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Subcutaneous toxicity of a dual ionically cross-linked atelocollagen and sodium hyaluronate gel
Rat in vivo study for biological safety evaluation of the injectable hydrogel

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ABSTRACT

Hydrogels are commonly used in wound dressing, as they retain moisture, accelerate healing, and break down necrotic tissue. This process enhances patient comfort levels while simultaneously reducing pain caused by dead tissue. The purpose of this study was to investigate the in vivo toxicity of a dual hydrogel consisting of type I atelocollagen cross-linked with sodium hyaluronate hydrogel used for wound dressing.

Porcine type I atelocollagen was cross-linked with sodium hyaluronate to form the hydrogel. For subcutaneous implantation, 0.5 ml of dual hydrogel was injected into two different sites of twenty rats per group. High density polyethylene rods were implanted subcutaneously to serve as a control material. Hematological assessment, blood biochemistry, histopathological, and histological evaluations were scored and graded after 4 weeks. A bioreactivity rating was used for evaluation of subacute toxicity.

Differences observed in blood chemical analysis and hematological analysis between control and test groups were within normal variations and considered unrelated to the test article implantation. No significant implantation-related lesions were observed in any of the major organs of all test animals. The overall histopathological index of the test article implantation sites was evaluated as 0. The bioreactivity rating was evaluated as non-irritant after 4-week subcutaneous implantation.

Overall, these results indicate that the dual hydrogel of type I atelocollagen and sodium hyaluronate is biologically and chemically safe for clinical application as a wound dressing.

1. Introduction

Hydrogels have been broadly used in biomedical and pharmaceutical industries for many decades [1]. Polyvinyl alcohol (PVA) which is a synthetic polymer has been used for articular cartilage repair [2–5]. The vinylpyrrolidone based copolymer (PVP) has been used as a vehicle for dispersing and suspending drugs [7]. Artificial polysaccharides such as hydroxyethyl starch (HES) have been used for plasma volume replacement [8]. Hydrogels are also useful for wound healing because they can provide moisture to the wound.

Various types of open wounds such as abrasions, cuts, lacerations, punctures, and avulsion can seriously affect patient quality of life. Traditional dry dressing treatments have limited therapeutic effects because dry dressings are not optimal in all wound types with different bleeding conditions such as arterial bleeding, venous bleeding, and capillary damage [9,10].

Wet wound dressings promote more rapid formation of collagen at the wound site, which is necessary for healing of complicated wounds [11]. The initial goal of using a wound dressing is to stop bleeding and initiate clotting [12]. The dressing should absorb excess blood, plasma, and other fluids. The wound dressings should also protect skin ulcers, burns, or wounds from irritation to facilitate proper healing. Some materials can also relieve itching and pain arising from various skin conditions [13,14].

To treat the wide spectrum of wounds, different types of wound dressing are used, such as cloth, foam, hydrocolloid, and hydrogel. Hydrogel dressings add moisture to the wound, accelerate healing, and break down dry and necrotic tissue. This process enhances patient

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Table 1
Hematological values of rats (n = 20; male = 10, female = 10).

Group (Male)		WBC	WBC Differential Counting (%)					RBC	Hb	HCT	MCV	MCH	MCHC	Retic	PLT	PT	APTT
		(K/ul)	NE	LY	MO	EO	BA	(M/ul)	(g/dl)	(%)	(fL)	(pg)	(g/dl)	(%)	(K/ul)	(sec)	(sec)
G1	Mean	7.18	13.7	81.8	2.3	1.0	0.1	7.81	15.4	45.2	57.9	19.8	34.1	2.03	1165	18.5	21.0
	S.D.	2.08	3.2	3.5	0.4	0.3	0.0	0.32	0.5	1.1	1.5	0.6	0.6	0.33	80	3.7	2.6
G2	Mean	6.78	9.9	86.8*	1.4*	0.9	0.1	7.95	15.8	47.1	59.2	19.8	33.5	1.94	1098	20.3	19.9
	S.D.	1.70	2.9	2.8	0.4	0.2	0.0	0.28	0.6	2.2	1.9	0.5	0.4	0.31	80	3.2	1.3
Group (Female)		WBC	WBC Differential Counting (%)					RBC	Hb	HCT	MCV	MCH	MCHC	Retic	PLT	PT	APTT
		(K/ul)	NE	LY	MO	EO	BA	(M/ul)	(g/dl)	(%)	(fL)	(pg)	(g/dl)	(%)	(K/ul)	(sec)	(sec)
G1	Mean	4.56	12.0	83.6	2.1	1.1	0.1	7.72	15.0	43.4	56.2	19.5	34.7	1.87	1107	15.4	15.7
	S.D.	1.10	5.5	6.1	0.7	0.3	0.1	0.20	0.4	0.8	1.5	0.8	0.6	0.57	42	0.4	1.1
G2	Mean	4.61	11.9	83.7	2.2	1.1	0.1	7.90	15.1	44.5	56.4	19.1	33.9*	1.42	1244	15.7	18.1*
	S.D.	1.10	3.4	3.9	0.6	0.4	0.1	0.45	0.3	1.2	2.1	0.8	0.3	0.15	241	0.2	0.8

Significant differences as compared with control: *p < 0.05.

Table 2
Blood chemical values of rats (n = 20; male = 10, female = 10).

Group (male)		TP	ALB	ALP	AST	ALT	BUN	T-CHO	TG	GLU	CA	IP	CK	Na	K	Cl
		(g/dl)	(g/dl)	(U/L)	(U/L)	(U/L)	(mg/dl)	(mg/dl)	(mg/dl)	(mg/dl)	(mg/dl)	(mg/dl)	(IU/L)	(mmol/L)	(mmol/L)	(mmol/L)
G1	Mean	5.8	3.9	452	117	24	13.3	56	25	132	10.3	8.2	746	142.7	4.79	101.4
	S.D.	0.3	0.1	115	12	2	1.5	18	12	13	0.3	0.2	223	1.1	0.34	1.3
G2	Mean	5.9	3.9	567	97*	24	15.2	48	23	132	10.3	8.5	388*	142.9	4.73	101.7
	S.D.	0.2	0.1	142	13	3	3.6	9	8	5	0.1	0.5	129	0.9	0.38	1.1
Group (female)		TP	ALB	ALP	AST	ALT	BUN	T-CHO	TG	GLU	CA	IP	CK	Na	K	Cl
		(g/dl)	(g/dl)	(U/L)	(U/L)	(U/L)	(mg/dl)	(mg/dl)	(mg/dl)	(mg/dl)	(mg/dl)	(mg/dl)	(IU/L)	(mmol/L)	(mmol/L)	(mmol/L)
G1	Mean	6.1	4.1	339	73	18	16.0	48	10	143	10.3	7.3	190	141.2	4.29	104.3
	S.D.	0.2	0.1	90	6	1	1.4	8	2	13	0.3	0.4	81	1.6	0.23	1.7
G2	Mean	6.1	4.0	303	74	18	17.7	52	14	144	10.1	7.0	250	141.2	4.48	103.7
	S.D.	0.2	0.1	56	7	2	2.9	16	9	11	0.1	0.2	71	1.1	0.17	1.2

Significant differences as compared with control: *p < 0.05.

comfort levels while simultaneously reducing the pain caused by dead tissue [15]. Hydrogel wound dressings are therefore commonly used in a wide range of wounds.

Natural biopolymers such as hyaluronic acid and collagen have been used to formulate a variety of hydrogels for cosmetic and pharmaceutical applications [16–19]. Hyaluronan or hyaluronic acid (HA) is a natural linear polymer with promising biomedical and cosmetic applications [20]. It has multiple functions including space-filling, lubrication, and regulation of the hydro-physiologic environment of critical cellular activities including cell growth, migration, and differentiation [22–24]. Sodium hyaluronate (403.31 g/mol) has a lower molecular weight than HA (846.8 g/mol) or hydrolyzed HA and is more easily absorbed into skin pores [25]. HA works at the surface level to smooth and hydrate the skin, and sodium hyaluronate does the same on a deeper level and helps reduce signs of aging. Sodium hyaluronate is a powerful humectant that attracts and holds water, hydrating the skin and keeping it moist. However, it is limited by poor mechanical properties and rapid degradation in vivo [26,27]. Collagen dressings are useful for chronic wounds, controlling incidence of bioburden and reducing occurrences of wound infection. Despite these advantages of collagen as a biomaterial for wound dressing application, matrix metalloproteinase-induced degradation of collagen-based dressings raises concerns in terms of dressing forming-stability, non-controllable swellability, and poor hydrated mechanical properties [28]. Atelocollagen is a low-immunogenic derivative of collagen obtained by removal of N- and C- terminals telopeptide components, which are known to induce antigenicity in humans [29]. Commercial type I atelocollagen-based hydrogel for wound dressing such as Aquacel® and Mepilex® and type I atelocollagen hydrogels had higher compressive modulus and comparable water uptake with respect to commercial dressings [30].

A dual hydrogel (Juvencoll, D-med, Seoul, South Korea) combining the benefits of sodium hyaluronate and type I atelocollagen is likely to be more effective than either one. Sodium hyaluronic acid binding peptide-polymers have been studied for osteoarthritis treatment [31,32]. Sodium hyaluronate reduces the friction in the joints and enhances function. Collagen stimulates proteoglycan synthesis which helps in regenerating cartilage and lowers pain and inflammation [33,34].

The objective of this study was to assess the safety of a hydrogel composite of Type I atelocollagen and sodium hyaluronate. This composite hydrogel of collagen and sodium hyaluronate for wound dressing was evaluated for subacute toxicity and reactivity in vivo.

2. Materials and methods

2.1. Preparation of type I atelocollagen and sodium hyaluronate hydrogel

3 % (w/v) acidic collagen type I gel (D-med, Seoul, Korea) extracted from porcine skin was dissolved with phosphate-buffered saline by using an overhead stirrer with pH adjusted between 6–8 with 10 M NaOH solution. 15 % (w/w) sodium hyaluronate A (D-med, Seoul, Korea) was dissolved with 0.2 N NaOH solution by using a magnetic stirrer. 1,4-Butanediol diglycidyl ether 0.3 % (w/w) was used as the crosslinking agent. 3.5 % (w/w) sodium hyaluronate B (D-med, Seoul, Korea) was dissolved with phosphate-buffered saline by using a magnetic stirrer. Both hyaluronate A (cross-linked) and B (uncross-linked) were mixed at a ratio of 30(A):70(B). The neutralized collagen and cross-linked sodium hyaluronate were then mixed at a ratio of 50:50. The mixed hydrogel was filled in syringes and stored at 4C° until use for the experiment.

Table 3
Number of animals with histopathological findings (n = 20; male = 10, female = 10).

Organs	Histopathological findings	Male		Female	
		G1	G2	G1	G2
Liver	No abnormalities	5	4	4	4
	Hepatocyte vacuolation minimal multifocal	–	1	1	–
	Inflammatory cell foci minimal multifocal	–	–	–	1
Kidney	No abnormalities	4	5	5	5
	Tubular basophilia minimal multifocal	1	–	–	–
Adrenal gland	No abnormalities	5	5	5	5
Heart	No abnormalities	5	5	5	5
Lung	No abnormalities	5	4	4	4
	Macrophage infiltration, alveolar minimal multifocal	–	1	1	1
Brain	No abnormalities	5	5	5	5
Spinal cord	No abnormalities	5	5	5	5
Spleen	No abnormalities	5	5	5	5
Testis / Ovary	No abnormalities	5	5	5	5
Trachea	No abnormalities	5	5	5	5
Esophagus	No abnormalities	5	5	5	5
Thymus	No abnormalities	5	5	5	5
Thyroid	No abnormalities	5	5	5	5
Stomach	No abnormalities	5	5	5	5
Intestine	No abnormalities	5	5	5	5
Bladder	No abnormalities	5	5	5	5
Eye	No abnormalities	5	5	5	5
Skin	No abnormalities	5	5	5	5
Muscle	No abnormalities	5	5	5	5
Femur	No abnormalities	5	5	5	5

2.2. Rat subacute toxicity model

This animal study was approved by The institutional animal care and use committee of Korea Testing and Research Institute. A total of 40 rats (Sprague Dawley specific-pathogen-free rats, male (n = 20, 225.04 ~ 243.06 g), female (n = 20, 155.51 ~ 176.45 g), Orient Bio Co., Ltd., Korea) were used for subcutaneous implantation. After clipping the fur before the day of implantation, implantations in the dorsal subcutaneous region of each animal were carried out using a needle under isoflurane anesthesia. 0.5 ml of test or control material was implanted in 2 sites for each animal. High-density polyethylene rods were used as a control material and were implanted subcutaneously in group #1 (G1, n = 20; female: n = 10, male: n = 10). Atelocollagen-sodium hyaluronate gels were implanted as the test material in group #2 (G2, n = 20; female: n = 10, male: n = 10).

2.3. Hematological examination

After implantation, animals were individually observed once a day for 4 weeks. The animals were fasted overnight prior to blood sampling and euthanasia. Blood samples were collected from the posterior aorta under isoflurane anesthesia. Approximately 2.0 mL of blood sample was added to an EDTA tube containing EDTA (K2EDTA 3.6 mg, BD, USA), and then analyzed by a hematological analyzer (ADVIA 2120i, Siemens, USA). A blood sample was placed in A 9NC Citrate tube (Buffered Sodium Citrate 0.109 M, 3.2 %, BD, USA) and centrifuged (300 rpm, 10 min). The plasma was analyzed with a coagulation analyzer (ACL ELITE PRO, Instrumentation Laboratory, USA) for prothrombin time (PT) and activated partial thromboplastin time (APTT) (Supplemental Table 1).

2.4. Blood biochemistry examination

To extract sera for blood biochemistry, blood samples were coagulated and centrifuged at 3000 rpm for 10 min. The biochemistry analyzer (TBA-120FR, Toshiba, Japan) was used for the assessment of blood biochemistry (Supplemental Table 2).

2.5. Histopathological examination

Internal organs (listed in Table 3) were collected and fixed with 10 % neutral buffered formalin (except for testis and epididymis which were fixed with Bouin's solution, and eyeballs which were fixed with Davison's solution). The implantation site was dissected along with adjacent tissue and inspected macroscopically before fixation in 10 % neutral buffered formalin. After fixation, tissue specimens were prepared using Hematoxylin & Eosin (H&E) stain and histopathological grade was scored using the grading system described in Supplemental Table 3.

2.6. Histological evaluation

The histological evaluation of the test and control material implantation was conducted, and the biological response parameters were recorded in 7 categories listed in Supplemental Table 4. Histological evaluation was measured using the scoring system described in Supplemental Table 5.

2.7. Evaluation of subacute toxicity results

The mean scores for each group were first calculated and then added to obtain the total score of the biological response divided by the number of the implantation sites. After calculation of the difference in mean scores between test materials and control materials, the bioreactivity rating was evaluated (Supplemental Table 6).

Table 4
Macroscopic findings of implantation sites in rats (n = 20; male = 10, female = 10).

Animal No.		1		2		3		4		5		6		7		8		9		10		Mean
G1	Inflammation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Encapsulation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Hemorrhage	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Necrosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Discoloration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Total	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G2	Inflammation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Encapsulation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Hemorrhage	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Necrosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Discoloration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Total	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 5
Microscopic findings of implantation sites in rats.

Group	Category	Animal number									
		1	2	3	4	5	6	7	8	9	10
G1 (Control)	Inflammation Polymorphonuclear leukocyte	0	0	0	0	0	0	0	0	0	0
	Lymphocytes	0	0	0	0	0	0	0	0	0	0
	Plasma cells	0	0	0	0	0	0	0	0	0	0
	Macrophages	1	1	1	2	1	1	1	2	2	1
	Giant cells	0	0	0	0	0	0	0	0	0	0
	Necrosis	0	0	0	0	0	0	0	0	0	0
	Sub-Total (x2)	2	2	2	4	2	2	2	4	4	2
	Neovascularization	0	0	0	0	0	0	0	0	0	0
	Fibrosis	0	0	0	0	0	0	0	0	0	0
	Fatty infiltrate	0	0	0	0	0	0	0	0	0	0
	Sub-Total	0	0	0	0	0	0	0	0	0	0
	Total	2	2	2	4	2	2	2	4	4	2
	Group Total	26									
	Traumatic necrosis	0	0	0	0	0	0	0	0	0	0
	Foreign debris	0	0	0	0	0	0	0	0	0	0
	No. sites evaluated	10									
G2 (Test)	Inflammation Polymorphonuclear leukocyte	0	0	0	0	0	0	0	0	0	0
	Lymphocytes	0	0	0	0	0	0	0	0	0	0
	Plasma cells	0	0	0	0	0	0	0	0	0	0
	Macrophages	1	1	1	1	1	1	1	1	1	1
	Giant cells	0	0	0	0	0	0	0	0	0	0
	Necrosis	0	0	0	0	0	0	0	0	0	0
	Sub-Total (x2)	2	2	2	2	2	2	2	2	2	2
	Neovascularization	0	0	0	0	0	0	0	0	0	0
	Fibrosis	0	0	0	0	0	0	0	0	0	0
	Fatty infiltrate	0	0	0	0	0	0	0	0	0	0
	Sub-Total	0	0	0	0	0	0	0	0	0	0
	Total	2	2	2	2	2	2	2	2	2	2
	Group Total	20									
	Traumatic necrosis	0	0	0	0	0	0	0	0	0	0
	Foreign debris	0	0	0	0	0	0	0	0	0	0
	No. sites evaluated	10									
	Average*	0.0									

Bioreactivity rating = 0.0 = Average (test – control).
* Average = Test (groups total score / No. sites evaluated) – Control (group total score / No. sites evaluated).

2.8. Statistical analysis

The student's *t*-test was used for comparing the mean values of hematology and blood chemical analysis. All the statistical analysis was performed by the SPSS (Ver 21.0).

3. Results

There were no abnormal clinical signs and dead animals were observed during the experimental period. The changes in body weight for all male and female groups were not statistically different compared to the control group, and general variations of body weight were observed for 4 weeks (Supplemental Table 7). In organ weights, statistical difference was not observed in all male and female groups compared to the control group (Supplemental Table 8). Any necropsy findings associated with implantation of the hydrogel were not observed throughout the main organs of all animals (Supplemental Table 9).

Hematologic analysis of male rats revealed differences in leucocyte count of monocyte (MO), and lymphocytes (LY) between control and experimental groups. For female rats, cell hemoglobin concentration (MCHC), and activated partial thromboplastin time (APTT) were significantly different between the two groups (Table 1). The blood chemical analysis also resulted in statistically significant differences in Aspartate aminotransferase (AST), and creatine kinase (CK) in male rats. However, no significant difference was observed in female rats (Table 2).

Few histopathologic lesions were observed in organs harvested from male and female rats of both groups (Table 3). In male rats, there was evidence of hepatocyte vacuolation in the liver and alveolar macrophage infiltration in the lung (Supplemental Fig. 1). In female rats, we observed inflammatory cell infiltration in the liver and alveolar macrophage infiltration in the lung (Supplemental Fig. 2). Their lesions were generally minimal in degree with no difference in frequency between control and test groups. All the lesions observed above were considered within the background levels for the species, sex and age of animals being tested in this study (Table 3).

On macroscopic evaluation, there was no abnormal macroscopic appearance associated with the hydrogel, and the macroscopic evaluation score was 0 (Table 4). Histological evaluation of the group implanted with the dual hydrogel revealed minimal macrophage infiltrations in all 10 test injection sites. This infiltration was similar to that observed in all 10 sites implanted with control material. The net bioreactivity rating of the test group relative to the control group was therefore 0 (Table 5).

4. Discussion

It is important to evaluate the biocompatibility of chemically and biologically formulated hydrogels for clinical use. The various synthetic polymers such as PVA, PVP, and HES have been known as biocompatible based on many cell and animal studies. Biological evaluation of these materials based on international standards such as the ISO10993 series is essential for clinical use. The biocompatibility and hemocompatibility of PVA based on ISO 10993-6 showed a low risk of hemolysis in in-vitro and in-vivo studies [6,35]. Another study about PVP showed controlled degradation effects by mixing ratio and temperature based on ISO

10993-13 [36]. HES studies showed the possibilities of hydrogel carrier systems for controlled drug delivery [37,38].

This study was performed to investigate the local effects and the systemic toxicity in SD rats 4 weeks after implantation of a wound dressing material composed of a dual hydrogel of atelocollagen and sodium hyaluronate. All animals survived the 4-week test period after implantation and no abnormal clinical signs related to the test substance implantation were observed in any animal during the test period.

There were no significant changes of body weights including organ weights compared to the control group suggesting that the implanted hydrogel did not affect any critical side effects in the animal body. Also, no necropsy findings in the hydrogel implanted area were not observed suggesting that the implanted hydrogel did not generate any specific disease or toxicity.

Differences observed in blood chemical analysis and hematological analysis between control and test groups were within normal variations, and these differences were considered unrelated to the test article implantation. No significant implantation-related lesions were observed in any of the major organs of all test animals. The overall histopathological index of the test article implantation sites was evaluated as 0.

Sodium hyaluronate has been in clinical use for several applications and is considered relatively safe for injection into synovial joints [21,31,32]. Collagen is the most abundant protein in the human body and several collagen-based injections and implants have been considered safe for clinical use [39–41]. In this study, we validated the safety of implantation of a dual hydrogel formulation composed of sodium hyaluronate and porcine collagen.

Based on these results, after 4 weeks of implantation, the test article comprising a dual hydrogel wound dressing did not induce significant toxic changes in rats. The overall bioreactivity rating was evaluated as "non-irritant." Our findings indicate that this cross-linked hydrogel of atelocollagen and sodium hyaluronate can be biologically and chemically safe for clinical application as a wound dressing. To our knowledge, this is the first report on in vivo toxicity of a dual hydrogel composed of atelocollagen and cross-linked hyaluronate for wound healing. Further studies are needed to determine the toxicity of different ratios of atelocollagen and sodium hyaluronate.

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Conflict of Interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Kwang-Il Lee: Data curation, Methodology, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Tae-Hoon Koo:** Conceptualization, Methodology, Project administration, Funding acquisition. **Peter Chen:** Formal analysis, Validation, Resources, Project administration. **Darryl D. D'Lima:** Conceptualization, Data curation, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2021.09.001.

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