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Research Article

DEVELOPING AN IN VIVO BIOASSAY FOR EVALUATION OF G-CSF BIOLOGICAL POTENCY

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ABSTRACT. Recombinant granulocyte-colony stimulating growth factor (G-CSF) is being widely used for the treatment of chemotherapy-induced neutropenia. The activity of recombinant G-CSF is regularly assessed using an *in vitro* bioassay based on the stimulation of leukemia cell proliferation. However, an accurate *in vivo* bioassay is also required to verify the efficiency of G-CSF in neutropenia treatment as well as to examine the response of the whole body to the recombinant protein. Therefore, in this study, we developed an *in vivo* bioassay for evaluating the biological potential of G-CSF using an immunosuppressed mouse model. We found that a single shot of 200 mg/kg cyclophosphamide was sufficient to induce immunosuppression in mice. The injection of G-CSF into the immunosuppressed mice at the dose of 5 µg/kg G-CSF was suitable to determine the biological activity of G-CSF. In addition, the daily multiple injections of 5 µg/kg G-CSF for 4 days in association with leukocyte counting for 7 days could be used to demonstrate the neutropenia treatment efficiency of recombinant G-CSF.

Keywords: *G-CSF, neutropenia, in vivo bioassay, immunosuppressed mouse model, cyclophosphamide.*

INTRODUCTION

Neutropenia, a condition in which the levels of neutrophils are abnormally low, is one of the common side-effects of cancer chemotherapy. Since neutrophils are among the first immune cells to defend from infection, patients with prolonged severe neutropenia are usually at a high risk of infections that might lead to sepsis and death [1, 2]. Therefore, development of neutropenia during chemotherapy often results in treatment delay or dose reduction, that may affect the efficiency of the cancer treatment and increase the cost of hospitalization [3].

One strategy to prevent and treat neutropenia is the use of recombinant granulocyte-colony stimulating factor (G-CSF). G-CSF functions in stimulating the production of neutrophils from precursor cells and enhancing the functions of mature neutrophils [4, 5]. Due to these functions, recombinant G-CSF was first trialed for the treatment of chemotherapy-induced neutropenia in 1988 and was approved by the Food and Drug Administration (FDA) in 1991 [6, 7]. Since then, G-CSF has been popularly used to reduce the duration and degree of neutropenia in cancer treatment.

In general, the activity of a recombinant protein must be strictly evaluated for the application in pharmaceuticals. In particular, an accurate method was developed for the

in vitro assessment of G-CSF biological activity, which is based on the stimulation of NSF-60 leukemia cell line proliferation [8, 9]. However, although having great advantages including low cost, time-saving, easy handling, allowing high-number replicates and automation, the *in vitro* bioassay has important limits as it cannot reflect the *in vivo* response as well as the efficiency and side effect of a drug [10]. Therefore, an *in vivo* evaluation of recombinant protein activity is also critical for its commercialization.

In addition, it has been previously reported that cyclophosphamide (CPA), an alkylating agent belonging to the oxazaphosporine group, can induce immunosuppression in animals and human [11-13]. Zuluaga et al. demonstrated that the treatment of CPA caused profound neutropenia ($\leq$10 neutrophils/mm^3) in mice [14]. Therefore, we here used CPA to generate an immunosuppressed mouse model. Based on this model, we subsequently optimized the dose and the schedule of G-CSF treatment in order to develop an *in vivo* bioassay for evaluation of recombinant G-CSF activity.

MATERIALS AND METHODS

Animals

Mice (*Mus musculus* var. Albino) were purchased from Pasteur institute, Ho Chi Minh city, Vietnam. Healthy male mice weighing ~25 g were used. The mice were housed in separate cages and fed ad libitum. All efforts were made to minimize animal suffering and to reduce the number of animals used and experiments in the study were approved by the university committee (562-2018-18-05).

Optimizing the condition to generate immunosuppressed mouse model

Optimizing the CPA injection dose: The experiment was carried out with twenty mice divided into 4 groups (n=5). The first, second, and third groups were injected with 100, 200, and 300 mg/kg body weight of CPA, respectively, whereas the fourth group were injected with saline solution and served as a negative control. The blood samples were collected from tail veins every day up to 10 days to determine the number of total leukocytes using Giemsa staining kit (Abcam, UK). A paired T-test was used for statistical analysis.

Optimizing the CPA injection schedule: The experiment was carried out with thirty mice divided into 6 groups (n=5). The first group were injected with a single shot of CPA with optimized dose (200 µg/ml) at day 0. The second group were injected with 50 µg/ml CPA every day within 4 days (days 0-3). The third group were injected twice with 100 µg/ml CPA, at day 0 and day 2. Three remaining groups were injected with saline buffer using the similar schedules and served as negative controls. The blood samples were collected from tail veins every day from day 0 to day 10 to determine the numbers of total leukocytes.

Optimizing the G-CSF concentration for in vivo bioassay

Twenty mice were divided into 4 groups (n=5) and injected CPA at the optimal dosage. Twenty four hours after the last CPA injection, the first three groups were injected with 1, 5, and 10 µg/kg body weight of the marketed G-CSF product Neupogen (Amgen Inc., USA). The fourth group were injected with saline solution as a control. The blood samples

were collected from tail veins every 4 hours within 24 hours after injection and the number of total leukocytes were counted.

Evaluating The Efficiency Of G-CSF in Neutropenia Treatment

Ten immunosuppressed mice were divided into 2 groups (n=5). The first group were injected with 5 µg/kg Neupogen every day for 4 days and the second group were injected with an equal volume of saline solution every day for 4 days. The blood samples were collected from tail veins every day for 7 days since the first G-CSF injection and the number of total leukocytes were counted.

Statistical Analysis.

The data at each time point were analyzed using one-way analysis of variance (ANOVA) with Tukey test on GraphPad Prism 7.04 software.

RESULTS AND DISCUSSION

Generating An Immunosuppressed Mouse Model Using CPA

In order to generate immunosuppressed mice, we first determined an appropriate dose of CPA, which could be used to efficiently suppress the immune system but not severely affect the physical condition of mice, so that the mice would acquire neutropenia but be healthy enough for further experiments. Therefore, in this experiment, CPA was injected into 3 groups of healthy mice at three doses of 100 mg/kg, 200 mg/kg, and 300 mg/kg. We found that 10 days after injection, all mice exhibited normal behaviors and appearance, and no signs of hair loss and blindness were observed. In addition, from day 1, the total leukocyte numbers in all CPA-injected mice were remarkably decreased whereas those of the control group did not change, indicating that these doses were sufficient for immunosuppression (Fig. 1, Table 1). However, from day 7 to day 10, the number of leukocytes gradually increased to reach the normal levels, suggesting the recovery of immune system during this time. When comparing the total leukocytes numbers of four groups, we also found that the effects of the two doses 200 mg/kg and 300 mg/kg were similar and higher than the dose 100 mg/kg. Therefore, the dose 200 mg/kg was chosen for further experiments.

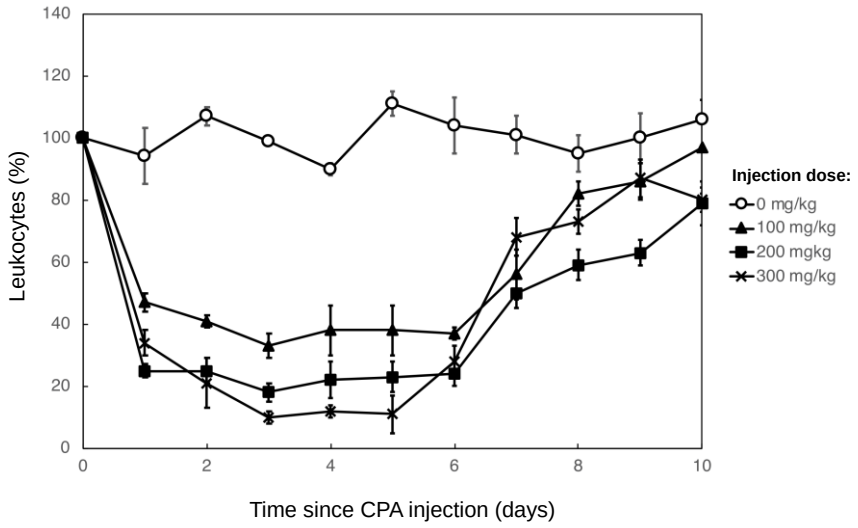

Fig 1. *The changes in leukocyte numbers in mice injected with different doses of CPA. The data were shown as mean ± SD.*

Table 1. *The changes in leukocyte numbers in mice injected with different doses of CPA*

	CPA dose			
	0 mg/kg	**100 mg/kg**	**200 mg/kg**	**300 mg/kg**
Day 0	100 ± 0^{0a}	100 ± 0^{0a}	100 ± 0^{0a}	100 ± 0^{0a}
Day 1	94 ± 9^{1a}	47 ± 3^{1b}	25 ± 2^{1c}	34 ± 4^{1c}
Day 2	107 ± 3^{2a}	41 ± 2^{2b}	25 ± 4^{2c}	21 ± 8^{2c}
Day 3	99 ± 1^{3a}	33 ± 4^{3b}	18 ± 3^{3c}	10 ± 2^{3c}
Day 4	90 ± 2^{4a}	38 ± 8^{4b}	22 ± 6^{4b}	12 ± 2^{4b}
Day 5	111 ± 4^{5a}	38 ± 8^{5b}	23 ± 5^{5c}	11 ± 6^{5c}
Day 6	104 ± 9^{6a}	37 ± 2^{6b}	24 ± 4^{6c}	28 ± 5^{6c}
Day 7	101 ± 6^{7a}	56 ± 8^{7b}	50 ± 5^{7b}	68 ± 6^{7b}
Day 8	95 ± 6^{8a}	82 ± 4^{8b}	59 ± 5^{8c}	73 ± 4^{8d}
Day 9	100 ± 8^{9a}	86 ± 6^{9b}	63 ± 4^{9c}	87 ± 6^{9b}
Day 10	106 ± 1^{10a}	97 ± 15^{10a}	79 ± 7^{10b}	80 ± 4^{10b}

Different superscript numbers indicate different time point
Different superscript letters (a, b, c, d) indicate significant differences at each time points ($P<0.05$)

Since CPA is an extremely toxic agent, the amount of CPA for one injection must be minimized to lower its effects on the physical health of mice. Therefore, we considered whether the multiple shots of low G-CSF dose would be better than the single injection of high dose. Hence, we next optimized the schedule for CPA injection, in which group 1 were injected once with full amount of CPA (200 mg/kg) at day 0, group 2 were injected twice with one-half the full amount of CPA (100 mg/kg) at day 0 and day 2, and group 3 were injected four times with one-fourth the full amount of CPA (50 mg/kg) at days 0-3. Accordingly, the total amount of injected CPA for three groups were equal but the CPA amount for one injection was different. Groups 4, 5, 6 were injected with saline buffer using the same schedule for groups 1, 2, 3, respectively. We found that after CPA injection, the leukocyte numbers of group 2 and 3 were lower than those of group 1, indicating that the multiple-injection schedules could trigger immunosuppression more efficiently than did the single-injection schedule (Fig. 2, Table 2). However, we unexpectedly found that nearly 70% of mice in group 2 died whereas mice in group 3 showed hair loss and slow movement during the observation period. We supposed that the very low neutrophil numbers in group 2 and group 3 might severely affect the health of mice. Therefore, the single injection of 200 mg/kg CPA was the most suitable to induce immunosuppression, and the neutropenic mouse could be obtained after 1 day since injection.

Table 2. *The changes in leukocyte numbers in mice given different schedules of CPA*

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Day 0	100 ± 0^{0a}	100 ± 0^{0a}	100 ± 0^{0a}	100±0	100±0	100±0
Day 1	85 ± 4^{1a}	98 ± 2^{1a}	84 ± 2^{1a}	108±2	128±2	111±2
Day 2	34 ± 1^{2a}	73 ± 8^{2b}	43 ± 4^{1b}	103±7	115±7	104±7
Day 3	33 ± 4^{3a}	28 ± 5^{3a}	23 ± 3^{3a}	105±1	124±1	144±1
Day 4	21 ± 4^{4a}	24 ± 3^{4a}	21 ± 4^{4a}	121±9	148±9	150±9
Day 5	15 ± 4^{5a}	30 ± 9^{5a}	7 ± 5^{5b}	99±7	123±7	113±7
Day 6	11 ± 3^{6a}	40 ± 2^{6b}	7 ± 3^{6b}	108±3	139±3	112±3
Day 7	5 ± 3^{7a}	32 ± 1^{7b}	16 ± 2^{7c}	106±5	134±5	113±5
Day 8	5 ± 3^{8a}	88 ± 1^{8b}	33 ± 4^{8c}	110±5	116±5	119±5
Day 9	21 ± 0^{9a}	122 ± 9^{9b}	45 ± 3^{9c}	116±8	128±8	139±8
Day 10	20 ± 0^{10a}	109 ± 2^{10b}	86 ± 3^{10c}	110±6	129±6	138±6

Schedules included: 1 shot of 200 mg/kg CPA at day 0 (group 1), 2 shots of 100 mg/kg CPA at day 0 and day 2 (group 2), 4 shots of CPA at days 0-3 (group 3). Mice in groups 4-6 were injected with saline solution using the same schedules given to groups 1-3, respectively.
Different superscript numbers indicate different time points
Different superscript letters (a, b, c) indicate significant differences at each time points ($P<0.05$)

Previously, Hattori *et al.* demonstrated that the treatment of 100 mg/kg CPA to mice induced the highest response to G-CSF [15]. Qi *et al.* suggested using one injection of 200 mg/kg CPA to establish proper immunosuppressed mice models [16]. Huyan *et al.* showed that a good immunosuppressive mouse model could be generated using two injections of 150 mg/kg CPA at 2-day intervals [17]. In the current study, we found that single injection of 200 mg/kg CPA was sufficient for generating a immunosuppressed model. The slight difference between the optimized doses of CPA in these studies might come from the different environmental, feeding, or housing conditions. However, all these studies showed that a vast reduction of leukocytes was observed at day 1 after CPA injection, supporting our suggestion of obtaining immunosuppressive mice after 1 day since injection for further experiments.

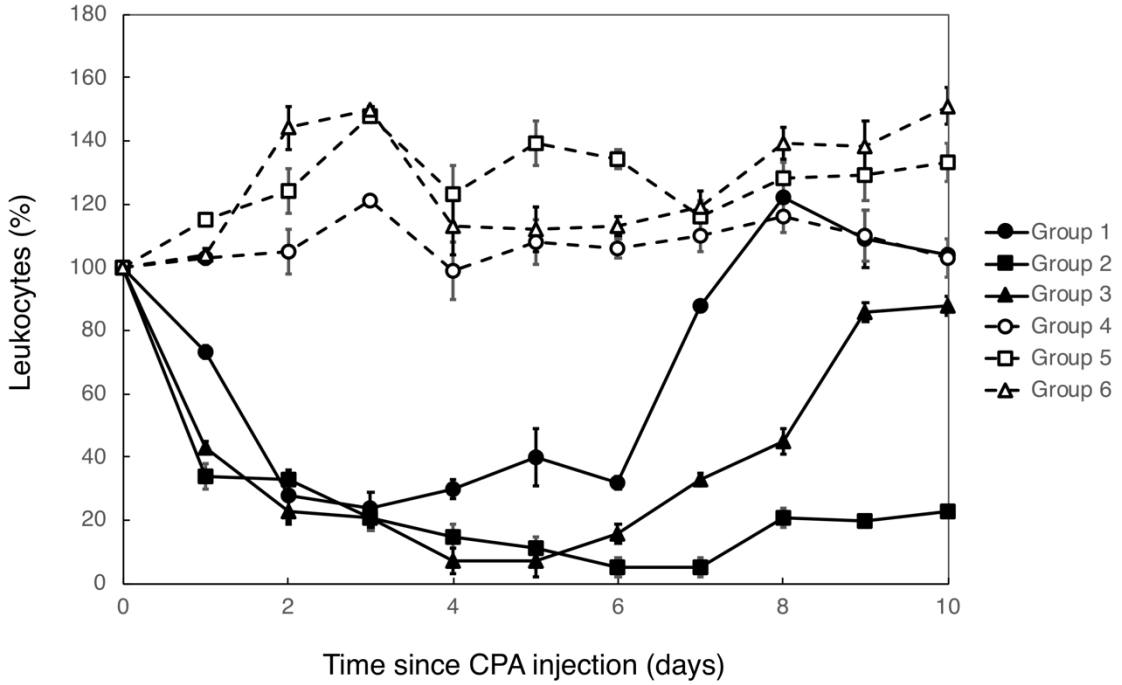

Fig. 2. *The changes in leukocyte numbers in mice given different schedules of CPA injection. Schedules included: 1 shot of 200 mg/kg CPA at day 0 (group 1), 2 shots of 100 mg/kg CPA at day 0 and day 2 (group 2), 4 shots of CPA at days 0-3 (group 3). Mice in groups 4-6 were injected with saline solution using the same schedules given to groups 1-3, respectively. The data were shown as mean ± SD.*

Optimizing The G-CSF Concentration For in vivo Bioassay

G-CSF induces the production of neutrophils in a dose-dependent manner. Thus, an appropriate G-CSF injection dose, which is high enough to remarkably induce the production of neutrophils but not cause any side-effects in mice, must be determined to develop an efficient *in vivo* bioassay.

To optimize the injection dose of G-CSF, the immunosuppressed mice were injected with G-CSF at the doses of 1 µg/kg, 5 µg/kg, and 10 µg/kg and the total leukocytes were counted every 4 hours in 24 hours after injection. We found that all three doses of G-CSF could increase the number of total leukocytes (Fig. 3, Table 3). Importantly, the dose of 5 µg/kg showed significantly higher effects than the doses of 1 µg/kg and 10 µg/kg. In the group injected with 5 µg/kg G-CSF, the total leukocyte number reached the maximal level by 12 hours after G-CSF injection but slightly decreased thereafter. In addition, the mice injected with 5 µg/kg behaved normally, implying that this dose can be used for *in vivo* evaluation of G-CSF biological activity using immunosuppressed mice.

Table 3. *The changes in leukocyte numbers in immunosuppressed mice after injected with different doses of commercial G-CSF drug*

	G-CSF dose			
	0 µg/kg	**1 µg/kg**	**5 µg/kg**	**10 µg/kg**
-24h	100 ± 0^{-24a}	100 ± 0^{-24a}	100 ± 0^{-24a}	100 ± 0^{-24a}
0h	29 ± 8^{0a}	46 ± 6^{0a}	18 ± 4^{0a}	19 ± 2^{0a}
4h	9 ± 7^{4a}	44 ± 10^{4a}	36 ± 6^{4a}	50 ± 10^{4a}
8h	7 ± 3^{8a}	48 ± 3^{8b}	68 ± 11^{8c}	34 ± 5^{8d}
12h	6 ± 2^{12a}	42 ± 12^{12b}	96 ± 6^{12c}	42 ± 2^{12b}
16h	12 ± 5^{16a}	54 ± 8^{16b}	96 ± 6^{16c}	45 ± 6^{16b}
20h	12 ± 4^{20a}	54 ± 8^{20b}	88 ± 4^{20c}	45 ± 6^{20b}
24h	12 ± 7^{24a}	54 ± 8^{24b}	88 ± 4^{24c}	45 ± 6^{24b}

Different superscript numbers indicate different time points
Different superscript letters (a, b, c, d) indicate significant differences at each time point (P<0.05)

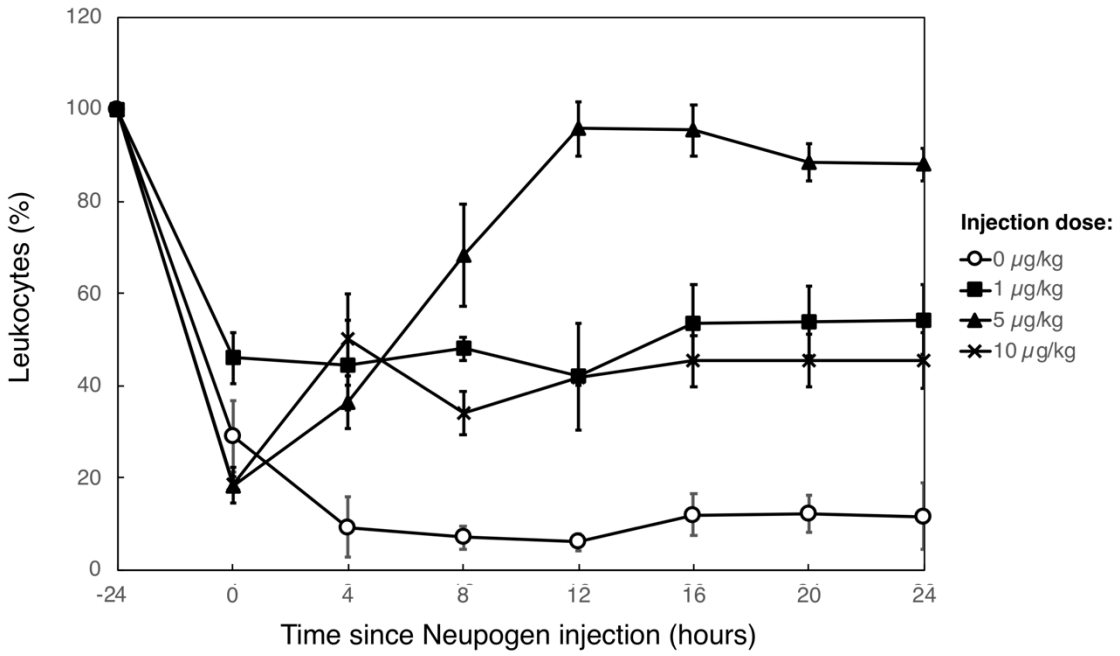

Fig. 3. *The changes in leukocyte numbers in immunosuppressed mice after injected with different doses of commercial G-CSF drug (Neupogen). The data were shown as mean ± SD.*

Development Of A Procedure To Evaluate The Neutropenia Treatment Efficiency Of Recombinant G-CSF

Since the effects of G-CSF decreased after 12 hours of G-CSF injection, the single injection of G-CSF is not sufficient to examine the effect of G-CSF in neutropenic mice. Additionally, according to Amgen Inc., G-CSF should be administered for at least 4 days to efficiently treat neutropenia [18]. Therefore, a G-CSF injection schedule at the dose of 5 µg/kg in 4 consecutive days was used to further evaluate the effects of G-CSF in neutropenia treatment.

We found that upon G-CSF treatment, the total leukocyte numbers were elevated to the normal level as that in mice before immunosuppressed (day -1) (Fig. 4, Table 4). The leukocyte numbers were maintained at normal level up to 7 days, indicating that the injection of G-CSF into immunosuppressed mice at the dose of 5 µg/kg for 4 days could be used to evaluate the effectiveness of using recombinant G-CSF for neutropenia treatment.

In a similar study, Hattori et al. treated mice with 100 mg/kg CPA to induce immunosuppression and 24 hours later, mice were injected daily for 4 days with 0.2 µg, 1 µg, and 5 µg of G-CSF [15]. They found that the neutrophil levels were gradually increased, peaked at day 4, and decreased thereafter. However, for an unknown reason, our result showed a different response, in which the total leukocyte numbers were peaked at day 1 and maintained for at least 7 days when we daily injected the mice with 5 µg/kg G-CSF (~1.25 µg G-CSF for one mouse). However, despite the slight difference between these results, both the study of Hattori et al. and our study suggested that the treatment of CPA followed by 4 day-injection of G-CSF into mice might be an useful method for *in vivo* evaluation of G-CSF.

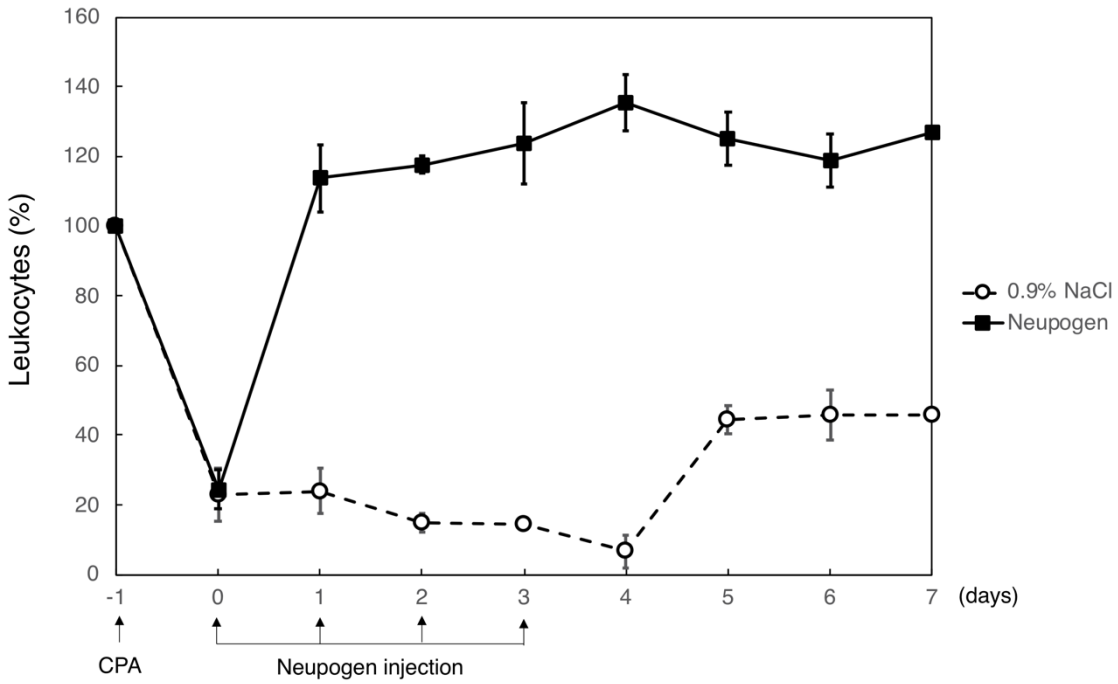

Fig 4. *The changes in leukocyte numbers in immunosuppressed mice after given a 4-day G-CSF treatment therapy. The data were shown as mean ± SD.*

Table 4. *The changes in leukocyte numbers in immunosuppressed mice after injected with different doses of commercial G-CSF drug*

	G-CSF dose	
	0 µg/kg	**5 µg/kg**
Day -1	100 ± 0^{-1a}	100 ± 0^{-1a}
Day 0	23 ± 8^{0a}	24 ± 8^{0a}
Day 1	24 ± 9^{1a}	114 ± 9^{1b}
Day 2	15 ± 2^{2a}	118 ± 4^{2b}
Day 3	15 ± 2^{3a}	124 ± 5^{3b}
Day 4	7 ± 4^{4a}	135 ± 7^{4b}
Day 5	46 ± 5^{5a}	125 ± 7^{5b}
Day 6	46 ± 5^{6a}	119 ± 2^{6b}
Day 7	46 ± 5^{7a}	127 ± 10^{7b}

Different superscript numbers indicate different time points
Different superscript letters (a, b) indicate significant differences at each time point ($P<0.05$)

CONCLUSION

Based on the results from this study, we suggested a procedure for *in vivo* evaluation of recombinant G-CSF biological activity. Specifically, the healthy mature mice are injected with 200 µg/ml CPA to induce immunosuppression. After 1 day of CPA injection, the total leukocyte number must be checked to verify the reduced neutrophil counts. The immunosuppressed mice are then injected with G-CSF at the dose of 5 µg/kg body weight and the total leukocyte numbers should be monitored during 24 hours after G-CSF injection (Fig. 5A). If the leukocyte number can be recovered to the normal levels within 24 hours from the G-CSF injection, we suggest that the recombinant G-CSF is considered to have biological activity. In addition, to demonstrate the efficiency of G-CSF in neutropenia treatment, the immunosuppressed mice are injected with G-CSF at the dose of 5 µg/kg body weight every day in 4 days and the total leukocyte numbers are checked up to 7 days (Fig. 5B). It can be concluded that the recombinant G-CSF can be effectively used for neutropenia treatment if the leukocyte counts increase to the normal levels and maintain up to 7 days since the first injection.

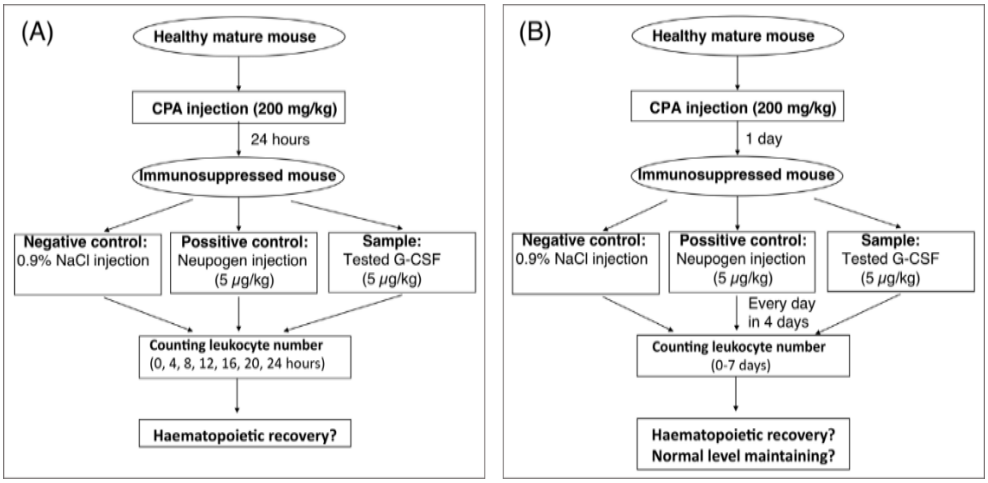

Fig. 5. *The suggested in vivo bioassay to evaluate the effects of recombinant G-CSF on neutrophil production (A) and its potential in neutropenia treatment (B).*

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