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Influence of the timing of switching a protein-free to a protein-containing diet on the wound healing process in a rat all-layer skin defect

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Short running title : Protein influence of diet on wound hearing

ABSTRACT

We prepared full thickness skin defects in rats fed a protein-free diet as a hypoproteinemia model, then switched the animals to a diet containing a normal protein level 1, 6, or 12 days after wounding (inflammatory, granulation, and rearrangement phases of the wound healing process) to examine whether improvement in the low-protein state promotes subsequent wound healing. The interval until wound healing in rats fed a normal protein diet was significantly shorter, while that in rats continuously fed a protein-free diet was significantly longer than those of other groups. Early correction tended to accelerate wound healing. Although wound contraction in groups receiving a protein-corrected or protein-free diet remained similar until 15 days after wounding, thereafter the duration of the rearrangement phase was significantly longer in the protein-free group than in the other groups. The collagen level per unit of granulation tissue area during wound healing was significantly lower in the protein-free group than in the other groups. These findings indicate that protein correction at any time after wounding accelerates wound healing, although early correction is more effective, and reduces the duration of the rearrangement phase more than those of the inflammatory and granulation phases due to the deposit of collagen.

Key words : Protein-containing diet, Protein-free diet, Rat, Skin defect, Wound healing,

Key points

Introduction

- hypoproteinemia in the presence of an wound delayed a decrease in the wound area.
- the appropriate timing of hypoproteinemia control in accordance with the wound healing process is unclear.
- to examine whether improvement at an appropriate time during the wound healing in the low-protein state promotes subsequent wound healing

Materials and Methods

- two kinds of diets with 20% casein or 0% protein were used.
- 20 g daily diet was given and water was provided ad libitum.
- twenty percent casein group: After the 20% casein diet was given for 4 weeks, a wound was prepared (day of wound preparation: Day 0). Thereafter, the same diet was continued.
- zero percent casein group: After the 0% casein diet was given for 4 weeks, a wound was prepared. Thereafter, the same diet was continued.
- group A: After the 0% casein diet was given for 4 weeks, a wound was prepared. The 20% casein diet was given from 1 day after preparation (inflammatory phase).
- group B: After the 0% casein diet was given for 4 weeks, a wound was prepared. The 20% casein diet was given from 6 days after preparation (granulation phase).
- group C: After the 0% casein diet was given for 4 weeks, a wound was prepared. The 20% casein diet was given from 12 days after preparation (rearrangement phase).
- 2 x 1 cm rectangle wounds were made at 2 points on the left and right sides of the back of rats.
- weights of all rats and their wounds areas every day were measured.

- serum total protein and albumin were measured.
- the specimen level of collagen per 0.1 g wet tissue weight (at the wound center on the day of wound healing) was measured.
- wound sections were stained with H-E stain and Azan stain.

Results

- the body weights of rats fed with 0% protein diet decreased significantly comparing with those of rats faded with 20% casein diet.
- the body weights of rats fed with 20% casein diet changed from 0% protein diet increased dramatically.
- Serum total protein and albumin levels changed like the body weights before and after 20% casein diet.
- the interval of wound healing in the 20% casein group was significantly shorter than those in three groups of protein correction and 0% casein group.
- the interval of wound healing in 0% casein group was significantly longer than those in other four groups.
- the intervals of wound healing among in threes groups of protein correlation were not significant.
- the interval from the rearrangement phase (15 days or more after wound preparation) until healing in the 0% casein group prolonged significantly more than those in three groups of protein correlation.
- thicknesses of dermis in the three groups of protein correlation at the time of healing were nearly close to that of the 20% casein group.

Discussion

- the interval from protein correction until healing was similar regardless of the phase, suggesting the importance of protein correction in wound healing.
- a large amount of protein is required for pronounced collagen production in the rearrangement phase.
- the state of macroscopic healing differs from that of histological healing.

INTRODUCTION

The wound healing process is divided into 3 phases: inflammatory, granulation, and rearrangement phases, which overlap one another(1). In this process, 3 major nutrients, carbohydrate, protein, and lipids, as well as trace elements such as vitamin A/C and zinc, are required as cell components(2-4).Therefore, deficiency in these nutrients may delay wound healing. In clinical practice, postoperative hypoproteinemia sometimes delays the healing of a surgical wound. Actually, most patients undergoing invasive surgery and those with pressure ulcers during long-term admission show chronic malnutrition(5,6).The malnutrition-related development and deterioration of pressure ulcers have been reported, suggesting the necessity of nutritional intervention in the early stage(7).

Many animal experiments have been conducted to examine the relationship between proteins and wounds. Several studies reported that marked hypoproteinemia in the presence of an open surgical wound delayed a decrease in the wound area, and that histological findings included a thin epidermis and presence of residual inflammatory cells(8,9).Others indicated that incised/sutured wound healing was delayed in rats with hypoproteinemia, and that protein correction achieved as rapidly as possible normalized the tensile strength(10).In addition, the tensile strength was attenuated in rats fed a protein-free diet (11),and protein deficiency reduced myofibroblast activity(12).These studies suggest the marked influence of protein on wound healing; healing is delayed in the presence of hypoproteinemia, and protein correction should be performed as promptly as possible. However, no study has investigated the timing of hypoproteinemia control in accordance with the wound healing process: whether a normal protein intake leads to similar wound healing regardless of its phase, or whether the interval until healing is not shortened after a specific period. In this study, we prepared all-layer skin defects in rats fed a protein-free diet as a hypoproteinemia model, and switched the diet to a normal

protein diet at an appropriate time during the wound healing process to examine whether improvement in the low-protein state promotes subsequent wound healing.

MATERIALS AND METHODS

Materials

We used 55 eight-week-old male Wistar rats, ranging from 280 to 285 g in body weight. During the experimental period, 1 animal per cage was housed under the following conditions: room temperature, 25.0±2.0°C; and lighting cycle, 12 hours (8:45 to 20:45). For 1 week after arrival, these rats were acclimated with 0 or 20% casein diets: the former is employed during the growth period, and the latter contains cornstarch instead of casein and cystine, which are contained in the former. The calorie intake per g was similar between the two diets (Table 1). The daily dietary intake was established as 20 g based on the data provided by the manufacturer. Water was provided ad libitum.

Experimental procedures

Twenty percent casein group: After the 20% casein diet was given for 4 weeks, a wound was prepared (day of wound preparation: Day 0). Thereafter, the same diet was continued.

Zero percent casein group: After the 0% casein diet was given for 4 weeks, a wound was prepared. Thereafter, the same diet was continued.

Group A: After the 0% casein diet was given for 4 weeks, a wound was prepared. The 20% casein diet was given from 1 day after preparation.

Group B: After the 0% casein diet was given for 4 weeks, a wound was prepared. The 20% casein diet was given from 6 days after preparation.

Group C: After the 0% casein diet was given for 4 weeks, a wound was prepared. The 20% casein diet was given from 12 days after preparation.

Based on the findings described by Clark (1) and macroscopic findings obtained in the 0% casein group, we established 3 phases (inflammatory, granulation, and rearrangement phases) of the wound healing process in this group as 0 to 5 days, 6 to 11 days, and 12 days or more after wound preparation, respectively. In

accordance with the phases, we determined the day of protein correction in Groups A, B, and C, as described above.

Wound preparation methods

After inhalation anesthesia with diethyl ether, pentobarbital sodium at 30 to 40 mg/kg body weight was intraperitoneally administered to rats. After their hair was shaved, all-layer skin defects involving the subcutaneous tissue and cutaneous muscle were prepared using scissors by drawing rectangles measuring 2 x 1 cm from head-to-tail and mediolaterally, respectively, at 2 points on the left and right sides of the back.

Macroscopic observation and measurement of the wound area

To maintain a wet environment, hydrocolloid dressing was applied to the wound site. To prevent autoexfoliation, the trunk was rolled with adhesive, nonwoven fabric bandage, and fixed with a wristband. Hydrocolloid dressing was exchanged every day. The wound margin was traced with a transparent, polypropylene sheet, and photographs were taken every day from the day of wound preparation (Day 0) until complete wound healing. In all groups, rats showing linear, scar healing and a wound area approximately 0.05-fold that on the day of wound preparation were regarded as achieving wound healing. For wound margin tracing, we employed Adobe Photoshop Elements 2.0 sheets. (Adobe System Incorporated, San Jose, CA) .The data were input into a personal computer using a scanner, and the wound area was calculated using Scion Image Beta 4.02 software(Scion Corporation, Frederick, MD) for image analysis.

Blood collection

In all groups, blood was collected at the start of 0% casein diet ingestion, 2 weeks after it, and the day before and 1, 2, 3, and 4 weeks after wound preparation

(total: 7 times).

Measurement of collagen using tissue specimens

A specimen of the skin incised at the time of wounding was prepared. On the day of wound healing, the skin involving the wound site with scar formation was collected. The specimen level of collagen per 0.1 g wet tissue weight (at the wound center on the day of wound healing) was measured using Sircol dye reagent (Sircol Collagen Assay Kit Rat Standard: biocolor, Carrickfergus, Northern Ireland), as described on its package insert and by Lee et al.(13). The skin on the day of wound preparation was collected in 5 rats each in the 0 and 20% casein groups. The skin on the day of wound healing was collected in 5 rats in the 20% casein group and 4 rats each in the 0% casein group and Groups A, B, and C. We employed the following measurement methods: after fat, hair, and blood were thoroughly removed, rectangle-shaped skin specimens, measuring 2 x 1 cm, incised at the time of wound preparation, and those involving the scar site and its periphery, obtained at the time of wound healing were washed in physiological saline. Their wet weights were measured. These specimens were wrapped in aluminum foil, frozen in liquid nitrogen, and stored at -80°C in a freezer until measurement. For measurement, these specimens were thawed in a refrigerator for 20 to 30 minutes, and then 0.1 g of each specimen including the scar site was cut and placed in a bottle. Subsequently, 0.01 g of pepsin, corresponding to 1/10 of the wet tissue weight, was dissolved in 0.5 ml of 0.5 M acetic acid, placed in the bottle, and agitated with a magnet stirrer at room temperature (25°C) for 24 hours. The pepsin solution containing collagen was placed in a 1.5-ml Eppendorf tube, and centrifuged at 15,000 g for 60 minutes. The supernatant (100 μl) was used for analysis. It was mixed with 1 ml of Sircol dye reagent, agitated for 30 minutes, and centrifuged at 10,000 g for 15 minutes. The precipitate was separated, and the residual supernatant (Sircol dye reagent) was discarded. The

precipitate was dissolved in 1 ml of an alkaline reagent, and centrifuged at 10,000 g for 6 minutes. Using a spectrophotometer, we measured the absorbance at 540 nm. In each specimen, measurement was performed twice. The assay line was prepared by determining the absorbance via similar procedures using attached standard collagen at previously reported collagen levels (12.5, 25, and 50 μg).

Quantification of collagen using Azan-stained sections

Skin specimens collected on the day of wound preparation in each group and those involving the wound site and its periphery collected on the day of wound healing were fixed in 4% paraformaldehyde/0.1 M phosphate-buffered saline (pH 7.4) for 24 hours, and paraffin-embedded according to the standard method to prepare 5-μm paraffin sections. Sections involving the wound center were stained with hematoxylin and eosin. To examine collagen fibers, Azan staining was performed. Using a CCD camera, Azan-stained sections were photographed with Adobe Photoshop Elements 2.0 sheets, and input into a personal computer. We calculated the pixel count in a skin area 3 mm in width, involving the epidermis to subcutaneous tissue, using specimens collected at the time of wound preparation in the 0 and 20% casein groups. Subsequently, we selected collagen stained blue in the same extent, and calculated the pixel count. The rate of collagen per unit area (mm^2) was calculated by dividing the latter by the former. In sections prepared at the time of wound healing, we calculated the pixel count of the residual granulation tissue (scar). Subsequently, we selected the collagen fiber area stained blue in the granulation tissue, and calculated the pixel count. The rate of collagen per unit granulation tissue area (mm^2) was calculated by dividing the latter by the former. In addition, we measured the corium thickness at a site 1 mm from the wound margin in the same sections, and compared the results.

Analytical methods

The values are expressed as the mean±standard deviation. To compare the results among groups, we employed the Kruskal-Wallis test, one-way variance analysis, and Bonferroni's multiple comparison. Significance was tested using the t-test. We used Dr.SPSS software (SPSS Japan Inc., Tokyo, Japan) for statistical analysis. $P<0.05$ was regarded as significant.

Ethical consideration

All animal experiments conducted in this study were approved by the Kanazawa University Animal Experiment Committee, and carried out in accordance with its guidelines.

RESULTS

1. Body weight (Fig. 1)

The body weight was measured for 30 days after wound preparation. There were no significant differences in the body weight at the start of 0% casein diet ingestion among the 5 groups. There were significant differences in the body weight on the day of wound preparation between the 20% casein and other 4 groups. There were no significant differences among the other 4 groups. Until 12 days after wound preparation, the 20% casein group showed significant differences in comparison with the other 4 groups. The 0% casein group showed significant differences in comparison with Groups A and B. There was no significant difference between Groups A and B. However, Group C, in which the 0% casein diet was switched to the 20% casein diet, showed a significant difference in comparison with Group A, although there was no significant difference in comparison with Group B. There was no significant difference in the body weight 15 days after wound preparation between the 20% casein group and Group A. However, the former showed significant differences in comparison with the other groups. There were significant differences between Group A and Groups B/C. There were no significant differences between Groups B and C. The 0% casein group showed significant differences in comparison with the other 4 groups. Thereafter, all groups excluding the 0% casein group showed weight gain until 30 days after wound preparation. However, significant differences were similar to those 15 days after wound preparation.

2. Serum total protein and albumin levels (Fig. 2)

These parameters were measured until 4 weeks after wound preparation. At the start of 0% casein diet ingestion, there were no significant differences in the serum total protein level among the 5 groups. Two weeks after its start, the 0 and 20% casein groups showed significant differences in comparison with the other

groups. In the 20% casein group, the serum total protein level the day before wound preparation was about 1.3 times higher than those in the 0% casein group and Groups A, B, and C, showing significant differences. In the 20% casein group, the value 1 week after wound preparation was significantly lower than that the day before it, but returned to the normal value 3 weeks after it. In Groups A, B, and C, this parameter rapidly increased after the 0% casein diet was switched to the 20% casein diet, but returned to the value the day before wound preparation 10 to 14 days after switching.

The changes in the serum albumin level were similar to those in the serum total protein level described above.

3. Macroscopic findings

1) Macroscopic observation (Fig. 3)

On the day of wound preparation, the wound bed was red and smooth in the 20% casein group, and the volume of blood loss was larger than in the other groups. Furthermore, the difference between the wound bed and margin was marked, and the level of fat in the collected specimen was high.

The day after wound preparation, Groups A, B, and C, as well as the 0% casein group showed marked wound enlargement in comparison with the 20% casein group. Exudate and necrotic tissue had more extensively covered the wound bed. The wound angle had disappeared, making the wound's rectangular shape become oval. The difference between the wound margin and bed was unclear, and there was a space between them. The wound bed was not flat, showing a convexo-concave surface.

In the 20% casein group, the wound area was reduced 12 days after wound preparation. The granulation tissue on the wound bed was protruding, and the entire wound site showed advanced cutification.

In Group A, the wound area was reduced to 1/2 of that at the time of wound

preparation on 12 days after wound preparation. The wound margin was circumferentially enveloped by the epidermis.

In Group B, the level of exudate was decreased. Granulation tissue on the wound bed was protruding, with the disappearance of the difference between the wound margin and bed. At the wound margin, the epidermis was slightly observed on 12 days after wound preparation.

In Group C and the 0% casein group, the necrotic tissue had almost disappeared. At the wound margin, the epidermis was slightly observed on 12 days after wound preparation.

Linear scar healing in the head-to-tail direction was achieved 20, 25, 30, and 40 days or more after wound preparation in Groups A, B, and C, as well as in the 0% casein group, respectively. One rat in Group A, 1 rat in Group B, 4 rats in Group C, and 5 rats in 0% casein group were died of infection or after taking blood sample before the wounds achieved linear scar healing.

2) Wound area ratio

In calculating this ratio, we excluded rats in which healing was not achieved due to infection and those with a fatal outcome. The day after wound preparation, the wound area was increased in all 5 groups (Fig. 4). In the 20% casein group, it was 1.1 times greater than that at the time of wound preparation. In the other 4 groups, it was 2.1 times greater. In the former, 5 days after wound preparation, the value returned to that at the time of wound preparation. Eight days after wound preparation, the area ratio was reduced to 0.5. It was below 0.05 fifteen days after wound preparation; scar healing was achieved. Between 1 and 15 days after wound preparation, there were significant differences in the area ratio between the 20% casein and other groups. There were no significant differences in the area ratio among the latter groups until 15 days after wound preparation when corresponded to early stage of rearrangement in Groups A, B, C and 0% casein; the area ratio was reduced to approximately 0.5 in comparison with the

value at the time of wound preparation. In Groups A, B, and C, wound contraction was similar to that in the 0% casein group from the day of wound preparation until 15 days after wound preparation. Thereafter, the wound areas in Groups A, B, C and 0% casein were differently reduced.

The intervals from wound preparation until scar healing were 14.9±2.0, 22.0±2.3, 24.6±3.5, 27.8±3.1, and 36.8±10.4 days in Groups 20 % casein, A, B, and C, as well as in the 0% casein group, respectively (Table 2). There were significant differences between the 20% casein and other 4 groups. There were also significant differences between the 0% casein and other 4 groups. There were no significant differences among Groups A, B, and C. However, when the interval until the introduction of the 20% casein diet was prolonged, healing was likely to be delayed.

In Groups A, B, and C, the intervals from the introduction of the 20% casein diet until healing were 19.9±2.4, 18.6±3.5, and 16.8±3.1 days, respectively, showing no significant differences (Table 3). However, the intervals from the rearrangement phase (15 days or more after wound preparation) until scar healing in Groups A, B, and C, as well as in the 0% casein group were 7.0±2.3, 9.6±3.5, 13.1±3.1, and 21.8±10.4 days, respectively; there were significant differences between the 0% casein and other 3 groups(Table 4). Although there were no significant differences among Groups A, B, and C, wound reduction was most markedly accelerated in Group A, followed by Groups B/C and the 0% casein group. This suggests that the influence of a switch to the 20% casein diet is more marked 15 days or more after wound preparation (rearrangement phase).

4. Histological findings (Fig. 5)

On the day of wound preparation, the dermis thickness in the 0% casein group was 0.5-fold that in the 20% casein group, and the cutaneous muscle thickness was 0.8-fold. There was no adipose tissue. On the day of wound healing,

epidermis formation was observed above the regenerative scar granulation tissue in all groups. In the 20% casein group, the density of the scar granulation tissue was high. In particular, collagen fibers were dense, and some blood vessels were substituted for neovascularization. In the 0% casein group, the border between the normal and scar granulation tissues was unclear. The collagen bundle of the granulation tissue was less dense than in the other groups. There were no marked differences among Groups A, B, and C.

5. Collagen level

We compared the collagen level per 0.1 g wet tissue weight among the 5 groups. On the day of wound preparation, there was no significant difference in the collagen level between the 0 and 20% casein groups. On the day of wound healing, there were no significant differences among the 5 groups (Table 5).

We measured the collagen level per unit skin area (mm^2) using Azan-stained sections. On the day of wound preparation, there was a significant difference between the 0 and 20% casein groups ($p=0.000$). In Azan-stained sections, we determined the collagen level per unit granulation tissue area. On the day of wound healing, there were no significant differences among the groups excluding the 0% casein group. However, in the 0% casein group, the collagen level was significantly lower than in the other groups ($p<0.05$) (Table 6).

We compared the dermis, which was measured using Azan-stained sections. On the day of wound preparation, there was a significant difference between the 0 and 20% casein groups ($p=0.008$). We measured the thickness of normal corium at a site 1 mm from the wound margin on the day of wound healing. There were no significant differences among the groups excluding the 0% casein group. However, in the 0% casein group, the corium thickness was significantly lower ($p<0.05$) (Table 7).

DISCUSSION

Relationship between the timing of protein correction and wound healing

In this study, we investigated the influence of the timing of protein-containing diet introduction on the wound healing process. After a protein-free diet was given to rats for 4 weeks, all-layer defects were prepared. We evaluated the effects of switching it to a standard diet containing protein with respect to 3 phases of the wound healing process. There were also significant differences in the interval until wound healing between the 20% casein and other groups. There were also significant differences in the interval until wound healing between the0% casein and other groups. There were no significant differences in the interval from protein correction until healing among the 3 phases. However, the early introduction of the protein diet slightly shortened the interval. Previous animal experiments regarding the relationship between nutrition and wound healing have shown that hypoproteinemia delayed the healing(8,9),and that the tensile strength at the skin incision/suture site was reduced in animals with persistent hypoproteinemia(10).In addition, collagen fiber formation was less marked in animals fed a protein-free diet(11).These findings suggest that hypoproteinemia delays wound healing. In our experiment, there were also significant differences in the interval until wound healing between the 20% casein and other groups, indicating that hypoproteinemia related to the ingestion of a protein-free diet delays the wound healing process. Furthermore, the above studies reported the efficacy of protein correction early after wound preparation. However, no study has examined the phase of the wound healing process in which protein correction should be started. In this experiment, protein correction promoted wound healing regardless of the phase in comparison with a group without protein correction. As a result, the interval until healing was shortened.

The interval from protein correction until healing was similar regardless of the phase, suggesting the importance of protein correction in wound healing. Previous studies in humans regarding the efficacy of protein correction indicated that the pressure ulcer area in a group with super-high-protein supplementation was significantly reduced in comparison with a group with high-protein supplementation(14),and that protein supplementation in malnutrition patients normalized the albumin level after 2 months(15).According to others, in the presence of malnutrition, wound healing was delayed, increasing the incidence of pressure ulcers(16).There was a correlation between the presence or absence of pressure ulcers and the albumin level, which reflects nutrition, with a high correlation coefficient, suggesting that a decrease in the serum albumin level increases the risk of pressure ulcer development(17).Our data support the results of these studies.

Phase in which protein correction influences the wound healing process

In the 20% casein group, the wound area the day after wound preparation was 1.1-fold that on the day of wound preparation. Seven days were required until it reduced to 1/2 of the initial area. Healing was achieved 15 days after wound preparation. In 4 groups in which a protein-free diet was given before wound preparation, the wound area increased 2-fold or greater the day after wound preparation. In these groups, 14 days were required until it reduced to 1/2 of the initial area. In 3 groups in which the 20% casein diet was introduced during the wound healing process, wound contraction was noted, as demonstrated in the 0% casein group. It is known that the appearance of myofibroblasts and their binding to peripheral collagen in the granulation phase rapidly reduce the wound area via their contraction(18,19).However, wound contraction similarly occurs even when the timing of protein correction differs, or in the absence of protein. Therefore, protein may not influence myofibroblastic contractility. This issue should be

further reviewed. In addition, there were differences in the wound healing process among these 4 groups 15 days or more after wound preparation (rearrangement phase). In the 0% casein group, the interval until wound healing was significantly prolonged in comparison with the other 3 groups, in which the 20% casein diet was introduced during the wound healing process. Although there were no significant differences among these 3 groups, a delay in the introduction of the 20% casein diet slightly prolonged the interval until healing. This suggests that a large amount of protein is required for pronounced collagen production in the rearrangement phase, as indicated for marked collagen deposition in this phase(1).In addition, this is not consistent with the findings described by Fujii (8,9): in a hypoproteinemia group, healing stops in the resting phase of the wound healing process, the reduction phase is short, and healing is delayed in the cutification phase. This issue should also be further investigated.

Changes in the collagen level

Macroscopically, there was no marked difference in the state of wound healing between the 0 and 20% casein groups. To compare the tissue level of collagen, we measured the collagen level per 0.1 g on the days of wound preparation and healing. There was no significant difference. Collagen is the main component of skin, and so the skin weight may reflect the collagen weight. However, the switch from a protein-free diet to a low-protein diet promoted collagen deposition in the skin. As histological findings on light microscopy included differences in the tissue thickness and density, we quantified collagen fibers using Azan-stained sections. In skin specimens collected on the day of wound preparation, there was no significant difference in the rate of collagen fibers between the 0 and 20% casein groups. In scar granulation tissue specimens collected on the day of wound healing, there were no significant differences among the 4 groups other than the 0% casein group. The amount of collagen fibers was similar to that on the day of

wound preparation. However, the rate of collagen fibers on the day of wound healing in the 0% casein group significantly differed from those in the other 4 groups. This is consistent with the finding that the granulation tissue distribution of collagen in the 0% casein group was less dense than in the other groups. A study reported that a dermis tissue repair-related increase in the collagen level enhanced the tensile strength of the wound(20).In this study, in the 0% casein group, in which protein correction was not performed, collagen fibers were histologically less dense than in the other groups with protein correction, and wound healing was macroscopically achieved, but the tensile strength may have been reduced. These findings suggest that the state of macroscopic healing differs from that of histological healing.

CONCLUSION

1. The interval of wound healing in 20% casein group was significantly shorter than those in three groups of protein correction and 0% casein group. The interval of wound healing in 0% casein group was significantly longer than those in other four groups.
2. There were no significant differences in the interval from protein correction until healing among the starting points of protein correction, that is, the inflammatory, granulation, and rearrangement phases after wound preparation. However, early correction slightly accelerated wound healing.
3. There were no significant different in the area ratio among three groups of protein correction and a 0% casein group until 15 days, when was the early stage of rearrangement, after wound preparation.
4. Protein correction did not shorten the interval from the day of wound preparation until the early stage of rearrangement phase in which the wound strongly constricted, as 0% casein diet. However, in the rearrangement phase, it shortened the interval until wound healing more than 0% casein diet.
5. The serum total protein and albumin levels were increased within about 1 week regardless of the timing of protein correction in the wound healing process.
6. The scar granulation tissue level of collagen at the time of wound healing reached that in the 20% casein group. In the absence of protein correction, histological findings of the wound site revealed less dense collagen fibers.

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REFERENCES

1 Clark RAF, Denver MD. Cutaneous tissue repair:Basic biologic considerations. Ⅰ J Am Acad Dermatol 1985;13:701-25.

2 Cartwright A. Nutritional assessment as part of wound management. Nurs Times. 2002;98:62-3.

3 Gidden F, Shenkin A. Clinical micronutrients. Nurs Times. 1997;93:45-6.Review.

4 Ruberg RL. Role of nutrition in wound healing. Surg Clin North Am. 1984;64:705-14.

5 Raffoul W, Far MS, Cayeux, MC, Berger MM. Nutrition status and food intake in nine patients with chronic low-limb ulcers and pressure ulcers: importance of oral supplements. Nutr. 2006;22:82-8.

6 Garretsen MK, Melis GC, Richir MC, Boelens PG, Vlaanderen L. Perioperative nutrition. Ned Tijdschr Geneeskd.2006;150:2745-49. Review.

7 Houwing RH, Rozwndaal M, Wouters-wesseling W.A randomized, double-blind assessment of the effect of nutritional supplementation on the prevention of pressure ulcers in hip-fracture patients. Clin Nutr.2003;22:401-5.

8 Fujii H. Influence of hypoproteinemia on wound healing in rats. Experimental hypoproteinemia in rats induced by low casein diets. Japanese Stomatological Society.1982a; 28:1433-48. (in Japanese)

9 Fujii H. Influence of hypoproteinemia on wound healing in rats. Macroscopical, histological and cell kinetical studies on wounds healing. Japanese Stomatological Society.1982b;28:1449-62. (in Japanese)

10 Yokogawa T. The Effects of Hypoproteinemia on the Skin Incision Suture Wounds in Rats －With Special Reference to Changes in Time Lapse of their Tensile Strength of the Tissue－.J Oral Sci.1984;58:467-85.

11 Enomoto T. The Effects of Protein-Free Diet on the Hearing of Skin Incision Suture Wounds in Rats-with Special Reference to Weight, Blood Components,

Tensile Strength and Histopathological Findings for Different Postoperstive Feeding Starting Times-.Nihon Univ. J Dent.1987J;61:429-38.

12 Modolin M, Bevilacqua RG, Margarido NF, Lima-Gocalves E. Effects of protein depletion and repletion on experimental open wound contraction. Ann Plast Surg. 1985;15:123-6.

13 Lee CG, Homer RJ, Zhu Z, Lanone S, Wang X, Koteliansky V, Shipley JM, Gotwals P, Noble P, Chen Q, Senior RM, Elias JA.Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth facter β_1.J Exp Med.2001;194:809-22.

14 Chernoff RS, Milton KY, Lipschitz DA. The effect of a high protein formula on decbitus ulcer healing in long term tube fed institutionalized patients. J Am Diet Assoc. 1990;90:A-130.

15 Fueda T., Sato T., Suzuki K., Takami M, Ohtomo H, Iida T. Usefulness of protein supplementation in elderly malnutrition patients with pressure ulcers. Clin Nutri.2004;104: 455-9. (in Japanese)

16 Yamanaka E. Nutrition Support and Pressure Ulcer. Jpn JPU. 2005;7:28-33.

17 Mino Y, Morimoto S, Okaishi K, Sakurai S, Onishi M, Okuro M, Matsuo A, Ogihara T. Risk factors for pressure ulcers in bedridden elderly subjects: Importance of turning over in bed serum albumin level. Geriatr Gerontol Int. 2001;1:38-44.

18 Singer II, Kawka DW, Kazazis DM, Clark RA. In vivo co-distribution of fibronectin and actin fibers in granulation tissue: immunofluorescence and electron microscope studies of the fibronexus at the myofibroblast surface. J Cell Biol. 1984;98:2091-106.

19 Gabbiani G. The myofibroblast in wound healing and fibrocontractive diseases. J Pathol.2003;200:500-3.Review.

20 Doillon CJ, Dunn MG, Bender E, Silver FH. Collagen fiber formation in repair tissue: Development of strength and toughness. Coll Relat Res. 1985;5:481-92.

FIGURE LEGENDS

Figure 1: Body weight changes in each group (Day 0: the day of wound preparation). In rats fed a 0% casein diet, there was no weight gain. However, switching it to a 20% casein diet (arrow) rapidly increased the body weight (n=5 per group). Comparison of body weight values 30 days after wound preparation: *$p<0.05$

Figure 2: Changes in the serum albumin level in each group (Day 0: the day of wound preparation). In rats fed a 0% casein diet, the albumin level was decreased. However, switching it to a 20% casein diet (arrow) rapidly increased this parameter. Two weeks or more after wound preparation, there were significant differences between the 0% casein and other groups (*$p<0.05$).

Figure 3: Macroscopic findings from wound preparation until healing. On the day of wound preparation, the wound size was similar among the 5 groups. However, 3 days after wound preparation, it rapidly increased in the groups excluding the 20% casein group. Although the interval until healing differed among the groups, similar linear scar healing was achieved in all groups. Bar: 1 mm

Figure 4: Changes in the wound area. The mean relative area is shown, regarding the area on the day of wound preparation as 1. Despite the introduction of a 20% casein diet (arrow), the wound area did not immediately decrease to the value in the 20% casein group. The rate of decrease in the wound area was similar among Groups A/B/C and the 0% casein group until 15 days after wound preparation, that is, in the inflammatory to granulation phases. Thereafter, there were differences in the rate of decrease in the wound area in the rearrangement phase.

Figure 5: Tissue sections on Azan staining: In the skin (a) on the day of wound

preparation in the 20% casein group, a thick corium and subcutaneous fat were observed. However, in the 0% casein group, the skin (b) on the day of wound preparation was thin, and there was no subcutaneous tissue. In the former, the scar (c) at the time of wound healing consisted of solid collagen. In the latter, the scar (d) was slightly less dense. We measured the collagen level in the site surrounded by the line (refer to Table 6). D: dermis, GT: granulation tissue

Table 1

	20%Casein diet	0%Casein diet
Casein	20%	0%
L-Cystine	0.3%	0%
Cornstarch	39.7486%	60.0486%
Chemical cornstarch A	13.2%	13.2%
Sucrose	10%	10%
Soybean oil	7%	7%
Cellulose powder	5%	5%
AIN93G containing minerals	3.5%	3.5%
AIN93 containing vitamins	1%	1%
Choline bitartrate	0.25%	0.25%
T-butylhydroquinone	0.0014%	0.0014%
Kcal(per 100g)	377.2kcal	377.2kcal

Figure 1　Ingredients contained in 0 and 20% casein diets

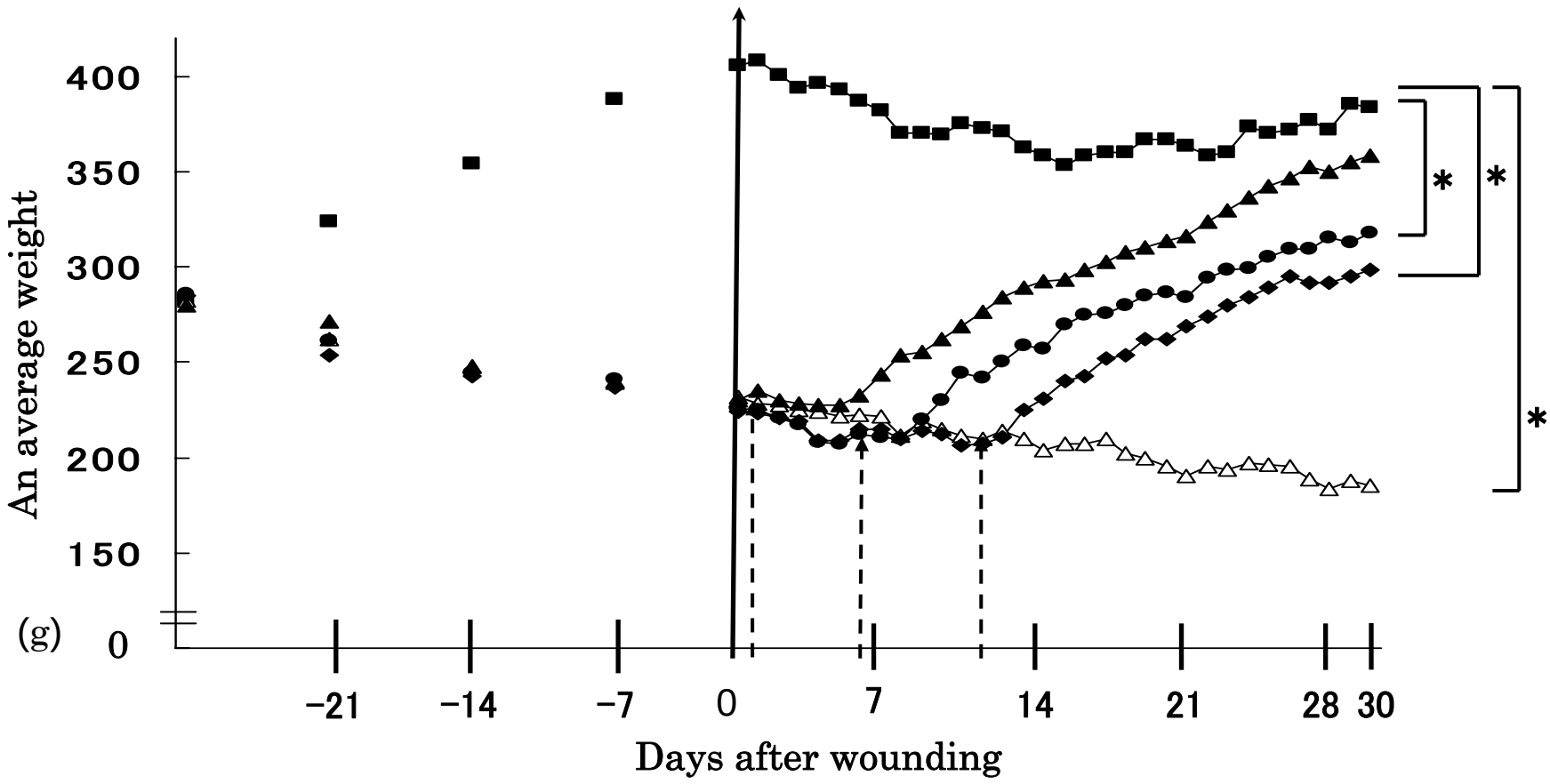


Figure 2

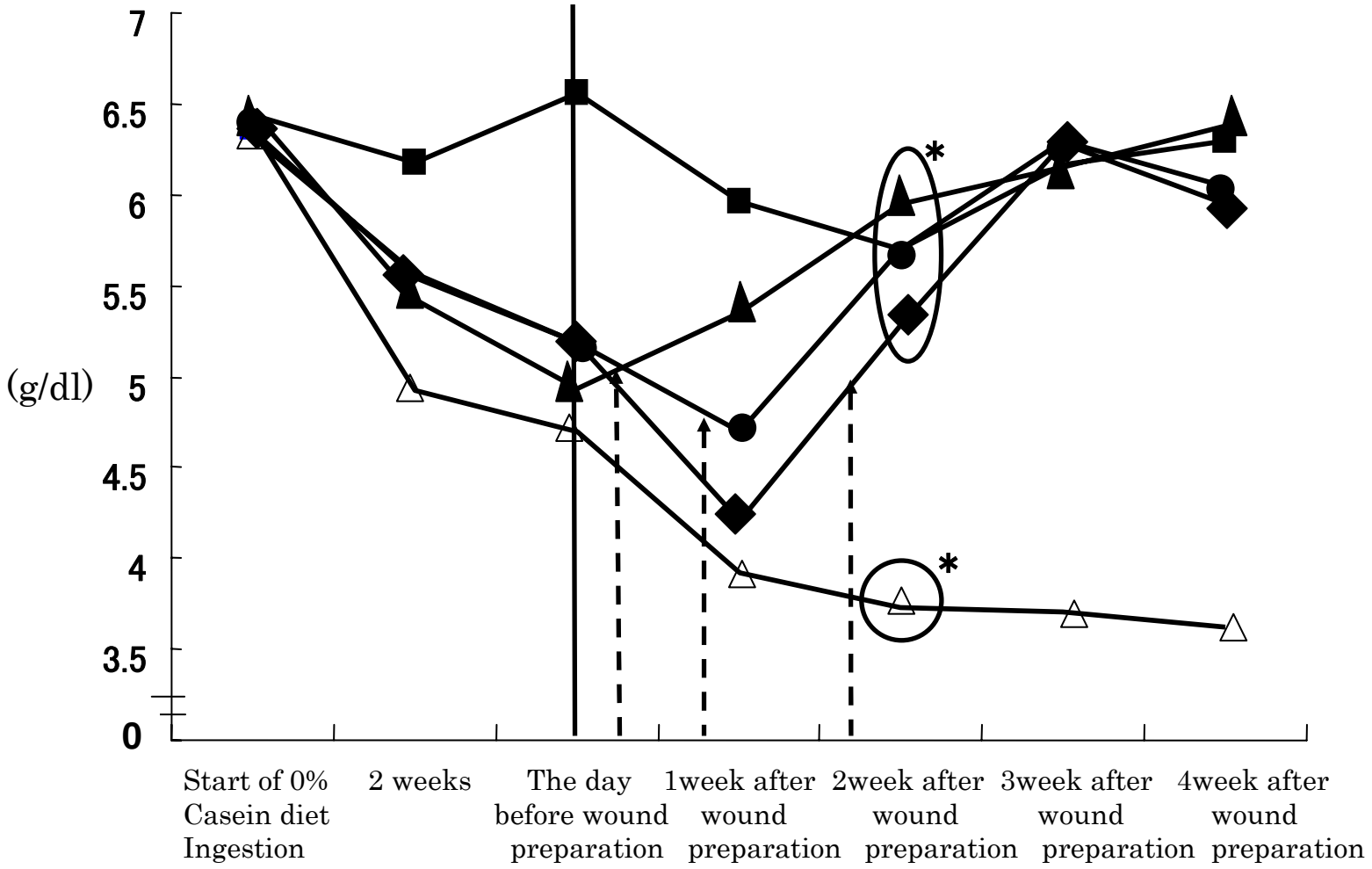

7
6.5
6
5.5
(g/dl) 5
4.5
4
3.5
0
*
*
Start of 0% Casein diet Ingestion
2 weeks
The day before wound preparation
1week after wound preparation
2week after wound preparation
3week after wound preparation
4week after wound preparation
■20%group(n=5)
▲A group(n=5)
● B group(n=5)
◆ C group(n=5)
△ 0%group(n=5)

Figure 3

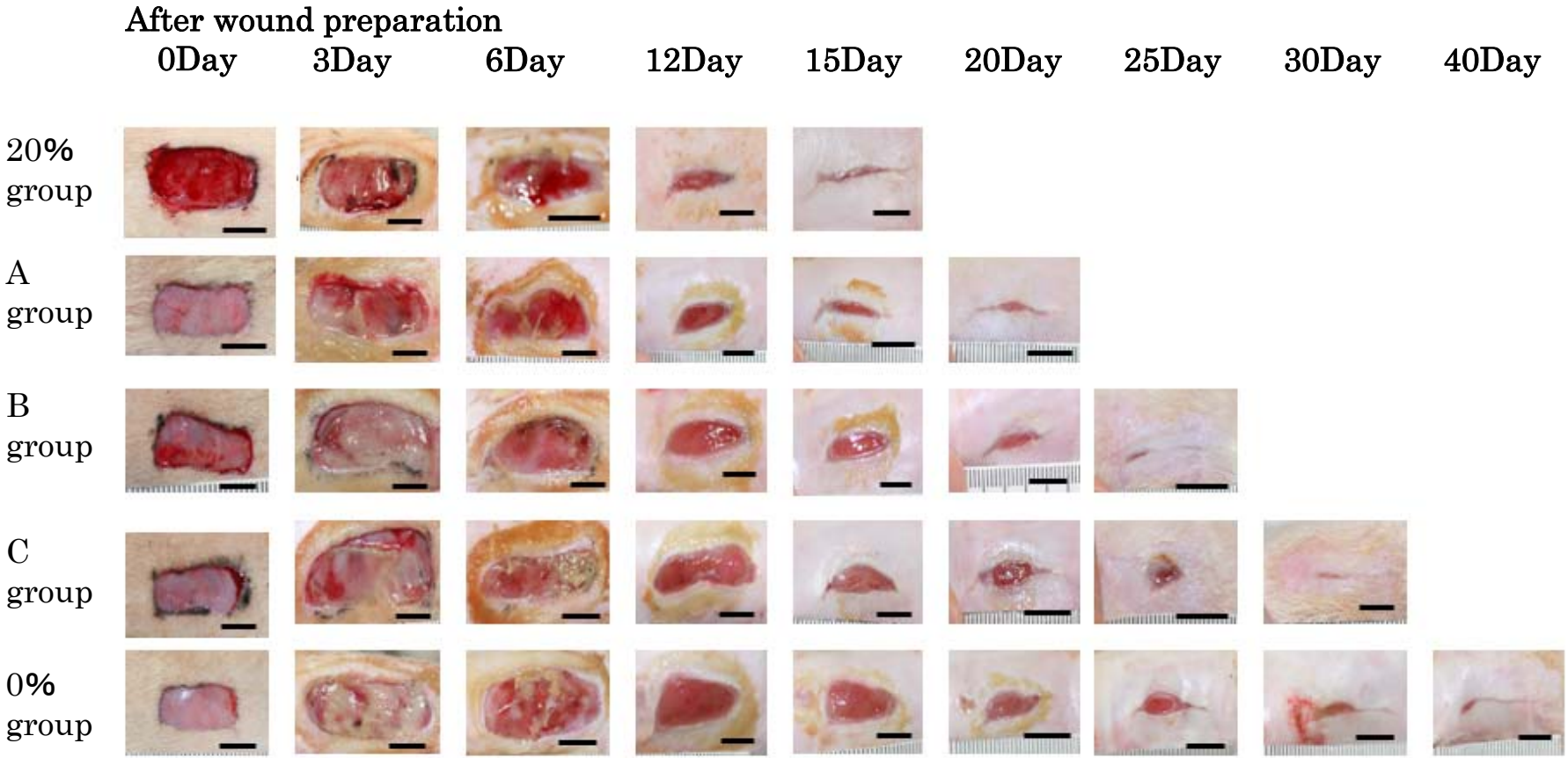

After wound preparation
0Day
3Day
6Day
12Day
15Day
20Day
25Day
30Day
40Day
20% group
A group
B group
C group
0% group

Figure 4

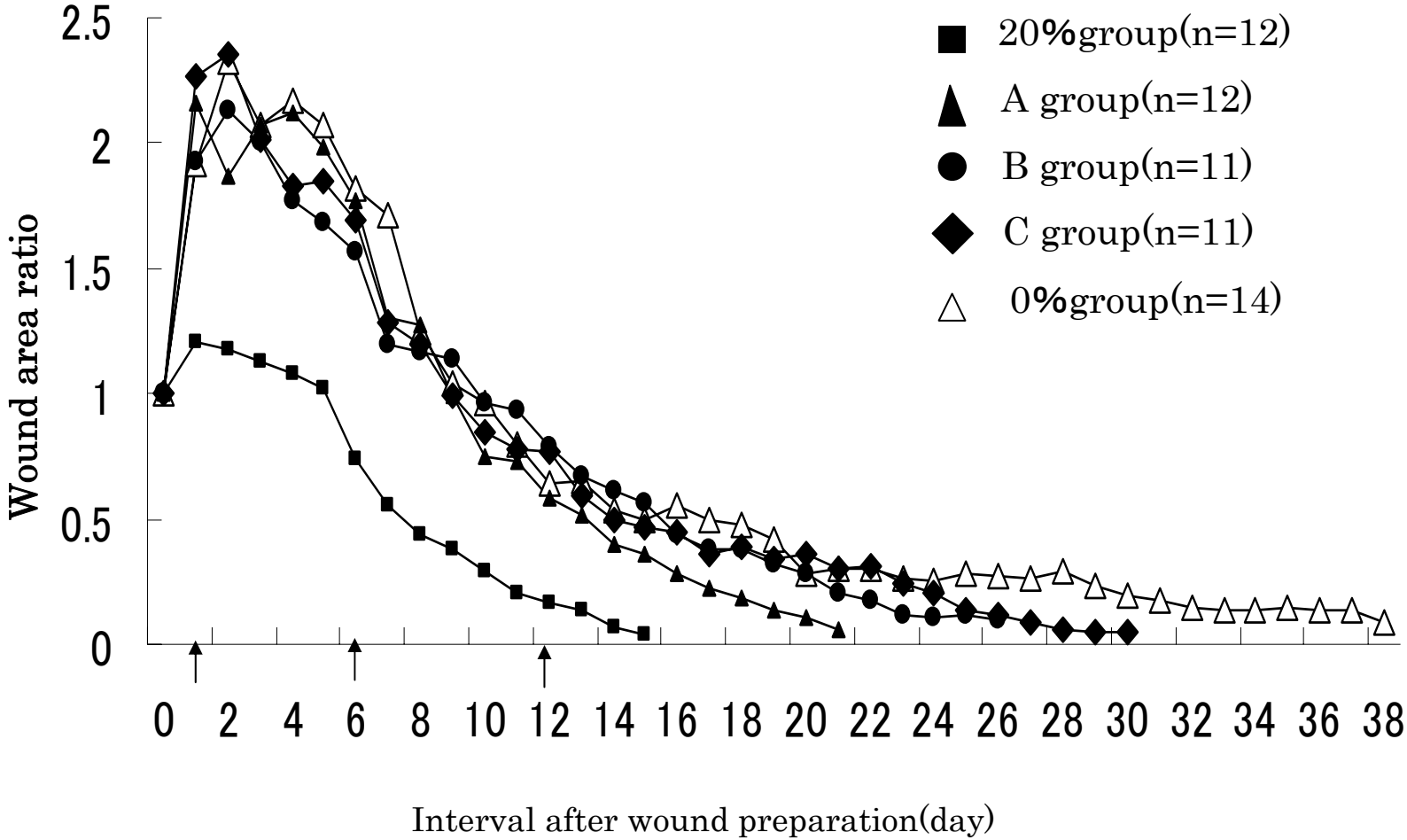
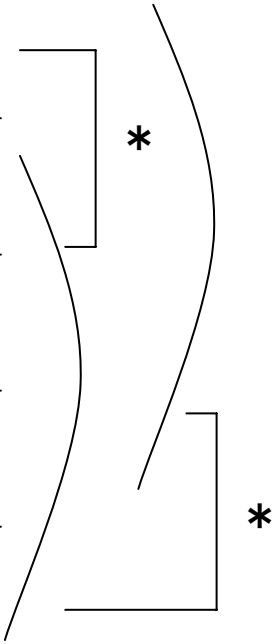

2.5
2
1.5
1
0.5
0
Wound area ratio
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38
Interval after wound preparation(day)
20%group(n=12)
A group(n=12)
B group(n=11)
C group(n=11)
0%group(n=14)

Table 2　　Interval from wound preparation until healing

	n	mean±standard deviation
20%group	12	14.9±2.0
A group	12	22.0±2.3
B group	11	24.6±3.5
C group	11	27.8±3.1
0%group	14	36.8±10.4



There were significant differences between the 20% casein and other groups, as well as between the 0% casein and other groups (*$p<0.05$).

Table 3 Interval from the introduction of a 20% casein diet until wound healing

	n	mean±standard deviation
A group	12	19.9±2.4
B group	11	18.6±3.5
C group	11	16.8±3.1

There were no significant differences among the 5 groups.

Table 4 Interval from 15 days after wound preparation until wound healing

	mean±standard deviation	
A group	7.0±2.3	
B group	9.6±3.5	
C group	13.1±3.1	*
0%group	21.8±10.4	

There were significant differences between the 0% casein and other groups ($p<0.01$). There were no significant differences among Groups A, B, and C.

Table 5　　Collagen level（μg）per 0.1g wet tissue weight

	n	mean±standard deviation（μg）
20%group, day of wound preparation	10	379.2±15.73
0%group, day of wound preparation	10	378.6±7.43

	n	mean±standard deviation（μg）
20%group, day of wound hearing	10	380.2±6.19
0%group, day of wound hearing	8	380.6±6.89
A group, day of wound hearing	8	382.8±7.07
B group, day of wound hearing	8	382.8±7.61
C group, day of wound hearing	8	379.3±5.91

There were no significant differences among the 5 groups.

Figure 5　　Quantification of collagen（pixel count）

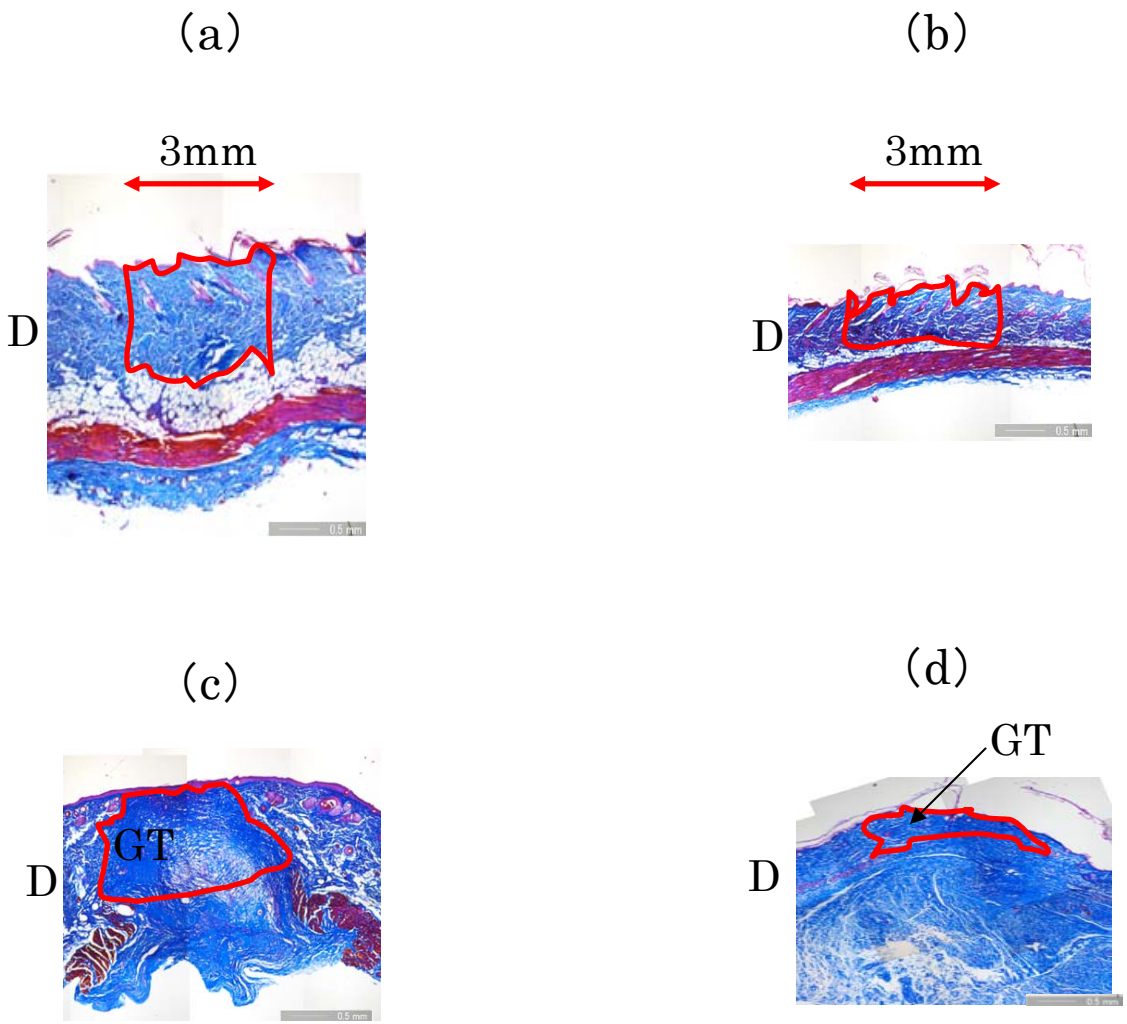


Table 6　Collagen level per unit area（mm^2）measured using Section on the days of wound preparation and healing

		n	mean±standard deviation（%）	
day of wound preparation	20%group	3	57.12±1.8	*
	0%group	3	34.04±1.86	

＊：$p<0.01$

Table 7 Dermis at a site 1mm from the wound margin measured using sections on the days of wound preparation and healing

		n	mean±standard deviation	
day of wound healing	20%group	3	44.13±3.83	
	A group	3	33.85±5.35	
	B group	3	31.27±1.93	
	C group	3	31.31±6.16	*
	0%group	3	17.34±10.93	

There were significant differences between the 0% casein and other groups ($p<0.01$). There were no significant differences among Groups 20% casein,A, B, and C.