrounding tissues with cauterization and ligation (using 7–0 Proline) of small arterial branches. The vessel wall was completely cleaned and the protective sheet surrounding the adventitia was removed. The artery was covered with a small piece of gauze and bathed with a few milliliters of 2% lidocaine to relax the vasospasm and dilate the artery to its normal diameter (fully dilated). The maintenance fluid was discontinued and allowed a 5 to 10 minutes stabilization period. A stable mean arterial pressure (MAP) of 60 mmHg or higher was maintained during this period before the surgery. The baseline data, including MAP and body temperature, were recorded. Preinjury (baseline) blood samples were collected from the arterial line. The artery was clamped proximally and distally and a 6 mm diameter arteriotomy was made on the anterior surface of the vessel about 2 to 3 cm from the bottom of the groin using a 6 mm vascular punch. The clamps were released, and free bleeding was allowed for 45 seconds. The shed blood was collected by suction, weighed, and recorded as pretreatment blood loss.

Wound treatment and resuscitation

Immediately after the free bleeding and while bleeding continued, either the test or control gauze was opened from the package and the material was packed in the wound within about 1-minute. The material was immediately covered with a folded laparotomy sponge or equivalent gauze and was manually pressed for 3 minutes against the wound with sufficient and constant pressure to occlude the artery and stop the bleeding. Fluid resuscitation was started once the 3 min compression was completed (5 minutes after injury). 500 mL of Hextend was infused (6% HES in balanced electrolyte solution + glucose *via* the jugular vein catheter at 33 mL/minutes) for 15 minutes to

raise and maintain the MAP between 60 and 65 mmHg. After completion of the Hextend infusion, the fluid resuscitation was continued with lactated Ringer's (LR) solution at 100 mL/minute as needed to maintain the MAP at the same level. A maximum of 10 L of LR solution was used. We extended the limit of resuscitation fluid to 10 L as in the mentioned reference study protocol.[17] After compression, the pressure was slowly released by lifting the hand, and was checked for the hemostasis at 3 minutes without disturbing the dressings. If no bleeding was apparent during this period, it was considered that initial hemostasis has been achieved. The time for the bleeding to stop following compression was recorded as the bleeding/hemostasis time. The shed blood was weighed during this period and reported as post-treatment blood loss.

The pigs were monitored for up to 2.5 hours or until death. The death was pronounced when the MAP fell below 20 mmHg and remained at these levels for at least 2 minutes. The survival time was recorded, and final blood samples (arterial) were collected for biochemical analysis. The surviving pigs were examined till the end of the study. The treated legs of anesthetized pigs were flexed and stretched five times to simulate walking to test the stability of the hemostasis produced by the test agent. At the end of the study, the hemostatic products were slowly removed from the wound, and the status of hemostatic clots and the patency of the vessel were examined visually. The animals were euthanized with an intravenous injection of euthanasia solutions.

Biochemical analysis and Statistical analysis

The baseline and final blood samples were collected for biochemistry, complete blood count and plasma coagulation tests. Standard clotting tests were prothrombin time, activated partial thromboplastin time and fibrinogen. Primary endpoints were pre and post-treatment blood loss, time to achieve stable hemostasis (no sign of bleeding through the dressing), occurrence of re-bleeding and survival rate and survival time. Data were expressed as the mean ± SEM and analyzed by t-test. A *p-value* < 0.05 was considered to be statistically significant.

RESULTS

Appearance

The total internal fibrous structure of Axiostat gauze dressing and QCG gauze dressing are depicted in Figures 1a and 1b, respectively. The SEM images illustrate both dressings has non-woven fiber networks with a diameter ranging from 10 to 20 µm. There were no particles adhering to the fiber surface of Axiostat gauze dressing, whereas in case of combat gauze fiber kaolin particles were observed.

Fluid Absorbency

Upon exposure to solution A, the AG dressing absorbed more than 30 times its own weight and retained over 60% of the absorbed liquid within its core structure. In contrast, the QCG dressing absorbed approximately 10 times its weight and could retain up to 20% of the liquid. A detailed comparison of the absorbency and fluid retention capacities of both dressings is presented in Table 2.

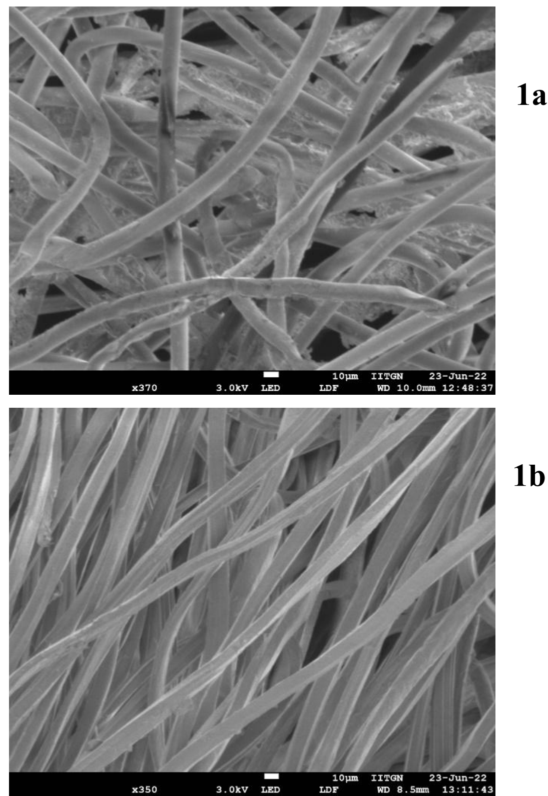

Figure 1: Appearance of test dressing. a. SEM image of the chitosan fibers in the Axiostat gauze dressing b. SEM image of the kaolin impregnated polyester based non-woven gauze in the QCG dressing. The scale bar represents 10 µm

Table 2: Results of fluid absorption capacity

Dressing	*Absorbency (times)*	*Fluid retention capacity (%)*
AG dressing	30 ± 2.15	60 ±0.68
QCG dressing	10 ± 1.76	20 ± 2.17

Particulate release from the topical hemostats

In the turbidimetry, it was observed that AG did not show any particles in the extract while QCG showed higher turbidity over the period of time (Figure 2). The extract of AG under the microscope showed negligible number of particles which was equivalent to the control ultrapure water. The extract of QCG showed particles that are in huge numbers (Figure 3).

Antimicrobial Efficacy

Barrier to bacteria

No microbial growth was observed in agar plates that had AG. In contrast, microbial growth was observed in plates with cotton gauze. The number of bacterial colonies present in the case of cotton gauze was above 300 which is considered as too numerous to count (TNTC). The results were consistent for all the tested organisms in all three plates for respective organisms. Based on the results of this study, it can be concluded that AG, has the property of a barrier to bacteria

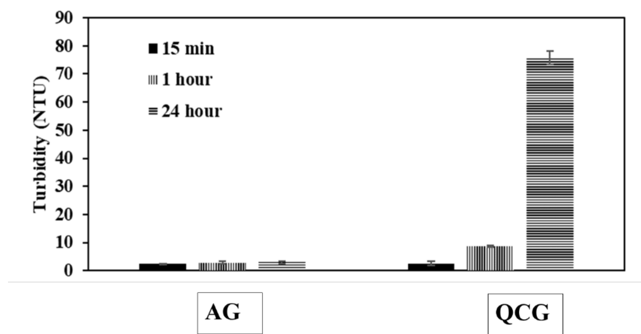

Figure 2: Comparison of the Turbidity level from the hemostat extracts

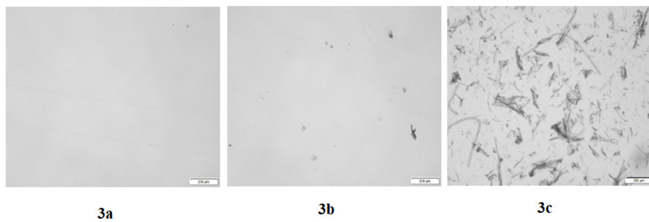

Figure 3: Particulate matter visualization in the hemostat extracts under the light microscope. Ultra-pure water (3a), AG extract (3b) and QCG extract (3c) under the microscope can be seen in the figure

and prevents the entry of microorganisms through its surface to the other side. AG showed a barrier to bacteria against the following strains of bacteria: *S. aureus, S. epidermidis, Micrococcus luteus, Pseudomonas aeruginosa, Escherichia coli, and Salmonella abony*. Table 3 shows the results with respective bacterial strains.

Log reduction result

After 24 hours of contact time, AG dressing showed more than 99.99% reduction of viable microorganisms against all the pathogenic microbial strains that causes wound infection. The mean value of microbial log reduction is summarized in Table 4.

The presence of chitosan molecule is responsible for showcasing the antimicrobial properties of AG dressing. Chitosan exerts its antimicrobial effects through several established mechanisms. Chitosan's positively charged amino groups interact with the negatively charged components of microbial cell membranes containing lipopolysaccharides. This interaction disrupts membrane structure, leading to increased permeability and leakage of cell contents, which can kill the microbes.[20,21] Chitosan can also interfere with microbial enzymes that are crucial for their metabolism and growth. This interference hampers microbial proliferation and contributes to its antimicrobial properties.[22,23]

***In-vitro* Hemostatic Efficiency**

The results indicated that the dressing achieved complete hemostasis in under 3 minutes and formed a strong blood clot that did not release any free erythrocytes when placed in excess water. The blood clotting index (BCI) of AG and QCG were found to be 0.14 ± 0.03 and 2.35 ± 0.36, respectively. Less BCI value indicates better hemostatic performance including strong and stable blood clot formation.

In-vivo hemostasis

The baseline hemodynamic and hematological parameters, including body weight, temperature, MAP, hemoglobin, platelet count, prothrombin time, activated partial prothromboplastin time and fibrinogen measured before the surgery were within normal ranges and were similar in both AG and QCG (Table 5).

Similar parameters were measured at the end of the experiments. The core temperature and pH remained unchanged, while, as expected, the hemodynamic parameters changed significantly at the conclusion of experiments as compared to the baseline values (Table 6). The 30 to 40% reduction in hemoglobin, platelet count, and fibrinogen concentration measured in post-treatment animals was partly because of the pretreatment (45-second initial free bleeding) and post-treatment blood losses and partly because of the hemodilution caused by fluid resuscitation, which was necessary to maintain the MAP at threshold levels.

The representative images showing the injury model and dressing application are shown in Figure 3. After isolating the femoral artery, it was treated with 2% lidocaine to dilate the artery. Bleeding was initiated and the bleeding started immediately once the clamps holding the blood flow were removed. Free bleeding was allowed for 45 seconds and the blood was collected by suction to determine the pretreatment blood loss. The pretreatment blood loss in both AG and QCG groups was similar.

The hemostatic dressings were applied within one minute after the free bleeding period and immediately packed with laparotomy sponges, followed by manual compression. The

Table 3: Microbial plate observation after 48 hours of incubation

S. No.	*Bacterial strain*	*Gram stain*	*Sign of microbial growth*					
			AG			*Sterile cotton gauze*		
			Plate - 1	*Plate - 2*	*Plate - 3*	*Plate - 1*	*Plate - 2*	*Plate - 3*
1	*S. aureus*	Gram-positive	No	No	No	Yes	Yes	Yes
2	*Staphylococcus epidermidis*		No	No	No	Yes	Yes	Yes
3	*M. luteus*		No	No	No	Yes	Yes	Yes
4	*P. aeruginosa*	Gram-negative	No	No	No	Yes	Yes	Yes
5	*E. coli*		No	No	No	Yes	Yes	Yes
6	*S.abony*		No	No	No	Yes	Yes	Yes

Table 4: Results of microbial log reduction after 24 hrs. of incubation for AG dressing

Tested microorganisms	*Log reduction*
S. aureus	5.27
E. faecalis	4.81
M. luteus	5.06
S. epidermidis	4.32
A. baumannii	5.61
P. aeruginosa	5.03
P. mirabilis	5.28
S. marcescens	5.14
Methicillin-resistant *S. aureus*	5.04
C. albicans	5.10
A. niger	4.77

manual compression continued for 3 minutes, and hemostasis was checked by slowly releasing the pressure. Hemostasis was confirmed by observing that no blood is oozing from the periphery of the dressing after releasing the manual compression. In some animals treated with AG immediate hemostasis was observed when compression was released at 3 minutes. While in the case of QCG group, none of the animals showed immediate hemostasis. All animals showed eventual hemostasis within 15 to 20 minutes. The study continued for three more hours to check the survival rate and survival time. At the end of the 3 hours, the hemostatic efficacy of test and control dressings is presented in Table 7.

The comparison of hemodynamic and hemostatic parameters between AG and QCG groups was presented in Table 7. The average pretreatment blood loss was similar in both groups, indicating that the injury and bleeding response were identical in both groups and total pretreatment blood loss for all animals was between 600 to 750 mL (Table 7). Immediate hemostasis was observed in 2/3 of animals treated with AG Gauze, whereas none of the animals treated with QCG showed immediate hemostasis when compression was released at 3 minutes. The remaining animals exhibited eventual hemostasis within 10-20 minutes.

At the end of the study, the leg of the pigs was flexed and stretched 5 times to simulate the walking action and to evaluate the stability of the hemostasis. The results showed that only one animal in the AG group maintained the hemostasis, while two animals in AG group and all animals in QCG exhibited active bleeding due to simulated walking action. The survival rate and survival time in both groups was similar, indicating that the hemostatic effects of AG and QCG are comparable (Figure 4).

DISCUSSION

Chitosan is well known for its property as a hemostatic biomaterial.[24-27] Topical hemostats containing chitosan has proven hemostatic ability in severe bleeding.[28-32] The most accepted mechanism of hemostasis for chitosan involves charge-based adhesion at the site of the wound and excellent absorption and locking mechanism clots the blood and stops the bleeding[33-35] AG topical hemostat has a positive charge

Table 5: Hemodynamic and hematological parameters of the swine before experimentation

Parameter	*AG*	*QCG*	*p value*
Body weight (kg)	41.1 ± 1.03	40.4 ± 1.0	0.78
Temperature (°C)	37.5 ± 0.18	37.7 ± 0.26	0.55
Mean arterial pressure (MAP) (mmHg)	78.1 ± 0.88	79.8 ± 2.41	0.55
Hemoglobin (g/dL)	9.4 ± 0.06	9.5 ± 0.06	0.17
Platelets (1,000/µL)	458.3 ± 30.18	436.0 ± 15.57	0.54
Prothrombin time (PT) (s)	11.9 ± 0.7	12.4 ± 0.95	0.69
Activated partial prothromboplastin time (aPTT) (s)	15.3 ± 0.42	14.9 ± 0.87	0.72
Fibrinogen (mg/dL)	258.3 ± 9.39	252.7 ± 6.01	0.61
pH	7.4 ± 0.01	7.4 ± 0.02	0.81

Table 6: Hemodynamic and hematological parameters of the swine after experimentation

Parameter	*AG*	*QCG*	***p** value*
Temperature (°C)	38.1 ± 0.22	38.1 ± 0.26	0.77
Mean arterial pressure (MAP) (mmHg)	63.5 ± 1.79	67.1 ± 1.7	0.29
Hemoglobin (g/dL)	8.0 ± 0.23	7.6 ± 0.18	0.20
Platelets (1,000/µL)	273.0 ± 34.02	236 ± 16.09	0.38
Prothrombin time (PT) (s)	11.3 ± 0.4	11.4 ± 0.37	0.87
Activated partial prothromboplastin time (aPTT) (s)	15.5 ± 0.36	15 ± 0.97	0.62
Fibrinogen (mg/dL)	222.1 ± 5.77	213.7 ± 3.71	0.30
pH	7.4 ± 0.01	7.4 ± 0.03	0.42

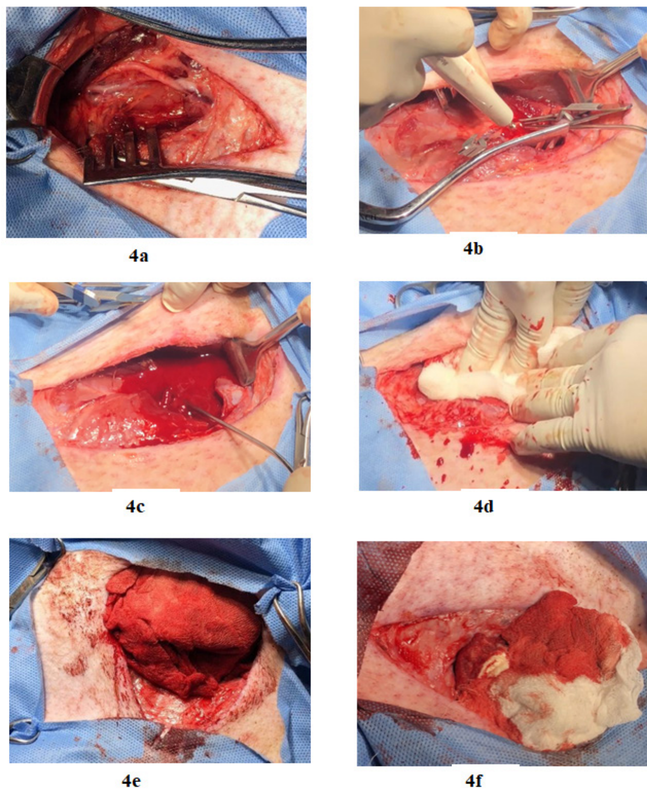

Figure 4: Representative images of vascular injury model in swine and application of test dressings. Isolated femoral artery (4a), after using a vascular punch (4b), free bleeding for 45 sec., blood was collected by suctioning to determine pretreatment blood loss (4c), application of hemostatic dressings. Axiostat gauze dressing was directly applied over the bleeding site and covered with a laparotomy sponge(4d), immediate hemostasis observed after releasing compression(4e), no re-bleeding at 180 minutes(4f)

Table 7: Hemostasis parameters in swine and study outcomes

Outcome	*AG*	*QCG*
Pretreatment blood loss (mL/kg)	16.3 ± 1.29	17.5 ± 1.24
Post-treatment blood loss (mL/kg)	7.1 ± 1.1*	36.2 ± 3.1
Total blood loss (mL)	966 ± 118*	2198 ± 149
Immediate hemostasis achieved	2/3	0/3
Eventual hemostasis achieved	3/3	3/3
Number of dressings used	1 per animal	1 per animal
Re-bleeding after simulated walking action	2/3	3/3
Survival rate	3/3	1/3
Survival time	>180 minutes	130 ± 26.46

AG – Axiostat rolled gauze; QCG – Quikclot combat gauze; **p-value* < 0.05

and blood components have a negative charge. Due to the attraction between positive and negative charges, it shows good bio adhesion. AG also initiates platelet activation via TLR receptor that helps initiate faster hemostasis and form stable blood clot formation.[16]

The present study has been designed to understand the hemostatic ability of chitosan gauze AG in comparison to a marketed kaolin-coated gauze QCG . Animal models serve as high reliability surrogates to study hemostasis. It is due to common molecular and cellular events similar to humans. The selection of a suitable animal model is important for extrapolation of the results to real clinical settings. There are many animal models that represent various classes of bleeding.[36,37] Our intention is to achieve severe bleeding in the animal and confirm the ability of the topical hemostatic agent at the end of the study. The leg of pigs was flexed and stretched 5 times to simulate the walking action and to evaluate the stability of the hemostasis. The results showed that only one animal in AG group maintained the hemostasis, while all other animals (two animals in AG group and all animals in QCG group) exhibited active bleeding due to simulated walking action. The survival rate and survival time in both groups was similar, indicating that the hemostatic effects of AG and QCG are comparable. The experimental data showed that AG offers better hemostasis efficacy in terms of faster hemostasis and reduction of overall blood loss with other benefits over QCG including antimicrobial efficacy and better fluid absorption.

Moreover, the importance of topical hemostat possessing antimicrobial attributes has grown significantly in both civilian and military trauma care settings. Zeolite or kaolin has no antimicrobial properties, so for kaolin gauze there is no protection for the bleeding site wounds from the risk of infection.[38] Chitosan's antimicrobial properties are poised to play an increasingly vital role in enhancing the benefits of chitosan-based hemostats like AG. AG dressing showed more than 99.99% reduction in the antimicrobial efficacy study. Chitosan's antimicrobial action arises from multiple mechanisms. It disrupts microbial cell membranes, inhibits enzyme activity crucial for microbial growth, and interferes with essential cellular processes. These multifaceted mechanisms contribute to its efficacy against bacteria, fungi, and even some viruses.[21,39,40]

In scientific literature based safety profile analysis, we found kaolin is a mineral that is insoluble in water and is present in particulate form which diffuses very rapidly. It's been shown that kaolin is also genotoxic, and the toxicity is size dependent.[41] The particles tend to diffuse out when the gauze touches the blood. Kaolin particles could be dislodged and cause emboli. In the particle release study, we observed the leaching of kaolin particles from QCG. Scientific evidence also showed that first generation kaolin impregnated dressing causes heat generation due to exothermic reaction of inorganic clay material.[42]

For the last few years marketed chitosan coated dressing has been shown to be more efficacious in controlling bleeding than QCG.[43] However, there is high risk of embolism in the case of chitosan granule or particle coated gauze and also the granules may flow in severe bleeding. AG is made up of 100% chitosan unlike the granules coated dressing. Thus, it is expected to work more efficiently than the simple chitosan

coated or chitosan impregnated gauze. Those risk of embolism are also not applicable for AG dressing as it does not contain any powder or granules.

CONCLUSION

The results of this study demonstrate that Axiostat gauze (AG) has a superior safety profile compared to QuikClot Combat Gauze (QCG), as indicated by the absence of particle release. AG also provided faster hemostasis, reduced overall blood loss, and greater fluid absorbency in comparison to QCG in a swine model. While AG achieved faster hemostasis, both AG and QCG eventually resulted in hemostasis in all animals, showing comparable overall hemostatic efficacy. In conclusion, AG is an effective hemostatic dressing with additional benefits, including antimicrobial properties and biocompatibility, making it suitable for managing severe traumatic bleeding. AG is recommended for use in both military and civilian pre-hospital settings as a strategic approach to effectively address critical bleeding.

Further research is needed to strengthen these findings, including animal studies with larger populations. Since this study was conducted under controlled conditions, which may not fully reflect real-world trauma challenges, future clinical research should explore a broader range of trauma scenarios, including various specific indications and diverse environmental conditions, to fully evaluate AG's performance.

DECLARATION

Anup Kumar, Animesh Agrawal and Leo Mavely are the current employees of Axio Biosolutions Private Limited, Ahmedabad, Gujarat, India.

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