NOTE *Surgery*

Influence of an Autologous Serum-Supplemented Medium on the Proliferation and Differentiation into Neurons of Canine Bone Marrow Stromal Cells

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ABSTRACT. We investigated the influence of autologous serum (AS)-supplemented medium on the proliferation and differentiation into neurons of canine bone marrow stromal cells (BMSCs). Canine BMSCs were cultured using α-MEM only, α-MEM with 10% fetal bovine serum (FBS), and 5, 10 and 20% AS-supplemented α-MEM. Growth of canine BMSCs was observed in all AS groups. The proliferation capacity of canine BMSCs in the AS groups was similar to that in the FBS group. No significant differences between the FBS and AS groups were observed in the percentage of the cells that changed to the neuron-like morphology and neuron-specific enolase-positive ratio after neuronal differentiation. Canine BMSCs cultured using AS-supplemented medium were able to proliferate and showed neuronal differentiation potency.

KEY WORDS: autologous serum, bone marrow stromal cell, canine, culture, neuron.

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Recently, spinal cord regenerative therapy using bone marrow stromal cells (BMSCs) has begun to be clinically applied as one option for the treatment of severe spinal cord injury (SCI) in human medicine [2, 9]. In the clinical application of BMSCs, the use of fetal bovine serum (FBS) during culturing has been called into question and many investigations into culture methods without FBS are underway [6, 12]. One proposed solution is the use of a culture medium supplemented with autologous serum (AS). The safety of the clinical application of BMSCs will improve if these cells can be cultured with AS-supplemented medium, thereby avoiding the use of FBS.

Human BMSCs have been successfully cultured and grown in AS-supplemented medium [3, 12, 15]. Most of these cases involved the use of a culture medium supplemented with 10% AS, with no significant differences reported in the morphology or proliferation capacity of BMSCs compared to culturing with media with 10 or 20% FBS [3, 10, 12, 15]. To the best of our knowledge, however, there have been no investigations into the neuronal differentiation potency of human BMSCs cultured with AS-supplemented medium; most such studies have focused on differentiation into bone and cartilage [7, 10, 12, 15].

Recently, a clinical trial of regenerative therapy for SCI using autologous BMSCs has also been performed in dogs [8]. At present, canine BMSCs are generally cultured in FBS-containing culture medium. Thus, even in dogs, concern over bovine-derived infectious diseases and an immunoreaction from heteroantigens is an issue that must be addressed for the clinical application of canine BMSCs. To the best of our knowledge, there have been no reports on the effect of AS-supplemented medium on the proliferation and neuronal differentiation of canine BMSCs. In addition, the optimum concentration when culturing canine BMSCs in AS-supplemented medium has yet to be determined. Therefore, in the present study, canine BMSCs were cultured using three different types of AS-supplemented media, and the effect of those media on the proliferation of canine BMSCs and their neuronal differentiation potency was examined.

Six male beagles were used in the present study. This study was conducted following the guidelines for the care and use of laboratory animals of the College of Bioresource Sciences of Nihon University. Bone marrow was collected from the humerus under general anesthesia, and mononuclear cells were separated by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich Inc., St. Louis, MO, U.S.A.). Following collection, the mononuclear cells were transferred to 25 cm^2 culture flasks (Corning Inc. Life Sciences, Lowell, MA, U.S.A.) and static cultured in an incubator at 5% CO_2 and 37°C using α-modified Eagle's minimum essential medium (α-MEM; Invitrogen Corporation, Carlsbad, CA, U.S.A.) with 10% FBS (Invitrogen). On the fourth day of culture, nonadherent cells were removed when replacing the culture medium, thereby isolating canine BMSCs. Canine BMSCs were collected using trypsin-EDTA (Invitrogen) once they reached 90–95% confluency.

The collected canine BMSCs were cultured using five different types of culture media in 6-well cell culture

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plates (Corning) with 1,500 cells per well (well area, 9.5 cm^2). The α-MEM supplemented with 10% FBS was used as the positive control (FBS group). The α-MEM without FBS or AS was used as the negative control (NS group). AS was separated from the subject dogs and was mixed with α-MEM to 5, 10 and 20% concentrations (5, 10 and 20% AS groups, respectively). The adherent cells were collected using trypsin-EDTA after 10 days of culture, and the number of cells was measured for each group using a NucleoCounter™(Chemometec A/S, Allerød, Denmark).

Canine BMSCs were differentiated into neurons using the method described by Woodbury *et al*. [13] in each group. After 8 hr of neuronal differentiation, the percentage of the cells that changed to the neuron-like morphology was measured. Following the measurement, these cells were fixed in 10% buffered formalin solution and immunostained using a polymer-based immunohistochemical detection system (EnVision™ Plus Kit; Dako Co., Ltd., Tokyo, Japan). The anti-neuron-specific enolase (NSE) mouse anti-human monoclonal antibody (Clone No. BBS/NC/VI-H14; Dako) was used as a neuronal marker. Positive cells were identified by their red color. Total 200 cells were evaluated by two investigators and the NSE-positive ratio was measured. Canine brain was used as positive control.

The data for this experiment are expressed as the means ± standard errors. The SigmaStat® 3.5 (Systat Software Inc., Chicago, IL, U.S.A.) statistical analysis software was used, and one-way analysis of variance (ANOVA) was performed for comparisons between groups. The Student-Newman-Keuls method was used for the post hoc test with $P<0.05$ indicating a significant difference.

The FBS group demonstrated the fastest tendency for cell adhesion among all groups. These cells were spindle-shaped and grew in a relatively uniform manner (Fig. 1A). In contrast, almost no canine BMSCs grew in the NS group (Fig. 1B). Proliferation of canine BMSCs was observed in all AS groups. The formation of colony-like cell masses was observed when canine BMSCs were cultured in AS-supplemented medium (Fig. 1C–E). The colony-like cell masses tended to grow larger and thicker with higher concentrations of AS (Fig. 1E). The number of adherent cells at 10 days of culture in the FBS group was $50.9 \pm 11.7 \times 10^4$, which was the largest among all groups (Fig. 2A). The number of adherent cells in the NS group was significantly lower than that in the other groups (Fig. 2A). No significant differences in the number of adherent cells were observed among the AS groups. However, the number of adherent cells in the 5% AS group ($23.1 \pm 10.7 \times 10^4$) was lower than those in the 10% ($38.0 \pm 18.4 \times 10^4$) and 20% AS groups ($39.8 \pm 11.2 \times 10^4$) (Fig. 2A).

In the NS group, almost all cells detached from the culture plate during neuronal differentiation, and no neuron-like cells were observed (Fig. 1B). Cells that changed to the neuron-like morphology were observed in all AS groups and showed the same tendencies in morphology as those in the FBS group (Fig. 1A, 1C–E). Excluding the NS group, no significant differences were observed among the groups in the percentage of cells that changed to the neuron-like morphology (Fig. 2B). Approximately 60% of canine BMSCs in the FBS and AS groups changed to the neuron-like morphology, with no observed differences related to the concentration of AS (Fig. 2B). Nearly all neuron-like cells showed positivity for NSE in the FBS and AS groups (Fig. 1A, 1C–E), with a portion of the other cells also testing positive. Excluding the NS group, no significant differences in the NSE-positive ratio were observed among the other groups (Fig. 2C).

These results suggest that it is possible to culture canine BMSCs using AS-supplemented medium. There was little difference in the proliferation pattern of canine BMSCs between culturing with AS-supplemented medium and culturing with FBS-containing medium. The culture of human BMSCs in an AS-supplemented medium has been reported with proliferation including colony-like cell mass formation [12]. The report indicated that colonies tended to grow larger and thicker with higher concentrations of AS [12]. The present study replicated the results of that previous study using human BMSCs, with the 20% AS group exhibiting the largest and thickest colonies (Fig. 1E).

It has been reported that the proliferation capacity of human BMSCs cultured in AS-supplemented medium was equal [1, 11, 12, 15] or slightly less [4, 5, 14] than that observed by culturing them in the medium with FBS. The present study found a slightly lesser proliferation capacity in canine BMSCs cultured with AS-supplemented media compared to the FBS group. However, there were no significant differences among these groups, and the present results suggest the possibility of acquiring an adequate number of canine BMSCs for clinical application using AS-supplemented culture media.

In this study, the number of adherent cells at 10 days of culture in the 5% AS group was lower than those in the 10 and 20% AS groups. The level of proliferation of human BMSCs has not been high enough in cultures conducted using 1 or 3% AS-supplemented culture media [12]. Thus, AS concentration levels of less than 10% are probably not desirable for the effective growth of canine BMSCs.

Anemia and shock from bleeding are common in dogs with traumatic SCI. For this reason, it may not be realistic to collect much blood and bone marrow from small breed dogs. Thus, it is necessary to consider a limit for the amount collected while using AS. In the present study, the proliferation capacity and neuronal differentiation potency of canine BMSCs in the 10% AS group were approximately the same as those in the 20% AS group. However, the proliferation capacity in the 5% AS group was lower than that of the other AS groups. Therefore, 10% may be the most realistic minimum concentration of AS for the medium used to culture canine BMSCs.

To the best of our knowledge, there have been no reports on the differentiation of BMSCs cultured with AS-supplemented medium into neurons. In the present study, when canine BMSCs were cultured in AS-supplemented media and were differentiated into neurons by the previously reported method [13], these cells changed to the neuron-like morphology and stained positive against NSE

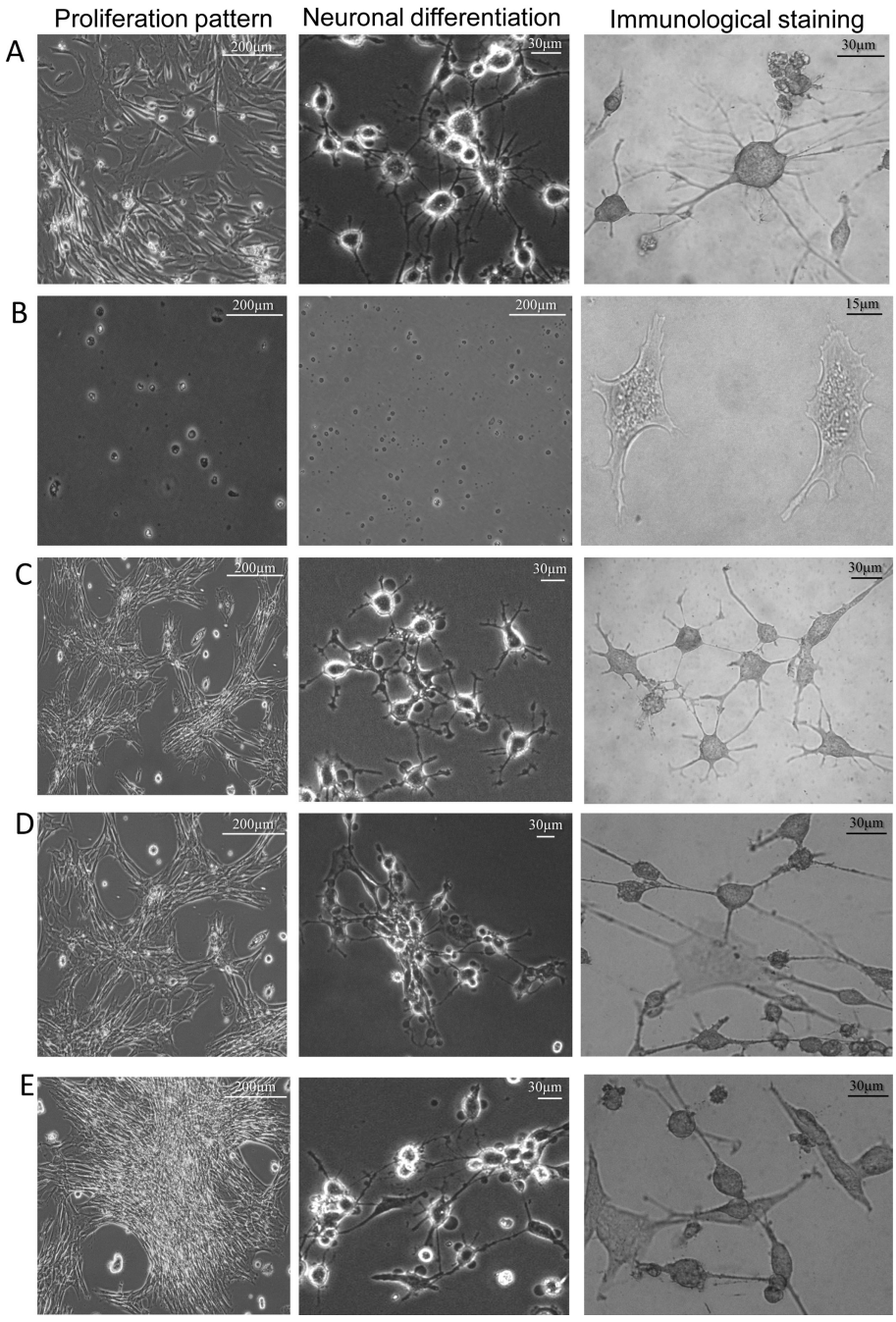

Fig. 1. Microscopic findings of canine bone marrow stromal cells (BMSCs) at 10 days of culture, after neuronal differentiation, and when immunological staining against NSE was performed. A: FBS group; B: NS group; C: 5% AS group; D: 10% AS group; and E: 20% AS group.

in all AS groups. These results indicate the high potential of canine BMSCs cultured in AS-supplemented medium to have neuronal differentiation potency. Furthermore, there were no significant differences in the percentage of cells that changed to the neuron-like morphology or NSE-positive ratio after neuronal differentiation in the FBS and AS groups. This study suggests the potential of canine BMSCs cultured in AS-supplemented medium for applications in spinal cord regenerative therapy.

In conclusion, this study suggests that the culture of canine BMSCs using an AS-supplemented medium is possible and that the proliferation capacity and neuronal differ-

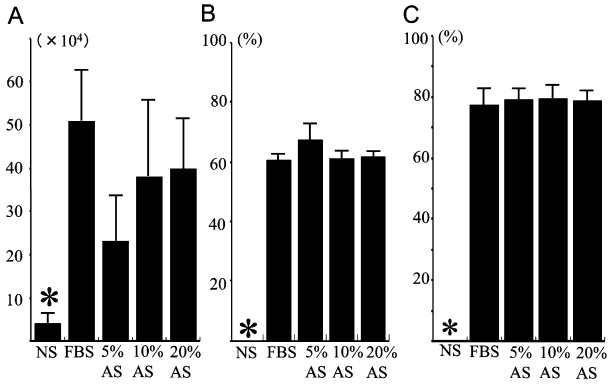

Fig. 2. The number of adherent cells at 10 days of culture (A), the percentage of cells that changed to the neuron-like morphology (B) and the NSE-positive ratio (C). The NS group exhibited significantly lower values than the other groups (*: $P<0.05$ vs. the other groups).

entiation potency of these cells are approximately the same as those of canine BMSCs cultured using FBS-containing culture medium. The optimum concentration of AS for culturing canine BMSCs was found to be 10%.

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