Evaluation of Liver and Kidney Toxicity in Rats Receiving Proteoglycans from Fish Cartilage for the Acceleration of Burn Wound Healing



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Abstract

OBJECTIVE: The aim of this study was to evaluate the toxicity of proteoglycan extracted from fish cartilage for the acceleration of wound healing.

MATERIAL AND METHODS: Second degree burn wounds were induced by an electrical hot plate measuring 2 cm in diameter and set at a temperature of 90 °C and placed on the back of rats for 10 seconds. Rats were randomly assigned to receive 1 gram of cream base (control group), 1% silver sulfadiazine (SSD), 1% proteoglycans (PG), 2% PG, a combination of 1% SSD + 1% PG, or a combination of 1% SSD + 2% PG applied to burn wounds to accelerate wound healing immediately after burning and once daily until day 27 post-burn. The toxicity of the rats' liver and kidney functions were evaluated on day 7, 14, 21 and 28.

RESULTS: An evaluation of liver function tests showed on day 28 that the level of aspartate aminotransferase, alanine transaminase, and alkaline phosphatase of rats treated with 1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were not significantly different when compared to the control group. An evaluation of blood urea nitrogen and creatinine levels, on day 28 showed the level of blood urea nitrogen and creatinine of rats treated with 1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were not significantly different when compared to the control group.

CONCLUSION: This study demonstrated that PG extracted from fish cartilage is safe without causing toxic effects to the liver and kidney for long term use.

Keywords: proteoglycans, toxicity, liver, kidney, fish cartilage, burn wounds

A burn wound is a type of skin injury caused by tissue damage after exposure to heat, chemicals, electrical, or radiation. A burn is one of the most common types of wound found in patients. There are up to 250,000 burn patients each year. Burns can occur in all age groups, from babies to the elderly, and are a problem in both developed and developing countries. The major causes of burn injury are classified as follows: flame exposure (55%), hot water exposure (scalds; 40%), and chemical and electrical exposure (5%). The incidence of burn injury is classified by age as follows: 16-64 years old (60%), 1-4 years old (20%), 5-14 years old (10%), and over 65 years old (10%).[1] The severity of burn injury depends on the percentage of the total body surface area damage (%TBSA) and depths of skin layer damage (degree of burn depth). It has been indicated that more than 30% of total body surface area damage could influence a change in physical systems such as cardiovascular, respiratory, and immune systems.[1]

Current treatment of burn wound depends on the severity of the burn: for example, drug therapy or surgery is applied to severe burn cases, but new approaches such as amniotic membrane, cytokine and gene therapy are increasingly used.[1-4] Medication is the most common and easiest way to treat burns, and 1% of silver sulfadiazine (SSD) cream is used as a gold standard for the treatment of burn wounds. When SSD is exposed to the wound, it will ionize to Ag+ and inhibit the enzymes involved in the electron transport chain and bacterial DNA replication process, resulting in the killing of bacteria. SSD is absorbed through the skin to the blood vessels and

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then transported to liver, where metabolism occurs and more than 50% is eliminated via the kidney system in the original chemical form.[5] A previous study showed that Ag+ of SSD is toxic to bone marrow, which leads to abnormal blood synthesis[5] and also affects liver and kidney.[6,7] Thus, the use of this drug in the patients with hepatic or renal impairment should be avoided.[6-8]

PG is a hybrid molecule composed of a central core protein by bonding it with polysaccharides (glycosaminoglycans or GAGs) with a covalent bond.[9] PG is the major component of cartilage, and makes up approximately 90% of dry weight.[10-12] It has been found that PG plays an important role in the wound-healing process.[2,13-14] Neelam et al.[13] studied the effect of PG extract of fish cartilage on L929 fibroblast cells. The results showed that PG extract promoted fibroblast cell activation by increasing cell proliferation and migration and increasing collagen synthesis, which is involved in wound healing[13] but this study is not an evaluation of toxicity. Bunman et al.[2] recently studied PG extract from fish cartilage for the acceleration of burn wound healing. The results suggested that PG extracted from fish cartilage can accelerate and facilitate wound healing in rats. The combination of 1% SSD and 1% or 2% PG seem to have high efficacy in accelerating and facilitating wound healing in rats. Further investigation of the safety profile of PG and the combination of 1% SSD and PG in detail is required.[2]

The aim of this study is to evaluate the toxicity of liver and kidney function in rats receiving PG from fish cartilage for the acceleration of burn wound healing in an *in vivo* model.

Materials and Methods

Drugs and chemicals: PG solution (Garguar Lab, Co., Ltd., Thailand), SSD powder (Sigma, USA.), Sodium pentobarbital (Nembutal; Tariqbrian Ltd, USA.).

Animals: Male Wistar (age 8 weeks, weighing 250-300 gram) were purchased from the National Laboratory Animal Centre, Mahidol University, Salaya, Thailand. The animals were housed in the Laboratory Animal Unit of the Faculty of Pharmaceutical Sciences, Chulalongkorn University under standard conditions of temperature 25 ± 2 °C, 50 - 60 % humidity, and a 12 hours/12 hours light/dark cycle. The rats were kept under laboratory conditions for one week prior to the start of the experiments and allowed food and water ad libitum. At the end of each experiment, the animals were sacrificed with carbon dioxide asphyxiation. Animal experiments in this study were carried out in accordance with the Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes of the National Research Council of Thailand. The animal use protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand (Protocol Approval No. 13-33-011).

Cream base preparation: Stearic acid, glyceryl monostearate, isopropyl myristate, sodium lauryl sulfate, glycerin, triethanolamine, uniphen P-23 and germaben II-E were dissolved in warm water and then mixed with other ingredients during the cream-forming process.

SSD and PG cream preparation: SSD powder, PG solution, stearic acid, glyceryl monostearate, isopropyl myristate, sodium lauryl sulfate, glycerin, triethanol-amine, uniphen P-23 and germaben II-E were used to formulate PG, SSD or SSD+PG creams. SSD powder and/or PG solution were dissolved in warm water and then mixed with other ingredients during the cream-forming process.

Electrical hotplate inducing second-degree burn wounds: The method to induce burn wounds was investigated using the method of Somboonwong,[15] which was modified from Zawacki.[16] Rats were randomly divided into 6 groups of 10 rats each: control rats were treated with cream base; positive controls were treated with 1% SSD cream; and 4 treatment groups received 1% PG cream, 2% PG cream, 1% SSD + 1% PG cream, and 1% SSD + 2% PG cream. Rats were anesthetized by intraperitoneal injection with sodium pentobarbital (60 miligrams per kilograms body weight). The hair on the back was shaved. Second-degree burn wounds were induced by placing an electrical hot plate, of a diameter of 2 cm, set at a temperature of 90 °C on a selected skin area of the back for 10 seconds. All wounds were cleaned and treated with 1 gram of cream base, 1% SSD cream, 1% PG cream, 2% PG cream, 1% SSD + 1% PG cream, or 1% SSD + 2% PG cream once daily and covered with sterile gauzes.

Evaluation toxicity

Blood collection and Preparation of plasma: Collect 0.5 ml of blood from lateral tail vein at 5 time points; baseline, on day 7, 14, 21, and 28.[17,18] The whole blood in the test tube was centrifuged at 3,000 revolutions per minute (rpm) for 10 minutes. The whole blood was separated into two layers: the upper layer which contains plasma (approximately 55% of whole blood) and the lower layer which contains erythrocytes (approximately 45% of whole blood). Plasma was stored at 2-8 °C and used within 24 hours for further analysis.[19, 20]

Evaluation of liver function tests: The liver function test of rats was evaluated by measuring three hepatic enzyme levels including aspartate aminotransferase, alanine transaminase and alkaline phosphatase. Twenty microliters of plasma were used for measurement of each enzyme level. The activities of aspartate aminotransferase, alanine transaminase and alkaline phosphatase were determined using assay kits which are commercially available diagnostic laboratory tests and light a ultraviolet (UV) spectrophotometer (I lab 150, Instrumentation laboratory, Italy).

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Evaluation of renal function tests: The renal function test of rats was evaluated by measuring levels of blood urea nitrogen and creatinine. Twenty microliters of plasma were used for measurement of each parameter. The concentrations of blood urea nitrogen and creatinine were determined using the assay kits and a UV spectrophotometer (I lab 150, Instrumentation laboratory, Italy).

Statistical analysis

Results are expressed as means±SD. Data were analyzed using one-way analysis of variance (ANOVA), followed by a Bonferroni *post hoc* test using SPSS for Windows, ver. 17. Values of $p < 0.05$ were considered to be significant.

Results

The level of aspartate aminotransferase enzyme: On day 7, 14 and 28 post-burn the levels of aspartate aminotransferase enzyme of rats treated with 1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were not significantly different when compared to the control group. On day 21 post-burn, the levels of aspartate aminotransferase enzyme of rats treated with 1% SSD, 1% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were not significantly different when compared to levels in the control group but were significantly lower than their own baselines ($p < 0.001$, $p < 0.001$, $p < 0.01$ and $p < 0.001$, respectively) (Table 1).

Table 1: The level of aspartate aminotransferase enzyme on day 7, 14, 21 and 28 post-burn.

Groups	The level of aspartate aminotransferase enzyme (U/L)				
	Baseline	Day 7	Day 14	Day 21	Day 28
Control (Cream base)	119.10 ± 8.94	118.80 ±17.52	97.20 ± 21.19	98.10 ± 14.91	139.11 ± 43.38
1% SSD	126.30 ± 16.57	101.90 ± 15.09	100.80 ± 24.10	84.60*** ± 12.94	164.20 ± 86.28
1% PG	129.70 ± 19.47	114.30 ± 29.08	101.60 ± 18.37	91.80*** ± 12.98	151.10 ± 35.32
2% PG	134.40 ± 21.34	114.30 ± 30.74	124.70 ± 26.80	108.90 ± 27.84	146.11 ± 46.59
1% SSD + 1% PG	123.10 ± 9.12	115.00 ± 27.90	110.70 ± 32.28	90.50** ± 9.95	143.20 ± 30.85
1% SSD + 2% PG	137.30 ± 28.51	99.70 ± 14.71	126.20 ± 46.04	99.90*** ± 15.36	180.87 ± 92.51

n = 10 for all groups, Data are shown as means ± SD.
**$p < 0.01$, ***$p < 0.001$ significantly different compared to baselines.

Table 2: The level of alanine transaminase enzyme on day 7, 14, 21 and 28 post-burn.

Groups	The level of alanine transaminase enzyme (U/L)				
	Baseline	Day 7	Day 14	Day 21	Day 28
Control (Cream base)	33.80 ± 9.07	52.10* ± 16.91	34.90 ± 8.46	43.40 ± 8.25	52.30 ± 24.33
1% SSD	32.80 ± 10.11	43.80 ± 4.61	42.40 ± 11.08	38.60 ± 5.21	56.00 ± 23.45
1% PG	39.30 ± 10.91	52.60 ± 20.06	43.30 ± 9.12	37.00 ± 2.36	57.22 ± 27.61
2% PG	41.20 ± 11.79	45.60 ± 8.85	50.50+ ± 11.28	45.40 ± 15.43	46.90 ± 19.53
1% SSD + 1% PG	38.00 ± 8.86	50.80 ± 10.36	42.80 ± 9.34	39.30 ± 7.48	41.40 ± 14.68
1% SSD + 2% PG	39.60 ± 6.47	43.80 ± 10.85	44.00 ± 9.50	40.50 ± 6.55	47.42 ± 10.93

n = 10 for all groups, Data are shown as means ± SD.
*$p < 0.05$ significantly different compared to baseline.
+$p < 0.05$ significantly different compared to cream base.

The level of alanine transaminase enzyme: On day 14 post-burn, the level of alanine transaminase enzyme of rats treated with 2% PG was significantly higher ($p < 0.05$) than the baseline. However, on day 7, 21 and 28 post-burn, the levels of alanine transaminase enzyme of rats treated with 1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were not significantly different when compared to the control group (Table 2).

The level of alkaline phosphatase enzyme: On day 7 post-burn, the levels of alkaline phosphatase enzyme of rats treated with 1% SSD, 1% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were not significantly different when compared to the control group but the levels of alkaline phosphatase enzyme of all groups were significantly higher than their own baselines ($p < 0.01$, $p < 0.001$, $p < 0.001$, $p < 0.001$ and $p < 0.01$, respectively). On day 14 post-burn, the levels of alkaline phosphatase enzyme of rats treated with 1% SSD, 1% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were significantly higher compared to the control group ($p < 0.05$, $p < 0.05$, $p < 0.05$ and $p < 0.001$, respectively). Moreover, the level of alkaline phosphatase enzyme of rats treated with combination of 1% SSD + 2% PG cream was significantly higher than baseline ($p < 0.05$). On day 21 post-burn, the level of alkaline phosphatase enzyme of rats treated with cream base and combination of 1% SSD + 2% PG were significantly higher than their baselines ($p < 0.001$ and $p < 0.05$, respectively).

However, on day 28 post-burn, the levels of alkaline phosphatase enzyme of rats treated with 1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were not significantly different when compared to the control group (Table 3).

Blood urea nitrogen: On day 7 post-burn, the levels of blood urea nitrogen of rats treated with 1% PG and combination of 1% SSD + 2% PG were significantly higher than the control group ($p < 0.05$ and $p < 0.01$, respectively). Moreover, on day 7 and day 14 post-burn, the level of blood urea nitrogen of rats treated with combination of 1% SSD + 2% PG were also significantly ($p < 0.05$) higher than 1% SSD. On day 21 post-burn, the levels of blood urea nitrogen of rats treated with cream base and 1% PG were significantly higher than their own baselines ($p < 0.01$ and $p < 0.01$, respectively) (Table 4).

Creatinine: On day 7 post-burn, the levels of creatinine of rats treated with 1% PG and combination of 1% SSD + 1% PG were significantly higher than the control group ($p < 0.05$ and $p < 0.05$, respectively). However, on day 14, 21 and 28 post-burn, the levels of creatinine of rats treated with 1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were not significantly different compared to the control group (Table 5).

Discussion

In addition, the liver enzymes including aspartate aminotransferase and alanine transaminase in the blood increase when the liver is damaged, injured or inflamed from alcohol or drugs. Aspartate aminotransferase has also been found in other cells besides liver cells such as cardiac cells and striated muscle cells. Thus, the level of this enzyme is not specific to liver dysfunction but can also be attributed to a disease like acute myocardial infarction. Therefore, alanine transaminase level is more specific than aspartate aminotransferase level as an indicator for the liver function test.[21] On day 21, the level of aspartate aminotransferase in rats treated with 1% SSD and PG increased significantly compared to baselines but were still within the normal range (82-127 U/L).[22] The level of alanine transaminase only in rats treated with 2% PG increased significantly compared to baselines but were still within the normal range (36-64 U/L).[22]

Alkaline phosphatase is an enzyme in the epithelial cells of the gall bladder. This enzyme increases when the patient has an obstruction of the bile duct, small biliary tract, stones, and some liver diseases. Alkaline phosphatase is also found in other organs such as bone, placenta and intestine.[23] On day 7, the level of alkaline phosphatase in rats treated with cream base increased significantly compared to baselines which may be due to dehydration from the second degree burn. The level of alkaline phosphatase of rats treated with 1% SSD and PG also increased on day 14 and 21 post-burn and decreased to normal range (82-112 U/L) on day 28.[22] The increase of all three liver enzymes levels (of less than three fold) are indicated as acceptable levels in clinical settings.[24-26]

Blood urea nitrogen (BUN) and creatinine are the waste products from the metabolism process which are excreted by the kidney to maintain the homeostasis. High level of BUN and creatinine indicates an impairment of renal function. The baseline levels of BUN of all groups were higher than normal levels (15.6-20.1 mg/dl) reported by Diloke B etal.[22] which was probably due to different housing environment of the animals. The level of BUN of rats treated with SSD appeared to decrease, while the BUN level of rats treated with PG appeared to increase compared to baselines on the early stages of wound healing (day 7). On day 7 post-burn, the level of BUN in rats treated with 1% PG was significantly higher compared to cream base and in rats treated with 1% SSD + 2% PG was significantly higher compared to cream base and 1% SSD. The levels of BUN of all groups reached their maximum levels on day 21 or 28 and dropped back to baselines at the end stage of wound healing (day 28). The decrease in BUN levels after day 21 post-burn was in accordance with other studies. The results indicated that the increase levels of BUN may partly be due to PG.

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Table 3: The level of alkaline phosphatase enzyme on day 7, 14, 21 and 28 post-burn.

Groups	The level of alkaline phosphatase enzyme (U/L)				
	Baseline	Day 7	Day 14	Day 21	Day 28
Control (Cream base)	138.30 ± 20.30	202.10 ** ± 50.65	183.00 ± 53.43	219.50 ** ± 53.66	153.80 ± 25.57
1% SSD	137.30 ± 25.52	220.70 *** ± 24.89	194.80 * ± 40.19	200.80 ± 50.71	160.90 ± 44.17
1% PG	152.20 ± 28.96	231.50 *** ± 29.89	211.50 * ± 44.02	200.40 ± 64.95	156.30 ± 33.01
2% PG	154.10 ± 36.75	206.40 ± 46.25	200.00 ± 32.00	198.10 ± 61.64	148.80 ± 27.12
1% SSD + 1% PG	146.30 ± 26.68	221.10 *** ± 46.45	205.90 * ± 35.00	208.10 ± 37.93	158.70 ± 20.47
1% SSD + 2% PG	143.60 ± 32.99	244.30 *** ± 35.15	237.30 *** + ± 43.38	210.60 * ± 27.68	160.20 ± 55.91

n = 10 for all groups, Data are shown as means ± SD.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ significantly different compared to baseline.
+$p < 0.05$ significantly different compared to cream base.

Table 4: The level of blood urea nitrogen on day 7, 14, 21 and 28 post-burn.

Groups	The level of blood urea nitrogen (mg%)				
	Baseline	Day 7	Day 14	Day 21	Day 28
Control (Cream base)	21.50 ± 2.07	22.50 ± 4.25	23.40 ± 1.96	37.34 ** ± 3.76	26.50 ± 3.66
1% SSD	34.10 ± 6.14	24.51 ± 6.73	29.90 ± 3.35	41.13 ± 12.91	27.80 ± 3.74
1% PG	24.40 ± 7.20	35.32 + ± 9.79	27.90 ± 3.35	38.89 ** ± 7.37	27.70 ± 3.74
2% PG	32.00 ± 5.66	33.81 ± 10.10	27.20 ± 4.32	36.55 ± 8.26	26.70 ± 2.26
1% SSD + 1% PG	30.40 ± 5.64	29.46 ± 10.51	28.20 ± 2.25	38.31 ± 9.09	26.60 ± 2.67
1% SSD + 2% PG	30.20 ± 5.07	37.59 ++, # ± 10.94	27.90 ± 2.81	28.35 # ± 10.55	29.90 ± 3.35

n = 10 for all groups, Data are shown as means ± SD.
**$p < 0.01$ significantly different compared to baseline.
+$p < 0.05$, ++$p < 0.01$ significantly different compared to cream base.
#$p < 0.05$ significantly different compared to 1% SSD.

Table 5: The level of creatinine on day 7, 14, 21 and 28 post-burn.

Groups	The level of creatinine (mg%)				
	Baseline	Day 7	Day 14	Day 21	Day 28
Control (Cream base)	0.54 ± 0.05	0.48 ± 0.04	0.50 ± 0.05	0.53 ± 0.05	0.54 ± 0.16
1% SSD	0.51 ± 0.05	0.50 ± 0.07	0.57 ± 0.05	0.60 ± 0.12	0.57 ± 0.13
1% PG	0.54 ± 0.05	0.58 + ± 0.05	0.56 ± 0.05	0.59 ± 0.05	0.51 ± 0.51
2% PG	0.54 ± 0.05	0.53 ± 0.05	0.55 ± 0.05	0.54 ± 0.05	0.53 ± 0.08
1% SSD + 1% PG	0.55 ± 0.05	0.57 + ± 0.05	0.55 ± 0.05	0.55 ± 0.05	0.49 ± 0.07
1% SSD + 2% PG	0.53 ± 0.07	0.55 ± 0.05	0.55 ± 0.05	0.52 ± 0.04	0.72 ± 0.07

n = 10 for all groups, Data are shown as means ± SD.
+$p < 0.05$ significantly different compared to cream base.

Baseline levels of creatinine in all groups were higher than normal levels (0.37-0.47 mg/dl) reported by Diloke et al.[22] which was also probably due to a different housing environment. In the early stages of wound healing (day 7 post-burn), the levels of creatinine in rats treated with 1% PG and combination of 1% SSD + 1% PG cream were significantly higher compared to cream base. However, the levels of creatinine of both groups decreased to baselines at the end stage of wound healing (day 28). The results indicated that the increased levels of creatinine may partly be due to PG. Altogether, these results indicated that the increase in BUN and creatinine levels were partly due to PG.

Conclusion

This study demonstrated that PG extracted from fish cartilage accelerated and facilitated wound healing as Bunman[2] and Neelam[13] studied without causing toxic effects to the liver and kidney in rats in long term use. Further investigation of the safety profile of PG and the combination of 1% SSD, 1% PG and 2% PG in detail is required in clinical settings.

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