NOTE *Surgery*

Effect of Compound of Gelatin Hydrogel Microsphere Incorporated with Platelet-Rich-Plasma and Alginate on Sole Defect in Cattle

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(Received 11 November 2011/Accepted 8 March 2012/Published online in J-STAGE 22 March 2012)

ABSTRACT. Platelet-rich plasma (PRP) was administered to cattle with hoof defects (10-mm drill hole) to promote sole defect healing. Two types of gels were prepared. One was a mixed gel [compound of gelatin microspheres (GM) incorporated with PRP and alginate] and was used for the test group, while the other was an alginate gel used for the control group. Each hole was filled with one of these gels. The drill hole depth, pressure pain, and hardness of the regenerated tissue were measured for 3 weeks after treatment. These measurements improved significantly in the test group than in the control group. The results suggest that a compound of GM incorporated with PRP and alginate had a hoof-regenerating effect in cattle.

KEY WORDS: cattle, drug delivery system, platelet-rich plasma, sole ulcer, tissue engineering.

doi: 10.1292/jvms.11-0495; *J. Vet. Med. Sci.* 74(8): 1041–1044, 2012

Sole ulcers are full-thickness breaks in the hoof epidermis. They are commonly seen in the center of the lateral hoof of the hind leg [13]. Although the prevalence of sole ulcers is generally low, it causes severe lameness and requires long-term treatment [13]. Healing of sole ulcers is similar to that of skin wounds. Therefore, common sole ulcer treatment methods are similar to wound treatment methods. However, few treatment methods promote hoof regeneration.

Platelet-rich plasma (PRP) is plasma with a platelet concentration twofold higher than the baseline concentration or more than 1.1×10^6 platelets/μl [5]. Platelets contain many growth factors within α-granules such as platelet derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1). Gelatin hydrogel microspheres (GM) have the potential as a biomaterial for controlled release of multiple growth factors present in PRP [11].

Drugs that are in a gel or paste form are most suitable for treating sole ulcers, because it is difficult to apply liquid drugs. PRP undergoes gelation after adding calcium [4]; however, the process takes some time. Alginate is a polysaccharide biopolymer derived from algae that immediately undergoes gelation after adding divalent cations such as calcium and magnesium [10]. This property did not affect addition of GM incorporated with PRP, and the mixture immediately gelled after adding calcium chloride. Furthermore, alginate is used as a dressing material and has shown safety and effectiveness for wound treatment [2].

The objective of this study was to investigate the hoof-regenerating effect of GM incorporated with PRP and alginate in cattle.

Six Holstein dairy cattle (age, 15.7 ± 11.8 months, mean ± SD) were used. All cattle had their hooves trimmed three times a year. All cattle were examined for the presence of any hoof lesions and confirmed to have no hoof lesions. They were kept in a sandy paddock throughout the study period. This study was approved by the Experimental Animal Committee of Obihiro University of Agriculture and Veterinary Medicine.

PRP was prepared according to established procedures [1]. Briefly, venous blood was collected from the jugular vein of each cow and mixed with the anticoagulant citrate dextrose. Blood was centrifuged at 1,500 rpm for 10 min, and the plasma and buffy coat layers were collected. The collected sample was then centrifuged at 3,000 rpm for 15 min. After discarding the upper layer of plasma, the lower layer (1 m*l*) and buffy coat were collected and used as PRP. There were $152.8 \pm 98.7 \times 10^4$ platelets/μl in PRP, and the platelet concentration factor was 4.2 ± 1.3 times.

GM was prepared from acidic gelatin by following established procedures [11]. Briefly, bovine gelatin (isoelectric point version 5.0, Nitta Gelatin Co., Kyoto, Japan) was dissolved in double distilled water, and the solution was crosslinked using glutaraldehyde, resulting in a gelatin hydrogel. The gelatin hydrogel was freeze-dried to yield GM. The water content of GM was 97.8% and the isoelectric point was 5.0.

In the test group, 640 μl/mm^3 of PRP and 380 μl/mm^3 of 2% alginate (Kimika Acid SA, Kimika Co., Tokyo, Japan) were used. First, PRP was mixed with 10 mg of GM. Then, the GM-containing PRP was mixed with 2% alginate and

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10% calcium chloride. The mixture changed to a gel after stirring. In the control group, 1,020 μl/mm^3 of 2% alginate was mixed with 10% calcium chloride to prepare the alginate gel.

After restraining the cattle in a treatment stall, the right hind leg was elevated, and the hoof was washed and disinfected. A 10-mm hole was drilled in the center of the lateral hoof (Fig. 1) using an industrial drill (NACHi 10.0 mm, Fujikoshi Co., Toyama, Japan). The drill hole penetrated the sole and drilling was stopped at the hoof dermis. The depth from the hoof surface to the dermis was measured, and the volume of the drill hole (mm^3) was calculated. The depth of the drill hole was measured along the wall of the drill hole using rounded-end wire and ruler. The depth was measured up to the first place after the decimal point and the deepest depth was regarded as depth at the beginning. Hereafter, drill hole depth was measured at the same site where the depth at the beginning was measured. Thereafter, the drill hole was filled with one of the above mentioned gels, and the hoof was covered with plastic film and bandaged (Coban, 3M, Tokyo, Japan). The bandage and plastic film were covered five times so as not to contaminate the wounds. The left leg was treated in the same manner as the right leg. Legs were randomly selected as control or test for each cow.

Bandages were changed and wounds were washed by saline at 1, 2, and 3 weeks after treatment. We measured drill hole depth, pressure pain, and hardness of the regenerated tissue. The drill hole depth was different for each cow at the beginning of the study. Therefore, we calculated the ratio of the measured depth to the ratio of depth at the beginning (%). Pressure pain and hardness of the regenerated tissue were scored on a four-point scale (Table 1). Both scores were defined as excellent for a score of 1 and worse for a score of 4. Both scores were evaluated by pressure using a metal bar (diameter: 5 mm) by the same veterinarian for all cattle. The veterinarian did not categorize the legs into the control or test group and could apply the same force consistently. The hole depth and the ratio of depth were expressed as mean ± SD and pain from pressure and hardness scores of the regenerated tissue were expressed as medians and ranges.

Differences in these results were determined by the Mann-Whitney U-test. $P<0.05$ was considered statistically significant.

The time course changes of drill hole appearance is shown in Fig. 2. In test group, hard tissue (score 1) was observed after 2 weeks. On the other hand, hard tissue (score 1) was not observed in control group. Furthermore, hard tissue (score 1) was observed wide area in the test group at 3 weeks.

The time course change in the hole depth is shown in Fig. 3. The hole depth rate of the test group was lower than that of the control group throughout the experimental period. Furthermore, hole depth and hole depth rate in the test group were significantly lower than those in the control group 1 and 2 weeks after treatment ($P<0.05$).

The pain from pressure and hardness scores of the regenerated tissues are shown in Table 2. The test group showed a significantly lower pain from pressure score than that of the control group at 1 week ($P<0.05$). Moreover, none of the cattle in the test group experienced pain 1 week after treatment, and lameness disappeared 1 week after treatment in both groups. The test group had a lower regenerated tissue score for hardness than the control group throughout the experimental period. Moreover, a significantly lower score was identified in the test group 3 weeks after treatment ($P<0.01$).

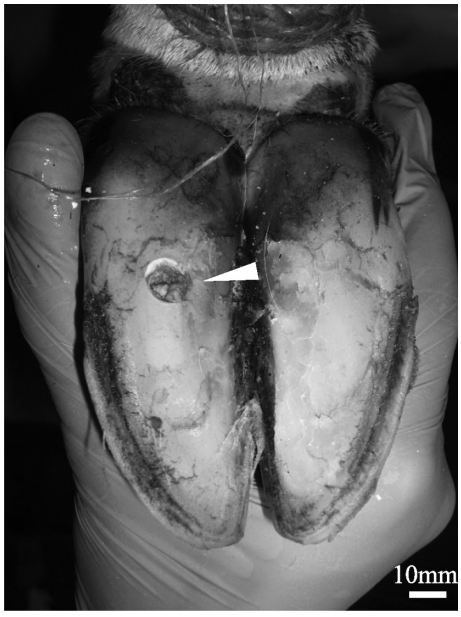

Fig. 1. A drill hole was made at the center of the outside hoof of the hind leg. The drill hole penetrated the hoof sole and drilling was stopped at the hoof dermis. Allow head indicates the drill hole.

Many factors such as laminitis, hard floors, high carbohydrate or protein ratios, age, and genetic predisposition are risk factors for sole ulcers [9, 12, 13]. The development of sole ulcers consists of two stages. The first stage is hemorrhage of the corium without a horn defect and the second is a penetrating horn. Sole ulcers are mainly caused by mechanical injury to the sole corium. Hard floors or deterioration of the digital cushion increases the possibility of mechanical injury [13].

Sole ulcer healing is poorly understood; however, it may be similar to wound healing [13]. First, epidermal cells regenerate and proliferate, and the ulcer defect site is covered with cells. Next, the cells start to cornify, and finally, the ulcer site is covered by a completely differentiated cornified epidermis. PRP has many growth factors, such as PDGF and TGF-β1, and has shown promoting effects on wound healing [4]. Growth factors secreted by PRP mediate the initial phase of wound healing. Furthermore, one study showed that wound with PRP was covered with stratified epithelium until 5 days [4]. Thus, PRP is considered to promote early stage of sole ulcer healing.

The platelet concentration in PRP used in this study was twofold higher than the baseline concentration, fulfilling the definition of PRP. Some studies have indicated that an ad-

Table 1. Evaluation criteria of pressure pain score and hardness of regenerate tissue score

Score	Pressure pain	Hardness of regenerate tissue
1	None	Same level of surrounding crow
2	Mild (vend crow when pressed)	Between score1 and score2
3	Moderate (move the leg when pressed)	Same level of the true skin
4	Severe (move the whole body when pressed)	None

vantageous effect from platelets is gained at concentrations of approximately 1×10^6 platelets/μl [14], whereas one study showed that a higher concentration may have an inhibitory effect on cell proliferation [8]. The number of platelets in this study ($152.8 \pm 92.7 \times 10^4$ platelets/μl) was higher than 1×10^6 platelets/μl. Thus, the preparative method for PRP used in this study was suitable for cattle.

Growth factors are unstable and have a very short half-life. Drug delivery systems (DDS) can be used to accurately deliver drugs with short half-lives, and GM is a material that could be used as a DDS. Growth factors combined with GM are stable materials and are continuously released by gradual hydrolysis of GM. Some studies have indicated that GM is available for PRP [7].

The hole depth and hole depth rate in the test group were significantly lower than those in the control group at 1 and 2 weeks. Moreover, the test group exhibited a lower hardness score for regenerated tissue than that of the control group at 3 weeks. Based on these results, the PRP promoted not only tissue proliferation but also cornification of the regenerated tissue, suggesting that the positive results were due to the effect of growth factors such as PDGF and TGF-β1. Furthermore, the hole depth of the test group improved significantly

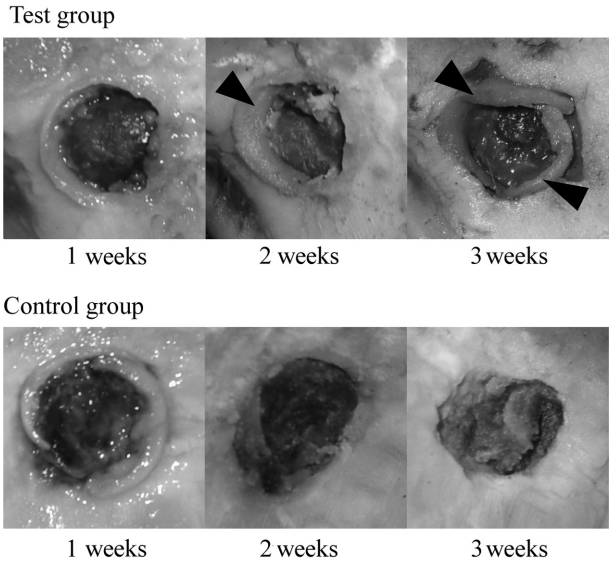

Fig. 2. Photographs showing the time course changes in hoof appearance (No. 5). Arrow head indicates the tissue evaluated as score 1 (hardness of the regenerate tissue). In test group, hard tissue was observed after 2 weeks.

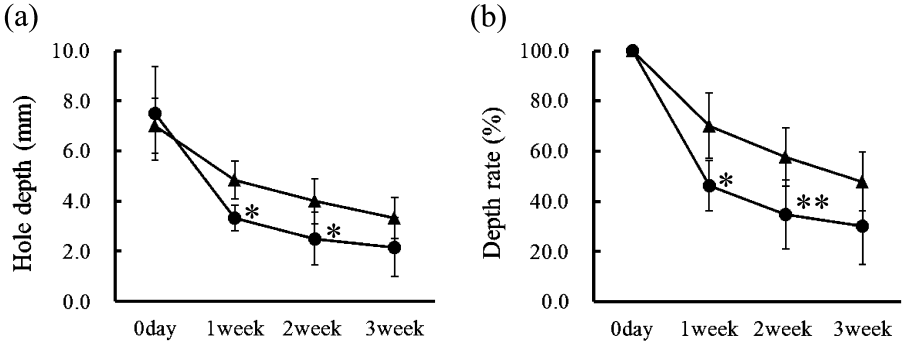

Fig. 3. (a) Mean value of the drill hole depth ± SD (mm). (b) Mean value of the hole depth rate ± SD (%). ● test group (n=6), ▲ control group (n=6), * indicates significant difference at $P<0.05$ and ** indicates significant difference at $P<0.01$.

Table 2. Time course change of hardness score of regenerate tissue and pressure pain score

		0 day		1 week		2 weeks		3 weeks	
		Test	Control	Test	Control	Test	Control	Test	Control
Hardness	Median	4	4	2.5	3	2	3	1**	2
	Range	4	4	1–3	2–3	1–3	2–4	1	1–2
Pain	Median	4	4	1*	2	1	1	1	1
	Range	4	4	1	2–3	1	1–2	1	1

*$P<0.05$, *$P<0.01$.

2 weeks after treatment, probably due to the continuous release of growth factors by the GM.

The pain from pressure score in the test group improved significantly at 1 week. Moreover, none of the cattle in the test group experienced pain 1 week after treatment, indicating that PRP injection resulted in pain reduction [6]. TGF-β1 is an anti-inflammatory cytokine [3]; hence, pain reduction observed in this study was due not only to promotion of cellular regeneration but also due to the anti-inflammatory effect of PRP.

We have shown that a combination of GM-incorporated PRP and alginate had a regenerating effect on the hooves of cattle. This method is expected to be used to treat sole ulcers in cattle.

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