

***In Vivo* Safety Evaluation of Granules and Dressing Hemostatic Agents from Radiation Processed Polymeric Materials**

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Granules and dressing hemostatic agents were developed from radiation-crosslinked carboxymethyl cellulose (CMC) and the combination of kappa-carrageenan (KC) and polyethylene oxide (PEO), respectively. Bioburden and sterility studies showed that 25 kGy irradiation dose was sufficient to achieve sterility in ten out of ten samples that were tested. The safety and biocompatibility of both granules and dressing hemostats revealed very promising results that support their suitability as medical devices for bleeding control. Extracts from CMC granules (CMC-G) and KC/PEO dressing (KP-D) hemostats injected into female and male Sprague-Dawley rats did not produce any systemic toxic signs like reduction in feed and water consumption and body weight. During the 14-d testing period, no rats in any of the treatment groups manifested behavioral, respiratory, and neurologic changes indicative of systemic toxicity. Hematology tests resulted in mean values within the published normal range. Blood chemistry assays gave normal alanine amino transferase, creatine, and blood urea nitrogen levels – indicating that the extracts were neither hepatotoxic nor nephrotoxic. Microscopic examination of the kidneys and liver revealed intact and normal structures with no inflammatory cells, fibrosis, or necrosis. No mortality occurred in all male and female test rats regardless of the treatment given; thus, the LD₅₀ for all treatment groups is zero (0). The skin irritation evaluation via intracutaneous injection of hemostat extracts generally did not induce erythema in four out of five rabbits, while edema was absent in all rabbits per treatment group throughout the 14-d test period. Both hemostat extracts had zero irritation score and is therefore classified as a non-irritant. The Guinea Pig Maximization Test (GPMT) of the Magnusson and Kligman method for skin sensitization potential classified both granules and dressing hemostats as weak sensitizers.

Keywords: hemostatic agents, irritation, safety, sensitization, systemic toxicity

INTRODUCTION

Uncontrolled hemorrhage was reported as the leading cause of preventable pre-hospital death after military and civilian trauma. It remained a persistent problem especially in anatomical areas where compression and tourniquet

cannot be applied (Granville-Chapman *et al.* 2011, Smith *et al.* 2012). In a study by the Division of Trauma of the Philippine General Hospital, one of the most frequent causes of death was uncontrolled bleeding – with the most common mechanisms including stab wounds, vehicular crashes, and gunshot wounds. From their statistics, 66% of trauma patients died within the first 24 h because of exsanguination (Consunji *et al.* 2011).

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The ability to control the flow of blood following vascular injury is critical to the survival of casualties of this kind of emergency situations. An anti-hemorrhagic agent, also known as hemostatic agent or hemostat, is a widely used biomedical device to arrest profuse bleeding. Different forms of hemostatic agents are commercially available, each adequately proven effective in their various applications. However, these hemostat products are all imported, hence not readily available as the costs are prohibitive. The most popular hemostat products – such as Celox, Combat Gauze, HemCon, Fibrin Sealant, and Modified Rapid Deployable Hemostat – are priced from USD 28 up to USD 500 for single-use packs, excluding shipment and exportation fees (Bennett and Littlejohn 2014). With the given lack of accessible and affordable hemostatic devices in the country, the healthcare sector remains in need of products to aid uncontrolled bleeding. In response to this challenge, our research team developed and prototyped two hemostatic agents (in granules and dressing forms) using of radiation technology, with the goal of translating the prototypes into commercial products to make it more accessible and affordable to local end-users. This will significantly contribute in the improvement of emergency response and possibly increase survivability in trauma victims in military battlefields, disasters, household accidents, and even in medical operations.

An ideal hemostatic agent must be safe and biocompatible, efficient to stop hemorrhage from actively bleeding large arteries and veins within 2 min, ready to use requiring no preparation, simple to apply and remove, lightweight and durable, has a minimum of two years shelf life and wide temperature storage stability, and inexpensive (Granville-Chapman *et al.* 2011, Smith *et al.* 2012). To develop hemostats conforming to these ideal characteristics, different natural and synthetic polymeric materials with known intrinsic bioactivity and biocompatibility such as KC, chitosan, CMC, polyvinyl alcohol, PEO, and polyvinyl pyrrolidone were screened for baseline clotting capability using the clotting assay described in the previous study. The best coagulative materials were observed to be KC and CMC, with blood clotting index significantly lower than that of the commercial hemostat product, Celox (Barba *et al.* 2016). KC has been used in wound dressing hydrogels because of its efficiency in absorbing pseudo-extracellular fluid solution (Silva *et al.* 2011, Singh *et al.* 2015), while carboxymethyl cellulose has been implicated to aid blood coagulation via physical, chemical, and physiological mechanisms (Wang *et al.* 2007). Among the synthetic polymers, only PEO had similar clotting capacity to Celox. PEO has been clinically used in the agglutination of blood by the osmotic drawing of water (Harmening 2012). These three polymers were selected as components for hydrogel-based hemostats developed by radiation crosslinking.

Radiation processing has been known to enhance polymer properties through the formation of crosslinking while simultaneously sterilizing the material for biomedical use. Furthermore, with no chemical initiators needed, the polymeric materials are simply mixed in water and remain free from toxic additives. Carboxymethyl cellulose can be radiation-crosslinked by itself at specific concentrations and irradiation dose (Fei *et al.* 2000, Fekete *et al.* 2014). KC is typically degraded upon exposure to ionizing radiation (Abad *et al.* 2014). As such, it was mixed with PEO – a radiation crosslinking polymer – to form an interpenetrating network (Tranquilan-Aranilla *et al.* 1999). Hence, the granules and dressing hemostat prototypes were based on radiation-processed CMC and KC/PEO, respectively. The chosen formulations were optimized in terms of concentration, blend ratio, particle size, and irradiation dose – which were assessed using *in vitro* clotting assays (Barba *et al.* 2016). The physico-chemical characteristic, *in vitro* efficacy, cellular toxicity, and shelf-life of the CMC-G and KC/PEO dressing hemostats were well described in our previous study (Barba *et al.* 2018). *In vitro* efficacy tests demonstrated their capability in clotting whole blood, adhering platelets, and accelerating clotting time. Cytotoxicity test results showed good cell viability for both hemostat forms, a preliminary indication of their biocompatibility. To assess further the safety of radiation-processed CMC-G (Figure 1A) and KP-D (Figure 1B) hemostats, samples were submitted for acute systemic cytotoxicity, skin irritation, and skin sensitization tests. The results are discussed in detail in this paper.

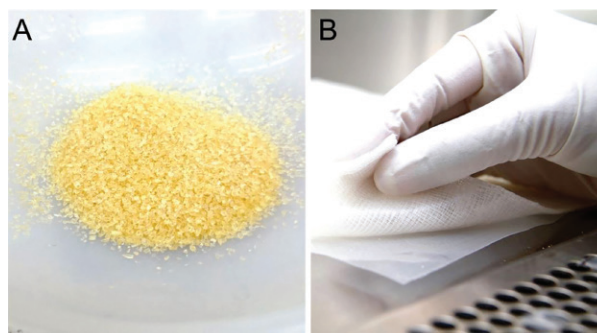


Figure 1. Developed hemostatic materials: (A) carboxymethyl cellulose granules (CMC-G) and (B) kappa-carrageenan/polyethylene oxide dressing (KP-D).

MATERIALS AND METHODS

Preparation of Hemostatic Agents

The granules were prepared from CMC (DS 0.8, MW 40 kDa, Celko 40000, CPKelco, Singapore) mixed with distilled water at 40% w/w to create a thick paste. After vacuum sealing in a foil pouch, the CMC paste was

crosslinked by gamma irradiation at a dose rate of 0.5 kGy/h until an absorbed dose of 40 kGy. The resulting hydrogel was oven dried at 50 °C, ground to a particle size range of 0.5–1.0 mm and packed into foil pouches at 5 g/pouch.

The dressing was fabricated by mixing KC (MW 920 kDa, Bengel WG-2000, Shemberg, Philippines) and PEO (MW 300 kDa, Sentry Polyox, DOW, Singapore) in distilled water to a final concentration of 2.5 and 5% w/w, respectively. The paste was spread onto three layers of cotton gauze and pressed to a uniform thickness of 1 cm. The final dressing form, with a dimension of 8 x 4 in and weighing 50 g, was vacuum-sealed in a foil pouch and subjected to one-step irradiation for crosslinking and sterilization.

Sterilization of hemostat products was carried out using gamma radiation from the Co-60 Irradiation Facility of PNRI. The hemostats in their final packaged form were exposed to a sterilization dose of 25 kGy.

Determination of Bioburden

Prior to sterilization, the bioburden of CMC-G and KP-D hemostats was determined in 10 replicates of each type. Using aseptic techniques, the samples were placed in an appropriate amount of diluent (1:10 sample: diluent) containing 0.1% buffered peptone water (DIFCO, Netherlands) and 0.85% sodium chloride. The samples were vigorously stirred into the buffer using a spatula. The initial solution (10^{-1}) was further diluted by a magnitude of ten (10^{-2} , 10^{-3} , 10^{-4}) by serial dilution. These were plated into Aerobic Count Plates (3M Petrifilm, USA) and incubated for 48 h at 35 ± 2 °C. The bioburden in terms of the number of colony-forming units (CFU) per gram of sample was calculated as follows:

$$\text{Bioburden (CFU/g)} = \frac{\text{counts} \times \text{dilution factor}}{\text{volume plated}} \quad (1)$$

Sterility Test

The sterility of CMC-G and KP-D was tested in 10 replicates. Tryptic soy broth or soybean casein digest medium (HiMedia, China) was prepared according to the manufacturer's specification. Using aseptic techniques, the samples were placed in bottles with enough medium to cover them. These were incubated for 14 days at 30 ± 2 °C. Microbial growth was indicated when the clear and transparent medium became turbid in appearance.

Safety Evaluation of Hemostatic Agents

The hemostatic agents were submitted to a battery of *in vivo* biocompatibility tests recommended for biomedical devices (Tian *et al.* 2015). Acute systemic toxicity, skin irritation, and skin sensitization potentials of extracts from CMC-G and KP-D hemostats were studied.

The Department of Basic Veterinary Sciences, College of Veterinary Medicine of the University of the Philippines Los Baños (UPLB) performed the acute systemic toxicity adapted from Auletta (2001) and intracutaneous irritation investigations in accordance with ISO-10993-10:2002 [Biological evaluation of medical products and devices – Part 10. Test for toxicity, irritation and hypersensitivity: Intracutaneous (intradermal) reactivity test]. All manipulations and procedures conducted in animals were approved by UPLB's Institutional Animal Care and Use Committee.

The skin sensitization potential in guinea pigs using the Magnusson and Kligman method was conducted by Palamur Biosciences Private Limited, India in accordance with the following regulatory references: OECD Guidelines for Testing Chemicals, Number 406 "Skin Sensitization" adopted on 17 Jul 1992; Biological evaluation of medical devices – Part 10: Test for irritation and skin sensitization (ISO 10993-10:2010); and Biological evaluation of medical devices – Part 12: Samples preparation and reference materials (ISO 10993-12:2012). The test facility was certified by the Committee for the Purpose of Control and Supervision of Experiment Animals and the study was approved by the Institutional Animal Ethics Committee.

Acute Systemic Toxicity

Thirty (30) male and thirty (30) female 8-wk-old Sprague-Dawley rats were individually caged in standard polycarbonate cages with stainless steel top and kept at 12 h light: 12 h dark cycle, 40-60% humidity and 22 ± 2 °C temperature. Commercial pellets and distilled water were provided *ad libitum* during the one-week acclimatization period. The rats were randomly allocated into three treatment groups with two replicates following a randomized complete block design: intravenous injection via the tail vein of 0.20 mL of (1) distilled water as the negative control, (2) CMC-G extract, and (3) KP-D extract. Blood was collected from the retro-orbital sinus of each rat using heparinized microhematocrit tubes at Day 1 (prior to injection) and at Day 14, which were analyzed to determine hepatotoxicity and nephrotoxicity using an automatic blood chemistry analyzer. The rats were monitored daily for body weight, feed intake, and water intake – as well as systemic toxic signs such as neurologic, respiratory, and behavioral changes. Morbidity and mortality rates were also monitored. After the test period, all rats were euthanized using pentobarbital sodium at a dose of 150 mg/kg body weight.

Gross observation of organs was done and then the liver, kidney, heart, spleen, and lungs were collected for histopathology.

Skin Irritation by Intracutaneous Toxicity Test

Fifteen (15) male, adult New Zealand rabbits were individually caged in a standard stainless rabbit cage and kept at 12 h light: 12 h dark cycle, 40–60% humidity, and 23 ± 2 °C temperature. Commercial pellets and distilled water were provided *ad libitum* during the 1-wk acclimatization period and throughout experimentation.

After the 1-wk acclimatization period, rabbits were randomly allocated into three treatment groups ($n = 5$ per group), namely: (1) distilled water as the negative control, (2) CMC-G extract, and (3) KP-D extract. Rabbits were anesthetized with pentobarbital sodium at a dose of 40 mg/kg body weight via the ear vein using a 1 mL disposable syringe and a gauge 30 needle (BD, USA). Hair in the mid-dorsum was clipped using a portable razor. The injection site was disinfected with sterile alcohol wipes and dried with sterile gauze. Each rabbit from the different treatment groups was injected with 0.2 mL of the distilled water, CMC-G, or KP-D extract intracutaneously using a 1 mL disposable syringe with gauge 26 disposable needle (Terumo, Japan). All solutions, including the negative control, were coded prior to administration to prevent bias.

Erythema and edema scorings following the ISO guidelines were done daily (refer to Table 1). Irritation scores were obtained from the sum of erythema and edema grades of each animal. The overall mean irritation score was calculated from the irritation scores at 24, 48, and 72 h after injection divided by 15 (three time points x five animal replicates) while the final score was obtained by subtracting the score of the control (distilled water) from

the test sample score. The requirements of the test are met if the final test sample score is 1.0 or less. Body weight was also monitored weekly using a digital top-loading balance.

After the 14-d test period, rabbits were anesthetized using pentobarbital sodium and a skin biopsy of the injection site was obtained using a biopsy needle for histologic processing and evaluation. Skin biopsy samples were fixed in 10% buffered formalin for at least 72 h, processed using the routine paraffin techniques, sectioned at 5 μ m in thickness using a rotatory microtome (American Optical, USA), and stained with H&E for microscopic evaluation.

Skin Sensitization by Guinea Pig Maximization Test

Two sets of eighteen (18) healthy young adult male guinea pigs (*Cavia porcellus*) acclimatized for 5 d were taken for randomization. Out of the 18 animals, three were eliminated in the randomization process, so that 15 animals were assigned into two groups in the main study. The first group (G1 control group) had five animals and the second group (G2 treatment group) had 10 animals.

Extracts of the CMC-G and KP-D were prepared by soaking a known weight in normal saline and kept in shaking water bath at 37 °C for 72 h. After completion of the incubation period, the solutions were filtered and used for dosing. Extraction ratios (mass/volume) were 0.1 g/mL and 0.2 g/mL for CMC-G and KP-D, respectively.

Preliminary tests were carried out to determine the high dose that causes mild or moderate skin irritation, as well as the highest non-irritant dose for both granule and dressing hemostats. Approximately 24 h before treatment (intradermal injections or topical applications), the application area was carefully clipped with a clipper to clean the site where the injections and topical applications were administered. Three (3) male guinea pigs were used – one each for intradermal induction, topical induction, and challenge tests. All scorings for skin reaction were done according to the Magnusson and Kligman grading scale (refer to Table 3).

For intradermal induction test, 0.1 mL of 1%, 5%, 7.5%, and 10% of the CMC-G extract in propylene glycol (vehicle) was intradermally injected at the scapular region of the guinea pig (Animal No. 1). In the case of KP-D extract using the same vehicle, 0.1 mL of 0.125%, 0.25%, 0.5%, and 1% concentrations were injected to the test animal. The test area was examined at 24, 48, and 72 h after injection for scoring erythema. For topical application and challenge tests, Animal No. 2 and No. 3 were applied with doses of 25%, 50%, 75%, and 100% concentrations of CMC-G and KP-D extracts in propylene glycol on both flanks. A filter paper (3 x 3 cm) was saturated with extract solution and applied to the test area. Degree of erythema and edema was evaluated

Table 1. Skin irritation scoring scheme^a.

Reaction	Irritation score	
Absent	0	
Erythema	Very slight erythema (barely perceptible)	1
	Well-defined erythema	2
	Moderate to severe erythema	3
	Severe erythema (beef redness) to eschar formation	4
Edema	Absent	0
	Very slight edema (barely perceptible)	1
	Slight edema (edges of area well defined by definite raising)	2
	Moderate edema (raised approx. 1 mm)	3
	Severe edema (raised more than 1 mm and extending beyond area of exposure)	4
	Maximum possible score for irritation	8

at 24, 48, and 72 h after patch removal. The observation period was 5 d.

Based on the pretest results, the concentrations of extracts in propylene glycol that were used for the main studies were as follows: (1) intradermal induction: 0.5% CMC-G and 10% KP-D; (2) topical induction: 100% CMC-G and KP-D, respectively; and (3) challenge test: 75% CMC-G and KP-D, respectively.

In the main study for the intradermal induction (Day 0), three pairs of intradermal injections of 0.1 mL volume were given in the shoulder region of the guinea pigs for both the treatment group and control group. The pairs of intradermal injections are enumerated in Table 2.

For the topical induction main study (Day 6), the scapular region of the animals (6 x 8 cm) was clipped prior to application. One week after the intradermal injections (Day 7), approximately 2 x 4 cm patch of filter paper (Whatman No. 1) was saturated with 0.2 mL and 200 mg of 100% CMC-G and KP-D extract solution, respectively and placed over the right scapular area of the test animals. The patches were covered with aluminum foil and secured with adhesive tape. The filter paper patches were removed after an exposure period of approximately 48 h. The guinea pigs in the control group were treated with distilled water only in the scapular region. The reaction sites were assessed for erythema and edema at 24 h observation after removal of the patch (Day 10).

The treatment and control groups were challenged 2 wk after the topical induction application. About 24 h prior to the application, hair was removed with a clipper from an area of approximately 5 x 5 cm on the left and right flank of each guinea pig. Two filter paper patches (approx. 5 x 5 cm for granules; 3 x 3 cm for dressing) saturated with 0.2 mL and 150 mg of 75% non-irritating concentration of CMC-G and KP-D extracts, respectively were applied to the left flank, while distilled water was applied on the right flank for all the animals using the same method. The patches were left in place for 24 h. At 48 and 72 h from the start of the challenge application, skin reactions were observed and graded as done previously.

For the entire duration of the study, all animals were routinely evaluated for clinical signs of pain and/or distress once daily. Based upon the percentage of animals sensitized (24 and 48 h reading), the hemostat extracts were assigned to one of the grades of allergenic potency according to the Magnusson and Kligman grading system (Table 3). As a reference, the positive control used was 2-mercaptobenzothiazole (75%).

Table 3. Magnusson and Kligman sensitization grading system^a.

Patch test reaction	Grading scale
No visible change	0
Discrete or patchy erythema	1
Moderate and confluent erythema	2
Intense erythema and swelling	3

Note: ^aISO10993-10 (2010)

Statistical Analysis

Data were presented as means \pm SD when applicable. Means of different treatment groups/parameters/samples were analyzed using ANOVA including post-tests such as Bonferroni test. Values were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Bioburden and Sterility of Hemostatic Agents

The safety of biomedical devices is a core goal of researchers, developers, or manufacturers. Sterility assurance is one of the key components in achieving that goal. For this study, gamma irradiation was utilized for sterilization of the hemostatic agents. Gamma irradiation is an internationally accepted and broadly used sterilization technology for biomedical devices. It is a physical method and considered a cold process because the temperature of the irradiated product does not increase significantly. It is most appropriate for sterilizing products that are sensitive to heat and chemical contamination (Aquino 2012,

Table 2. Injection scheme for the main study of intradermal induction.

Injection	CMC-G		KP-D	
	Treatment group	Control group	Treatment group	Control group
1	1:1 (v/v) FCA ^a / physiological saline	1:1 (v/v) FCA/ physiological saline	1:1 (v/v) FCA/ physiological saline	1:1 (v/v) FCA/ physiological saline
2	0.5% extract in propylene glycol	propylene glycol	10% extract in propylene glycol	propylene glycol
3	1:1 injection 1/ injection 2	1:1 injection injection 2	1/ 1:1 injection 1/ injection 2	1:1 injection 1/ injection 2

Note: ^a Freund's Complete Adjuvant

Lerouge and Simmons 2012). The established mechanism of action of gamma radiation in killing bacteria is through damage to DNA and consequently inhibiting bacterial division. The damage may be attributed to both the direct effect of radiation on DNA and the indirect effect arising from water-derived free radicals produced within the bacterial cells (Parsons 2012). The process is fast and requires only a single dose between 15–45 kGy to sufficiently destroy initial microbial load (Tipnis and Burgess 2018).

Prior to the sterilization process, the bioburden of the product was evaluated to characterize the population of viable microorganisms present in the device (*i.e.*, the total viable aerobic microorganisms). For CMC-G, an average bioburden of < 10 CFU/g or < 50 CFU/unit (5-g pouch) was observed. The KP-D, on the other hand, had an average bioburden of 250 CFU/g or 12,500 CFU/unit (8 x 4 in, 50 g). The higher bioburden value was expected because the moist nature of the dressing was conducive to microbial growth.

A dose between 10–60 kGy is typically used for medical and food industries in order to reduce bioburden by six orders of magnitude (Gradini *et al.* 2019). The hemostat samples were irradiated at 25 kGy, the most commonly validated dose used in medical devices (Simmons 2012). After irradiation, sterility of the samples was tested by soaking in tryptic soy agar (TSA) and incubating for 14 d. Microbial growth is indicated when the clear and transparent medium becomes turbid in appearance. For CMC-G, 10 out of 10 replicates were found sterile as indicated by the clear solution of TSA after 14 d. Though higher in bioburden, KP-D also achieved sterility at 25 kGy since no turbidity was observed in all the TSA media with test samples after 14 days. The sterile hemostats were submitted for safety evaluation studies.

Safety Evaluation of Hemostatic Agents

Biocompatibility can be defined as the ability of a material or device to perform an appropriate host response in a specific application (Zafar *et al.* 2019). Biocompatibility is governed not only of the material but also the interaction of the material with its environment (Perrotti *et al.* 2017). Biocompatibility tests are necessary for biomedical devices that contact the body and a wide range of tests are recommended by international standards and the Food and Drug Administration (FDA) guidance for devices that are under development in order to prove their biological safety. A biocompatibility test matrix [as per FDA 510 (K) G95-Memorandum and ISO 10993-1] has been developed as a guideline for biocompatibility evaluations (Tian *et al.* 2015). Depending on the biomedical device type, intended use, nature, and duration of contact with patients, there are at least three or more test recommendations that may be

performed (Bernard *et al.* 2018). The CMC-G and KP-D hemostatic agents were subjected to *in vitro* cytotoxicity during our previous study (Barba *et al.* 2018) and herein evaluated for acute systemic toxicity, skin irritation, and sensitization reactions.

Cellular toxicity or cytotoxicity investigation is the pilot step for the screening of material biocompatibility and safety. The acute adverse biological effects of extractables from the test material are performed on cultured mammalian cells (Li *et al.* 2015). *In vitro* methods – which provide rapid, reliable, and reproducible measurements – are available to evaluate the cytotoxicity of medical devices standardized by cell cultures. The methyl thiazolyl tetrazolium (MTT) assay is one of the most used assays to assess cytotoxicity by measuring cell viability based on the mitochondrial function of cells (Aslantürk 2017). In this colorimetric assay, mitochondrial dehydrogenase present in the living cells converts yellow water-soluble MTT to purple crystalline formazan. The amount of crystals formed is positively correlated to the number of cells and their activity, and the quantified colorimetric value of the absorbance (optical density) reflects the number of surviving cells (Vidal and Granjeiro 2017). Reduction of cell viability by more than 30% of the blank regards a material as cytotoxic according to ISO Standard 10993-5. From our previous study, we have reported that both granules and dressing hemostats were non-cytotoxic based on MTT assay. The cell viability values were 85% and 87% for CMC-G and KP-D hemostats, respectively (Barba *et al.* 2016).

Acute Systemic Toxicity

Acute systemic toxicity test identifies the potential effect of a medical device to cause adverse effects on the body's organs and tissues that are distant to the entry point after exposure to a single dose (Strickland *et al.* 2018). This test is required for materials that come in contact with blood or blood components or other internal tissues (Sastri 2013). The acute systemic toxicity was investigated by a single intravenous injection of the CMC-G and KP-D extract as well as the control substance (distilled water) through changes in physiological and hematology parameters.

The mean body weight, daily weight gain, daily and total feed, and water intake of male and female rats that received the treatments are presented in Figure 2. There was no significant difference in the physiological parameters measured during the 14-d testing regardless of treatment. Feed and water consumption of hemostat extract injected male and female rats fell within the normal average feed consumption of 10 g / 100 g body weight per day and average water consumption of 10 mL / 100 g body weight per day for Sprague-Dawley rats (Wolfensohn and Lloyd 2013).

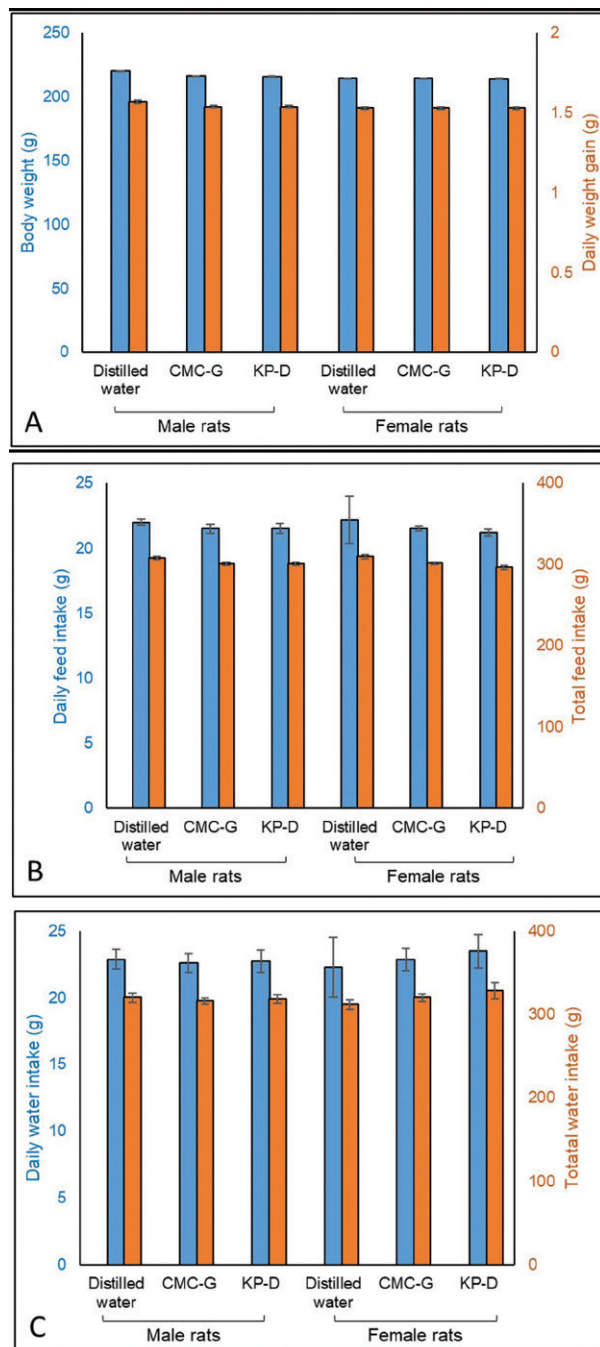


Figure 2. Physiologic parameters measured in male and female rats treated with distilled water and hemostat extracts: (a) body weight, (b) feed intake, and (c) water intake. Error bars represent \pm SD of $n = 5$. Comparison between sex is not statistically different using Student's T-test. No significant difference was found between control and treatments at $P < 0.05$.

Hematology test results are reflected in Table 4 and Figure 3. The total red blood cell (TRBC), total white blood cell (TWBC) counts, and packed cell volume (PCV) values at Days 1 and 14 were statistically comparable and the

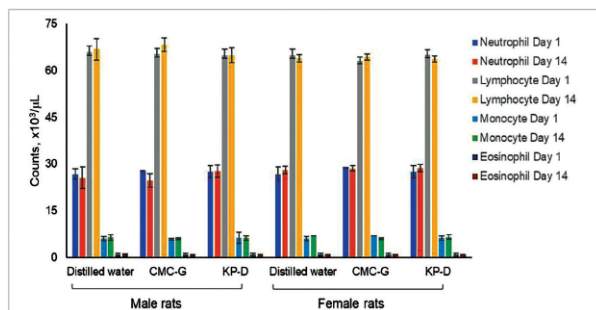


Figure 3. Relative WBC differential counts in male and female rats treated with distilled water and hemostat extracts at Day 1 vs. Day 14. Error bars represent \pm SD of $n = 5$. No significant difference was found between Day 1 and 14, and between control and treatments at $P < 0.05$.

obtained means fell within published normal TRBC, TWBC, and PCV range (Olfert *et al.* 1993). Relative white blood cell counts also did not significantly differ, and values for neutrophil, lymphocyte, monocyte, eosinophil, and basophil fell within the normal range (Thrall *et al.* 2012).

The blood chemistry profiles – given as mean alanine aminotransferase (ALT), creatinine and blood urea nitrogen (BUN) levels at Day 1 vs. Day 14 in male and female rats – are shown in Table 5. Blood ALT, creatinine and BUN levels of male and female rats from the different treatment groups did not significantly differ and the obtained results fell within the normal values for Sprague-Dawley rats. Comparison of mean ALT, BUN, and creatinine levels at Day 1 vs. Day 14 within the same treatment group was statistically comparable indicating that intravenous administration of the respective solutions did not cause any damage to the liver and kidneys. Moreover, comparison of mean ALT, BUN, and creatinine between male and female rats also did not significantly differ.

There were no observable gross abnormalities in the liver, kidneys, lungs, heart, spleen, and brain of the rats among the different treatments as shown in Figure 4. This was supported with microscopic examination of the kidneys and liver, which revealed intact and normal structures with no inflammatory cells, fibrosis, or necrosis (Figure 5).

Intravenous injection of the hemostat extracts in male and female rats did not produce any systemic toxic signs like reduction in feed and water consumption and body weight or abnormal hematology parameters. Blood chemistry assays showed that the extracts were neither hepatotoxic (normal ALT level) nor nephrotoxic (normal creatinine

Table 4. Hematology parameters (mean ± SD, n = 10) measured in male and female rats treated with distilled water and hemostat extracts at Day 1 vs. Day 14.

Treatment	TRBC count (x10 ⁶ /μL)		TWBC count (x10 ³ /μL)		Packed cell volume (%)	
	Day 1	Day 14	Day 1	Day 14	Day 1	Day 14
<i>Male rats</i>						
Distilled H ₂ O	6.56 ± 0.29	6.61 ± 0.22	6.17 ± 0.33	6.25 ± 0.26	40.80 ± 2.25	42.00 ± 1.63
CMC-G	6.78 ± 0.16	6.84 ± 0.12	11.93 ± 0.94	12.63 ± 0.49	39.65 ± 1.73	40.15 ± 1.31
KP-D	19.40 ± 0.55	19.38 ± 0.59	12.75 ± 0.99	12.89 ± 0.90	40.45 ± 1.34	41.23 ± 1.50
<i>Female rats</i>						
Distilled H ₂ O	6.03 ± 0.19	6.13 ± 0.23	6.16 ± 0.16	6.21 ± 0.18	40.00 ± 1.78	42.00 ± 1.58
CMC-G	6.07 ± 0.08	6.04 ± 0.12	6.13 ± 0.27	6.26 ± 0.30	39.75 ± 0.49	39.45 ± 0.83
KP-D	5.91 ± 0.14	6.00 ± 0.14	6.16 ± 0.23	6.11 ± 0.24	40.85 ± 1.25	40.60 ± 1.15

Note: Data at Day 1 vs. Day 14 per sex group and per parameter are not significantly different at *p* < 0.05 using ANOVA and Bonferroni test.

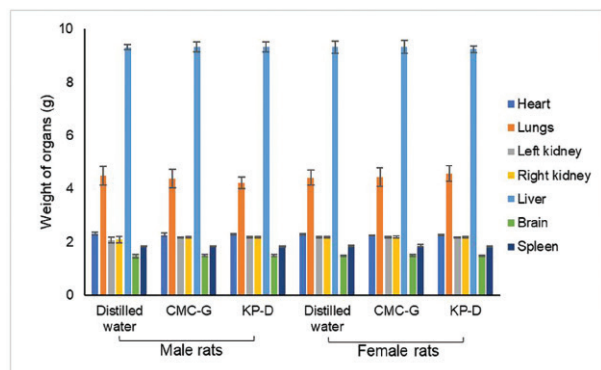


Figure 4. Weight of selected organs of male and female rats treated with distilled water and hemostat extracts. Error bars represent ± SD of n = 5. No significant difference was found between control and treatments at *P* < 0.05.

and BUN levels). During the 14-d testing period, no rats in any of the treatment groups manifested signs of toxicity or became sick after injection of their respective extracts. No behavioral, respiratory, and neurologic changes indicative of systemic toxicity was observed any rats from the three treatment groups. Both the gross and histopathologic examination revealed no abnormalities. In addition, no mortality occurred in all male and female test rats regardless of the treatment given. Thus, the LD₅₀ for all treatment groups is zero.

Intracutaneous Toxicity Test

Any chemical released or extracted from a medical device that contact the body may produce skin irritation. Such irritation is a local tissue response characterized by the usual signs of inflammation, redness, and swelling. Extracts from CMC-G and KP-D hemostats were intracutaneously injected at the mid-dorsum of the rabbits and changes in the skin area of injection site were examined for erythema, edema, and wound formation (Figure 6). Erythema or redness was not observed in all

Table 5. Blood chemistry parameters (mean ± SD, n = 10) measured in male and female rats treated with distilled water and hemostat extracts at Day 1 vs. Day 14.

Treatment	ALT (U/L)		BUN (mg/dL)		Creatinine (mg/dL)	
	Day 1	Day 14	Day 1	Day 14	Day 1	Day 14
<i>Male rats</i>						
Distilled water	19.47 ± 0.51	19.69 ± 0.54	11.93 ± 0.94	12.16 ± 0.74	0.35 ± 0.01	0.36 ± 0.02
CMC-G	19.22 ± 0.28	19.23 ± 0.30	11.93 ± 0.94	12.63 ± 0.49	0.34 ± 0.02	0.35 ± 0.02
KP-D	19.40 ± 0.55	19.38 ± 0.59	12.75 ± 0.99	12.89 ± 0.90	0.35 ± 0.01	0.36 ± 0.01
<i>Female rats</i>						
Distilled water	18.90 ± 0.42	19.10 ± 0.54	12.74 ± 0.66	12.33 ± 0.36	0.34 ± 0.02	0.34 ± 0.01
CMC-G	19.04 ± 0.48	19.30 ± 0.43	12.79 ± 0.94	12.33 ± 1.05	0.34 ± 0.02	0.35 ± 0.01
KP-D	18.89 ± 0.32	18.97 ± 0.27	12.70 ± 0.77	12.90 ± 0.96	0.33 ± 0.01	0.34 ± 0.02

Note: Data at Day 1 vs. Day 14 per sex group, per parameter and within the same row are not significantly different at *p* < 0.05 using ANOVA and Bonferroni test.

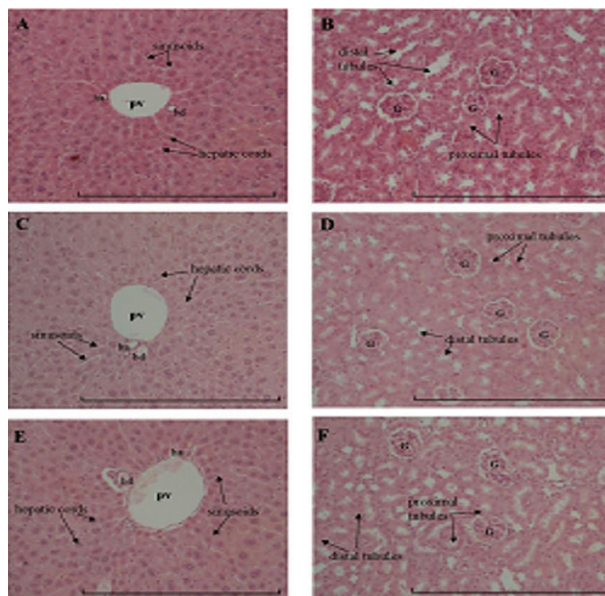


Figure 5. Photomicrographs of the kidneys and liver from representative rats treated with distilled water (A, D); CMC-G extract (B, E); and KP-D extract (C, F). Glomeruli and proximal and distal tubules of the kidney appear normal and intact. Liver hepatocytes in cord-like arrangement and sinusoids appear normal with no inflammatory cells, degeneration, and necrosis. Bar scale = 10 μ m, H&E, 40x. Pv = portal vein, ha = hepatic artery, bd = bile duct.

the rabbits injected with the control distilled water and in four out of five rabbits injected with CMC-G and KP-D extracts throughout the 14-d test period. One rabbit each from the hemostatic granule and dressing extract injected groups had very slight erythema 4 h after injection but the skin reaction was reversed by the end of Day 1. Thus, at 24 h and throughout the remaining test period, erythema was absent in all rabbits for all treatment groups (Figure 7). Edema or abnormal accumulation of fluid was absent in all rabbits regardless of treatment throughout the 14-d test period. Irritation scores for all treatments were zero



Figure 6. Gross pictures of representative rabbits with intracutaneous injections at the mid-dorsum during a 14-d period observation. The second column shows bleb formation immediately after injection. No visible erythema or edema was shown at 4 h, 24 h, 7 d, and 14 d post-injection.

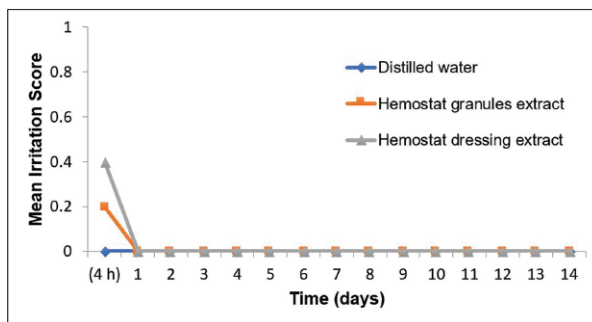


Figure 7. Mean irritation scores (sum of erythema and edema scores divided by the number of replicates) of the different treatments (n = 5) throughout the 14-d test.

and are therefore classified as non-irritants based on the Draize scale (Farage *et al.* 2011).

Skin biopsy samples from selected rabbits that received intracutaneous injections of distilled water, CMC-G extract, and KP-D extracts appeared normal – with intact epidermis and dermis (Figure 8). Intact hair follicles and glands were observed in the dermis of the rabbits from the different treatment groups. There were no signs of inflammation, degeneration, and necrosis. Another parameter monitored was the body weight of rabbits per treatment group. The mean weekly body weights are

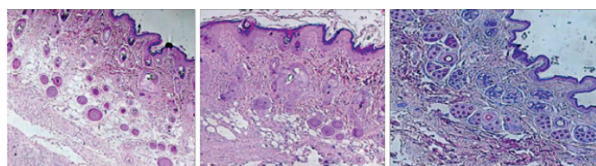


Figure 8. Skin section from representative rabbit from the different treatment groups showing intact epidermis and dermis with hair follicles and sebaceous glands. There was an absence of inflammation and growth on the injection site.

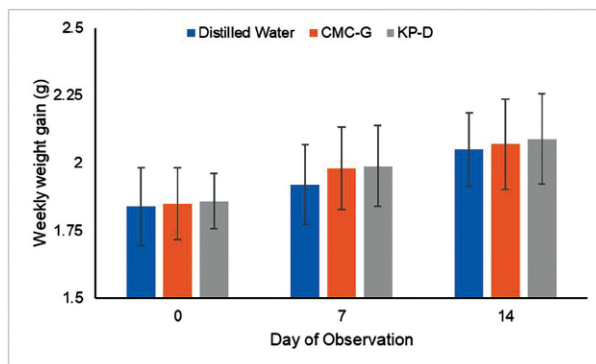


Figure 9. Weekly body weight of male rabbits intracutaneously injected with distilled water and hemostat extracts. Error bars represent \pm SD of n = 5. Continuous weight gain was observed during the 14-d period. No significant difference was found between control and treatments at $P < 0.05$.

shown in Figure 9. All rabbits regardless of treatment had a weekly increase in body weight. Statistical analysis showed comparable mean body weight for all treatment groups during the 14-d acute intracutaneous toxicity testing. These results show that injection of the hemostatic granule or dressing extract did not cause toxic effects that could have affected the body weight. In addition, rabbits in all treatment groups were consuming the average amount of feeds and water daily.

Skin Sensitization

The skin sensitization study was conducted to assess the allergenic potential of the hemostats when administered or applied to the skin of the guinea pigs. The study aimed to provide a rational basis for risk assessment of the sensitizing potential of the CMC-G and KP-D hemostats in humans.

For both CMC-G and KP-D hemostats, there were no observed toxic signs evident in the guinea pigs of the G1 control and G2 treatment groups. Also, no mortality was observed during the experiment. Normal body weight was observed among all animals in the G1 and G2 groups throughout the experiment period. While discrete or patchy erythema was observed on the hemostat treated animals during intradermal injection and topical induction, none of the animals showed skin reactions after challenge exposure with CMC-G and KP-D hemostats at 75% at 24- and 48-h observation of post patch removal. Based on such findings, the CMC-G and KP-D hemostats are classified as weak sensitizer – Grade 1 as per the Magnusson and Kligman (Table 3) classification since 0% sensitization rate was observed in the evaluation of challenge application

(Table 6). The hemostats can also be classified as the Category 1 of sub-category-1B of the Globally Harmonized System classification, since 50% response was observed in the evaluation of intradermal induction. The latter indicates that the products have the potential to produce sensitization in humans and should be properly labelled as such. Reputedly, the GPMT is one of the most sensitive methods in testing for sensitization such that in some case, it can overestimate sensitizing potential (ISO 10993-10). Clinical data should help confirm these findings. No skin reactions were observed in the animals from the control group and a 50% sensitization response was observed in the positive control (reliability check using 2-mercaptobenzothiazole).

CONCLUSION

CMC-G and KP-D hemostats developed through radiation processing were submitted for safety and biocompatibility evaluation. Microbial load assessment showed relatively low bioburden in the preparation of the hemostats, with sterility obtained at radiation sterilization dose of 25 kGy. Extracts from CMC-G and KP-D hemostats injected female and male Sprague-Dawley rats did not produce any systemic toxic signs like reduction in feed and water consumption and body weight. During the 14-d testing period, no rats in any of the treatment groups manifested behavioral, respiratory, and neurologic changes indicative of systemic toxicity. Hematology tests such as TRBC, TWBC, PCV, and differential white blood counts revealed mean values that fell within the normal range. Blood chemistry assays showed normal ALT, creatine, and blood urea nitrogen levels indicating that extracts were neither hepatotoxic nor nephrotoxic. There were no observable gross abnormalities in the organs of the rats among the different treatments supported with microscopic examination of the kidneys and liver, which revealed intact and normal structures with no inflammatory cells, fibrosis, or necrosis. In addition, no mortality occurred in all male and female test rats regardless of the treatment given; thus, the LD₅₀ for all treatment groups is zero. Intracutaneous injection of hemostat extracts generally did not induce erythema, while edema was absent in all rabbits per treatment group throughout the 14-d test period. Irritation score was zero, indicating that the hemostats are non-irritants. The skin sensitization or allergic potential assessed using the GPMT method classified both granules and dressing hemostats as weak sensitizers. Collectively, these findings showed that the CMC-G and KP-D hemostats are biocompatible and safe in terms of the above specifications and are fit for the next phase evaluation of its efficacy in animal model.

Table 6. Animal response during sensitization treatment of hemostat granules and dressing (*scored 1 on the Magnusson and Kligman grading system).

Parameters	Number of animals observed with discrete or patchy edema*	
	CMC-G	KP-D
Skin reaction after intradermal injection		
24 h at left shoulder region	5/15	4/15
24 h at right shoulder region	5/15	4/15
Skin reaction after topical induction		
24 h at right scapular region	6/15	5/15
Skin reaction after topical induction - challenge		
24 h at left flank	0/15	0/15
24 h at right flank	0/15	0/15
48 h at left flank	0/15	0/15
48 h at right flank	0/15	0/15

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