Experimental Studies on Polychromatic Labeling of Rats

〈*Original*〉

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Norihiro HASHIGUCHI[1], Masashi SHIMAHARA[1], Akio YAMAMOTO[1], Ryuzo FUKAWA[1], Koichi KONO[2], Takemasa WATANABE[2], Yukio ORITA[2], Tomotaro DOTE[2] and Kan USUDA[2]

Department of Oral Surgery, Osaka Medical College[1], *Department of Hygiene and Public Health, Osaka Medical College*[2], *2-7, Daigaku-machi, Takatsuki, Osaka 569-8686, Japan*

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ABSTRACT

This study was undertaken to select the most appropriate labeling agents for observing the turning point in the reconstructive healing phase of a condylar process fracture. Four different agents were used for labeling : tetracycline (TC), calcein (Cal), calcein blue (CB), and alizarin complexion (AC). Twenty-five 7-week-old SPF Wister female rats (Japan SLC Hamamatsu, Shizuoka, Japan) weighing 120-140g were used for the experiment. Each agent was administered once every 5 days, and the amount was adjusted according to the rat's body weight (BW) per 100g to determine the optimal amount to be injected. We consider that the four labeling agents (TC, Cal, CB and AC) satisfy the three conditions stated in our study for labeling when the dosages are properly considered, and are useful as labeling agents for the joints of the jaw. In observing labeling images for the observation of chronological changes in the bone where changes in reconstructive phenomena are brisk, the four labeling agents can be specifically utilized. We found the optimal concentrations for practical use to be : 2mg／100g BW for TC, 0.3mg／100g BW for Cal, 3mg／100g BW for CB, and 2mg／100g BW for AC.

INTRODUCTION

In observing the turning point in the reconstructive healing phase of a fracture of the condylar process by labeling, we, first of all, conducted studies on the labeling agents most appropriate for application.

The tetracycline family has been conventionally used as a labeling agent for observing the formation and growth of hard tissues (FROST et al, 1960; BOYNE, 1968; SUGA, 1965 et al). However, when labeling agents show the same color as the regions where the reconstruction is active, it is difficult to clarify the state of the callus during the time when the labeling agent is administered and also during the healing phase. In view of this situation, we studied the state of labeling and optimal concentrations of labeling agents in the condylar process by introducing the polychromatic multiple labeling method (bone tissue, time-description system) as employed by SUGA et al,(1973).

MATERIALS and METHODS

1. Materials

Tetracycline (TC), calcein (Cal), calsein blue (CB), and alizarin complexion (AC) were selected as labeling agents.

TC (crystal tetracycline chloride, WYETH

LEDERLE JAPAN LTD.) was dissolved in physiological saline at 2% (W/V). The other three agents were purchased from Wako Junyaku Co. and a 3% (W/V) solution of each was prepared in 3% KOH.

2. Methods

Twenty-five 7-week-old SPF Wistar female rats weighing 120-140g were used for the experiment. The rats were divided into five groups of five animals. Four groups of rats were each given one of the four kinds of labeling agents, and the other group was used as a control for measuring body weight. Each of the four labeling agents was hypodermally injected into the abdomen. **Table 1** shows the amount of each labeling agent injected. The labeling agents were administered every 5 days, and the amount administered was adjusted according to the rat's body weight (BW) per 100g to determine the optimal amount to be injected. The rats were killed 5 days after the final injection (**Table 1**). After death, the heads were cut off, fixed in a 10% formalin solution, dehydrated by alcohol, dipped in styrene monomer, and buried in rigolac^{R} (styrene resine). Next, 80-100 μm nondecalcified specimen slices were prepared in parallel, in the longitudinal direction of the condylar process, using a crystal cutter (MARUTO INSTRUMENT CO.,LTD.). The sliced specimens were prepared into ground slices of approximately 50 μm using a grinder (MARUTO INSTRUMENT CO.,LTD). The sliced specimens were microradiogrammed using a soft X-ray system with a tube voltage of 20 kVp, tube current of 4mA, photographing time of 12 minutes, and focusing point-film distance of 30cm. Finally, the labeling images of these ground slices were observed by an incident-light fluorescent microscope (OLYMPUS, Model BH-2).

During the experiment, body weight was measured and the injected region were observed periodically. The amount and method of administration were determined so as not to influence the entire body nor to disturb the calcification of the bone (TAKAHASHI et al, 1979). For TC, the total dosage was fixed at 21mg with a maximum single administration amounting to 6mg/100g BW ; for Cal, the total dosage administered was 2.8mg with each administration amounting to 0.7mg/100g BW; for AC, the total dosage was 15mg with a single administration of 5 mg/100 g BW ; and for CB, the total dosage was 20mg with each administration amounting to 2mg/100g BW.

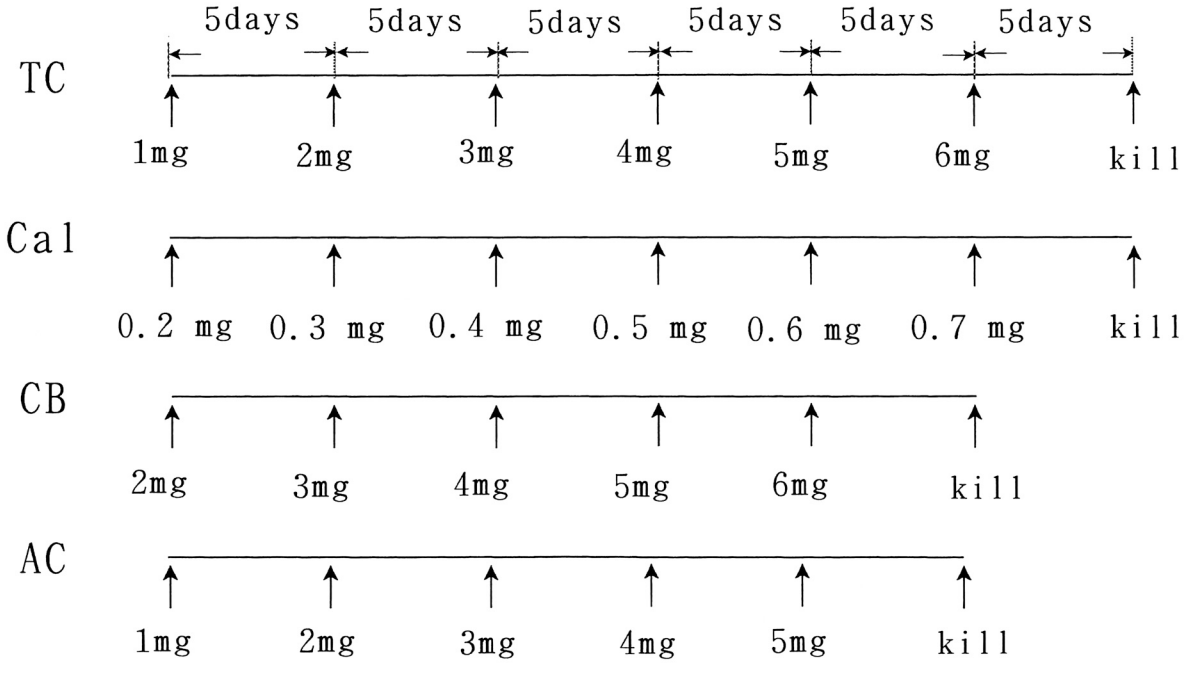


Table. 1 The amount (per 100g BW) of each labeling agents injectioned

RESULTS

1. Changes in Body Weight of Rats and in the Regions Injected with the Labeling Agents

The rats of the four experimental groups displayed the same trend toward an increase in body weight as those of the control groups, which findings suggested that there was no influence of the labeling agents on the growth of rats (**Table 2**).

A macroscopic examination showed that no changes occurred in the experimental groups injected with TC, Cal, or CB at any time, but transitory hardening of the region in all the rats in the group injected with AC over a dose of 4mg／100g BW. The hardening, however, disappeared 5 days later (**Fig. 1**).

2. Labeling Images in the Condylar Process under a Fluorescent Microscope

When the injected amount of TC was 1mg／100g BW, the labeling lines were found to be thinner than those by the other agents and detailed observation was therefore impossible. An increase in the amount of TC to 2mg／100g BW made yellow labeling lines clear and thus visible. Then, as the amount injected was increased from 2 to 6mg／100 g BW, the labeling lines became wide. When the amount injected exceeded 4mg／100g BW, light yellow labeling image appeared between the labeling lines, rendering the total image unclear. However, no changes were noted in the intensity of the fluorescence of the labeling lines (**Fig. 2**).

agent	solubility	change of resion injected (with naked eyes)	color	line clarity	The standard amount for injection /100 g BW	optimal amount for administration	disturbances of calcification
TC	good	nothing particular	yellow	clear	2mg	2mg	nothing
Cal	good	nothing particular	green	clear	0.3mg	0.3mg	nothing
CB	good	nothing particular	blue	clear	3mg	3mg	nothing
AC	good	induration (ephemeral)	red	clear	2mg	2mg	nothing

Table. 2 Characterristics of the labeling agents

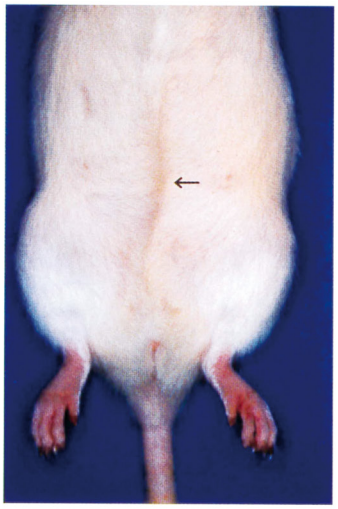


Fig. 1 Transitory hardening of the region where AC was injectied at a dose over 4㎎／100 g BW. An arrow indicates the region.

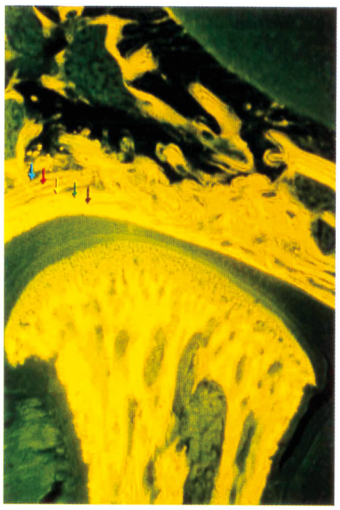


Fig. 2 Labeling lines observed in the TC group. In the TC group, the amount of TC to 2㎎／100g BW made yellow labeling lines clear and thus visible. AS the amount injected was increased from 2 to 6㎎／100g BW, the labelimg lines became wide. When the amount injected exceede 4㎎／100g BW, a light yellow labeling image appeared between the labeling lines, rendering the total image unclear.

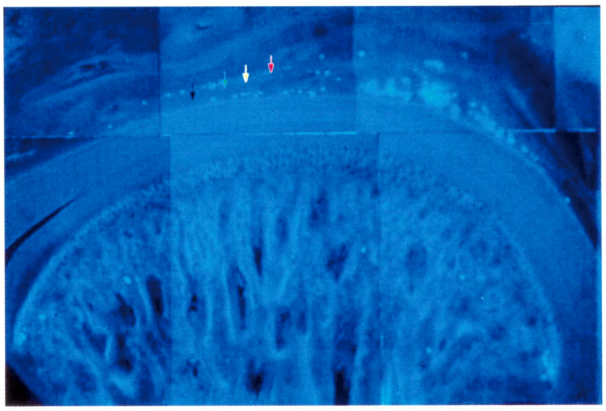
For the Cal group, green labeling lines were observed after the dose exceeded 0.3mg／100g BW, and as the amount injected increased from 0.3 to 0.7mg／100g BW, the width of the labeling lines increased. When the amount exceeded 0.5mg／100g BW, a thin green labeling image appeared between the labeling lines clearly. No changes were observed, however, in the intensity of the fluorescence of the labeling lines (**Fig. 3**).

In the CB group, blue labeling lines could be observed when the amount injected was over 2mg／100g BW. Administration over 3mg／100g BW produced a thin blue labeling image between the labeling lines, making the total image unclear.

No changes were observed, however, in the intensity of the fluorescence of the labeling lines(**Fig. 4**).

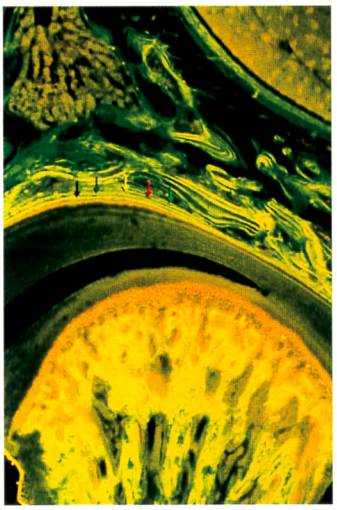
The labeling lines in the AC group were observed to be thinner at a dose of 1mg／100g BW when compared to those in the other groups, but detailed observation was impossible. In doses greater than 2mg／100g BW, a red labeling line became visible. As the amount injected increased to 5mg／100g BW, the intensity of the fluorescence and the width of the labeling lines increased. However, after exceeding 4mg／100g BW, a thin red labeling image appeared with a dose of over 4mg／100g BW, spoiling the clear total image (**Fig. 5**).

From the above results, the optimal concentrations of TC, Cal, CB, and AC for labeling the condylar process are considered to be 2mg／100g BW, 0.3mg／100g BW, 3mg／100g BW, and 2mg／100g BW, respectively.



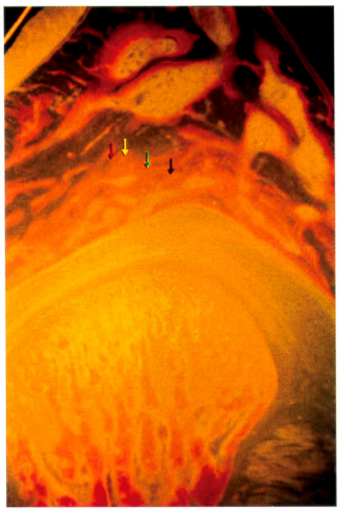
→ : 3㎎／100g BW　→ : 4㎎／100g BW
→ : 5㎎／100g BW　→ : 6㎎／100g BW

Fig. 4 Labeling lines observed in the CB group.
In the CB group, blue labeling lines could be observed over 2㎎／100g BW. Administration over 3㎎／100g BWproduced a thin blue labelling images between the labeling lines, making the total image unclear.



→ : 0.3㎎／100g BW
→ : 0.4㎎／100g BW
→ : 0.5㎎／100g BW
→ : 0.6㎎／100g BW
→ : 0.7㎎／100g BW

Fig. 3 Labeling lines observed in the Cal group.
In the Cal groups, green lines were observed after the dose exceeded 0.3㎎／100g BW, and as the amount injected from 0.3 to 0.7㎎／100g BW, the width of the labeling lines increased. When the amount exceeded 0.5／100g BW, a thin green labeling images appeared between the labeling lines clearly.



→ : 2㎎／100g BW
→ : 3㎎／100g BW
→ : 4㎎／100g BW
→ : 5㎎／100g BW

Fig. 5 Labeling lines observed in the AC group.
In the AC group, red labeling lines were observed to be thinner at a dose of 1㎎／100g BW, when compared to those in the other groups. In doses larger than 2㎎／100g BW, a red labeling line became visible. As the amount injected increased to 5㎎／100g BW, the intensity of the fluorescence and the width of the labeling lines increased.

3. Microradiogram Observation

Among all four labeling agents, no anomalous calcification was observed in any region at any time (**Fig. 6**).

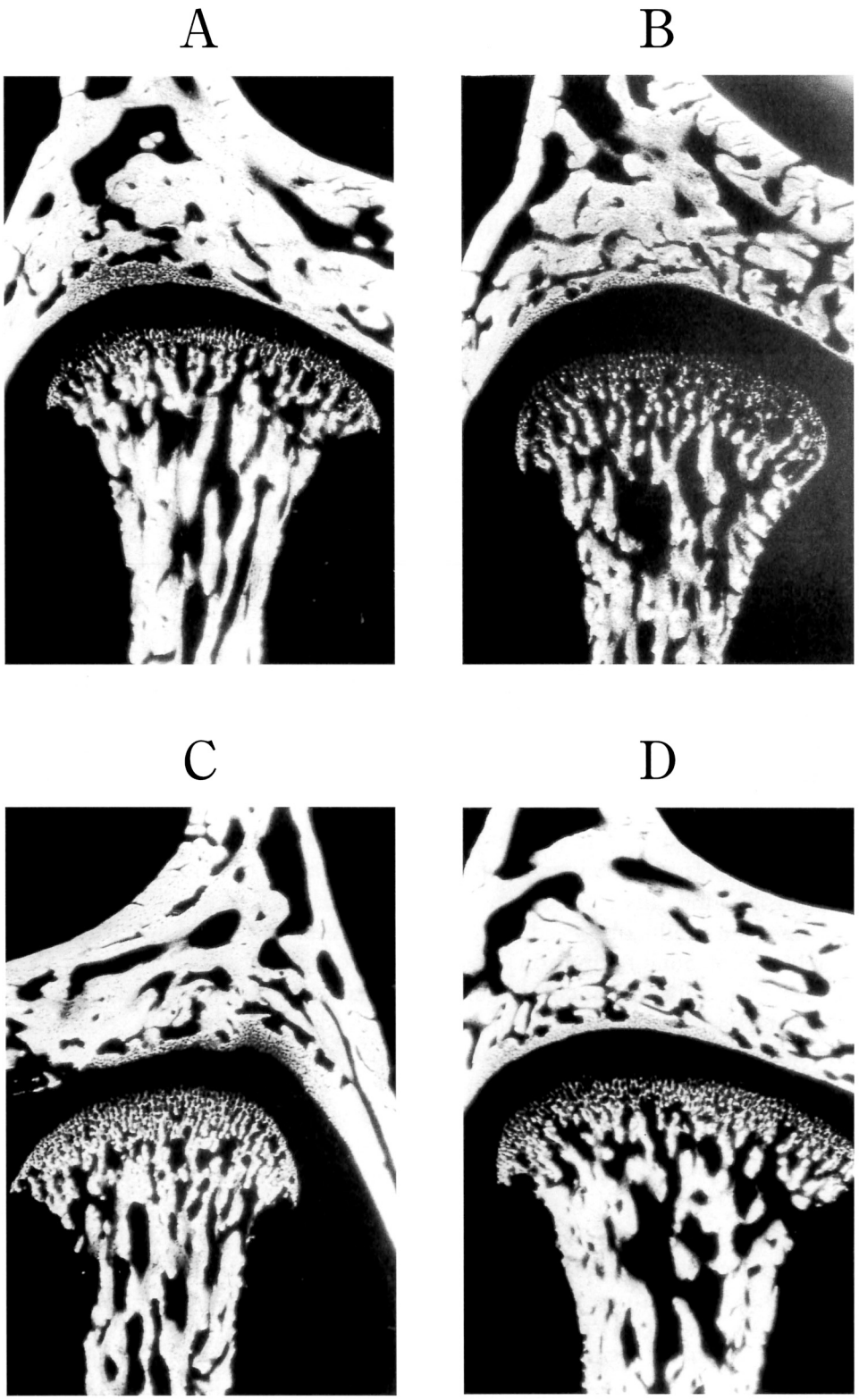


Fig. 6 Microradiogram of the sliced speciumens prepared from the TC (A), Cal (B), CB (C), and AC (D) groups.

DISCUSSION

Calcification of the bone occurs on three surfaces, specifically, the periosteum surface, the endosteum surface, and the osteon surface, forming layer plates. OKADA and MIURA (1938) began measuring the speed of calcification by depositing a marker in the calcification front. It was a time-describing method employing lead. Although this method was suitable for animal experiments, it presented difficulties because it was not applicable for the human body, and the osteoid and calcified bone could not be identified due to decalcification. MILCH et al, (1958) found that tetracycline-family antibiotics could be observed in the newly-formed bone under a fluorescent microscope because of the fluorescence transmitted. Later, tetracycline-family antibiotics were employed for time-description in bone and tooth tissues in the human body. Thus, numerous researchers (MILCH et al, 1958; FROST, 1963; BORDIER, 1964; SCHENK, 1969; MERZ et al, 1970) developed various methods to measure histological shapes of the bone.

The purpose of this study is to determine the optimum concentrations of various labeling agents when we observe the turning-point of the healing of the condylar process fracture chronologically. TAKAHASHI (1979) reported that labeling agents can disturb bone formation dose-dependently. Therefore, labeling agents should satisfy the following three conditions :

(i)Clear labeling lines should be observed with a minimal disturbance of bone formation.

(ii)Administration of the labeling agent should not cause a major change in either the injection region or the total condition of the body.

(iii)They should be easy-to-handle.

Table 2 indicates the results of our studies regarding these items

(1)Tetracycline (TC)

Many researchers have already reported that tetracycline is selectively incorporated and maintained in the calcification-progressing regions of ground bone substance (FROST, 1964; HARRIS et al, 1964). KEIL (1968) reported, from an experiment with mice, dogs, and monkeys, that the standard amount of tetracycline was 2 mg/100 g BW for observation of teeth and bones, while ONO (1973) reported that with TC injections of 1mg/100g BW, clear labeling lines with a strong fluorescent intensity could be observed in the calcification front of dentine and bone. In our study, like the report of ONO (1973), clear labeling lines could be seen with no abnormal findings in bone formation. Since TC satisfies the requirements of a good labeling agent, it is considered to be suitable for observing the condylar process fracture healing phase in rats.

(2)Calcein (Cal)

SUZUKI et al (1966) reported that an injection of 2, 4-bis fluorescent, an isomer of calcein, at a dosage of 25~100mg/100g BW into the abdominal cavity of mice, as an in vivo labeling agent of the bone, produced clear labeling lines with few occurrences of formation anomalies.

Our study showed that green labeling lines became visible at doses over 0.3mg/100g BW without any influence on the injection region and bone formation. Thus, Cal can be considered applicable as a good labeling agent.

(3)Calcein blue (CB)

RAHN et al (1970) employed CB as an in vivo labeling agent for hard tissues : preparing a solution with 2% $NaHCO_3$ of CB and injecting it into the veins of sheep and tame rabbits, and the abdominal cavities of rats, at a dosage of 30mg/100g BW. They then reported that blue labeling lines were obtained without any disturbance of the calcification of the bone. TAKAHASHI et al (1979) reported that labeling lines were obtained when the amount injected was over 4mg/100g BW and that the fluorescence was intensified along with an increase of the injected amount. They also stated that with an injected amount of 8mg/100g BW, slight disturbances of calcification could be recognized along the labeling lines. CB, however, tends to produce unclear labeling lines when radiated by ultraviolet rays for a long period of time under a fluorescent microscope. In view of this, it is considered that CB could be a labeling agent for hard tissues provided that the observation time is short and the amount of the injection is 4mg/100g BW. Under these conditions, very little calcification disturbance is generated, and photography is possible. Unlike the other labeling agents, regarding filter conditions for a fluorescent microscope in labeling line observation, observation with ultraviolet ray excitation is preferable. This makes

simultaneous observation with other labeling lines impossible.

(4)**Alizarin complexion (AC)**

RAHN et al (1970) used AC as an in vivo labeling agent for hard tissues. A 3% solution of AC in 2% $NaHCO_3$ was injected hypodermally into the abdomen of tame rabbits or rats, and into the veins of dogs and sheep at dosage of 10～30mg／10kg BW. The same solution was given orally to mice at a dosage of 30mg／kg BW. They reported that the influence of this agent on the entire body was low when the amount injected was kept at 20～30mg／kg BW.

No disturbance of bone calcification was generated and clear red labeling lines could be observed in the calcification front at the time of injection. TAKAHASHI et al (1979) reported that red labeling lines could be recognized with injections of over 2mg／100g BW. Fluorescence intensity increased along with an increase in the amount injected, and when the amount injected exceeded 4mg／100g BW, they could recognize a light calcification disturbance aligned with the labeling lines. In our study, transitory hardening was observed in the injection regions when the amount of the injection was over 4mg ／100g BW. However, no major influence on the entire bodies of animals was recognized and natural healing occurred during the experiment. In view of these results, it can be determined that AC providing clear labeling lines and minor disturbance may be used as a labeling agent for hard tissues.

From these tests, we consider that the four labeling agents satisfy the aforementioned three conditions for labeling when the dosages are properly selected and are useful as labeling agents for the joints of the jaw. TAKAHASHI et al (1979) stated that TC and Cal have been already used as labeling agents in many experiments for hard tissue and their excellent properties have been confirmed (ONO, 1978; OUCHI 1974; CHIBA 1976).

For observation under a fluorescent microscope, the same filter conditions of BV excitement can be used for TC, Cal, and AC labeling, but CB labeling requires an ultraviolet ray exciter. Because of this characteristic, CB is considered to be a good labeling agent for single labeling. Difficulties are encountered, however, when observation must be made simultaneously with TC, Cal, and AC. As mentioned above, to assess the formation status, each critical bone is different depending on the position. It is considered necessary to study the dynamics of the bone by adding a microradiogram and taking into account the differences in ground bone substances.

CONCLUSION

1. In observing labeling images for the observation of chronological changes in the bone where changes in reconstructive phenomena are brisk, the four labeling agents, TC, Cal, CB and AC can be utilized. The optimal concentrations for practical use are : 2mg／100g BW for TC, 0.3mg／100g BW for Cal, 3mg／100g BW for CB, and 2mg／100g BW for AC.
2. The labeling lines of CB tend to become unclear when CB is radiated with ultraviolet rays for a lengthy period under a fluorescent microscope. When the observation time is shortened, however, and the amount of injection is maintained at about 3mg／100g BW, there is very little disturbance in calcification. This makes photography possible and CB a practically applicable labeling agent for hard tissues.

The present study confirms that polychromatic labeling is a suitable method for observing the bone metabolism of a fracture in the condylar process.

Note. The experiments described in this paper were performed with adherence to the guidelines of the Japanese Association for Laboratory Animal Science for the use of experimental animals.

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