Effects of Fighting after Grouping on Plasma Cortisol Concentration and Lymphocyte Blastogenesis of Peripheral Blood Mononuclear Cells Induced by Mitogens in Piglets

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ABSTRACT. One litter (Group A) of three unacquainted groups of littermates (4 piglets/litter), 64.0 ± 0.8 days old, was moved to the pen of another litter (Group B) and they were housed together for 19 days after grouping (phase 1). The pigs in Group B violently attacked all the pigs in Group A for 9 hr after grouping. The remaining group was not grouped and used as controls. The plasma cortisol concentrations 1 hr after grouping were significantly higher than those 1 hr before and 24 hr after grouping, and the suppression of lymphocyte blastogenesis of peripheral blood mononuclear cells (PBMC) induced by mitogens was observed on 3, 8 and 19 days after grouping. After phase 1 ended, the pigs in Group A were returned to their own pen for 7 days, and then they were regrouped with the pigs in Group B and reared together for a further 14 days. Neither agonistic behavior nor change of plasma cortisol after regrouping was seen. Though the lymphocyte blastogenesis of PBMC induced by the mitogens on day 0 after regrouping was significantly lower in the pigs of Groups A and B compared to those in control pigs, a significant difference in lymphocyte blastogenesis among three groups was not seen on 7 and 14 days after regrouping. These findings indicate that fighting after grouping unacquainted litters increases plasma cortisol, and suppresses lymphocyte blastogenesis for 26 days after grouping. — KEY WORDS: fighting, grouping, lymphocyte blastogenesis, piglet, plasma cortisol.

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When animals encounter various stressors, the hypothalamic-pituitary-adrenal axis is activated and glucocorticoids from the adrenal cortex are released [4, 13, 15]. Therefore, a higher-than-normal concentration of plasma cortisol indicates that animals may be in a state of stress [1, 4, 19].

In pigs, it has been shown that early weaning [18], restraint [3] and administration of adenocorticotrophic hormone (ACTH) [16] raise the concentration of plasma cortisol and also reduce the lymphocyte blastogenesis in peripheral blood mononuclear cells (PBMC) induced by mitogens.

On the other hand, inter-pen moving and grouping pigs are common after weaning and during growing stages of meat production in modern swine management. Grouping pigs has been shown to elevate the concentration of plasma cortisol in many studies [2, 6, 12]. When pigs are grouped, there is also a rapid spread of infections among pigs, possibly owing to stress of pigs during agonistic behavior [5–11, 14] to establish a new ranking order [7–11] or wounds [5–8, 10, 11, 14], but reports as to the effect of grouping on the lymphocyte blastogenesis induced by mitogens are few [2, 5]. In a previous study [5], when two groups of unacquainted piglets were grouped for 9 days, the suppression of the lymphocyte blastogenesis of PBMC induced by mitogens was observed until the end of experiment, but this was not corroborated in a study reported by Blecha *et al.* [2].

The present study was therefore carried out to clarify the effects of fighting after grouping on the concentration of plasma cortisol and the period of suppression of the lymphocyte blastogenesis of PBMC induced by the mitogens as pokeweed mitogen (PWM), concanavalin A (ConA) and phytohemagglutinin (PHA) in piglets.

MATERIALS AND METHODS

Twelve (4 piglets/litter × 3 litters), Landrace × Large White piglets by the same sire, 50 ± 0.8 days of age on arrival at Kagoshima University, were used. The three litters had not been housed together in the same pen, but reared separately in three separate pens for 2 weeks.

This experiment consisted of two phases, i.e., phases 1 and 2.

Phase 1: At 64.0 ± 0.8 days of age and weighing 28.4 ± 1.2 kg of body weight, one group of 4 littermates (Group A) was moved to the pen where another group of 4 littermates (Group B) was housed, and they were reared together for 19 days after grouping (until 83 days old). The remaining group of 4 littermates was not moved and used as controls.

Phase 2: After phase 1 ended, the 4 littermates in Group A were returned to their original pen and reared for 7 days (84–90 days old). Then they were moved and regrouped with the same 4 littermates of Group B in phase 1, and all 8 pigs were housed together again for 14 days (91–104 days old). The remaining 4 littermates which had been used as controls in phase 1 were not moved and used again as controls.

After grouping in phases 1 and 2, behavior of the pigs, fighting time, and wounds on the individual pig were observed.

To determine the lymphocyte blastogenesis, blood samples were collected from all pigs via right external jugular venapuncture using heparinized tubes on Days 0, 1,

3, 8 and 19 in phase 1 and Days 0, 3, 7 and 14 in phase 2, respectively. On Day 0, the blood samples were collected 1 hr before grouping in both phases 1 and 2. To determine plasma cortisol, additional heparinized blood samples (about 2 m*l*) were collected from all piglets 1 hr after grouping in phase 1, and 1 and 24 hr after regrouping in phase 2, respectively. The elapsed times to collect the blood samples for all pigs under the restriction of a pig's nose were within 12 sec throughout the experiment.

The heparinized blood samples collected from pigs 1 hr before and 1 or 24 hr after grouping in phases 1 and 2 was centrifuged at 3,000 rpm for 10 min at 5°C. The concentrations of plasma cortisol were determined using a [^{125}I] radioimmunoassay kit (Eiken Kagaku, Tokyo).

Five m*l* of each blood sample with heparin was diluted (1:2) with 0.1 M phosphate buffer saline solution (PBS(-)), pH 7.2 and layered on 5 m*l* of Ficoll-Conray solution (9% Ficoll (Pharmacia Biotech, Uppsola) 2.05:33.3% Conray (Daiichi Phamaceutical, Tokyo) 1.00; specific gravity of 1.084 at 15°C) and centrifuged at 1,800 rpm for 30 min at room temperature. PBMC at the plasma-Ficoll-Conray interface were collected, placed in 15 m*l* of PBS (-), centrifuged at 1,550 rpm for 5 min and followed by 2 washings in PBS (-) to eliminate plasma and platelets. Cell concentrations were adjusted to 1×10^6 cells/m*l* of growth medium, i.e., RPMI-1640 medium with L-glutamine, supplemented with 1.2 mM pyruvic acid sodium, 25 mM HEPES, 24 mM $NaHCO_3$, gentamycin of 50 μg/m*l*, and 8% fetal bovine serum unactivated for 30 min at 56°C. Viability of cells (> 99%) was determined by 0.16% trypan blue dye exclusion.

The determination of lymphocyte blastogenesis was carried out using a method described by a previous report [5]. Briefly, isolated PBMC (200 μ*l*) suspended in growth medium were added in triplicate to a 96-well round-bottom cell culture plate and PWM 2.5 μ*l*/m*l* (final dilution of 1:400, Gibco Laboratories, Tokyo), Con A (IBF Biotechnics, Tokyo) 2.5 μg/m*l*, PHA 5 μ*l*/m*l* (final dilution of 1:200, Difco Laboratories, Tokyo) or growth medium without mitogens (controls) was added to cultures of 2×10^5 PBMC/well. Cells were incubated in a 5% CO_2 humidified incubator at 37°C. After 66 hr incubation, [^{3}H] thymidine (9.25×10^3 Bq/well) was added to all cultures, and plates were incubated an additional 6 hr under the same conditions. Cells were collected on glass fiber disks by using an auto-cell-harvester. The disks were dried at 37°C, then placed in vials containing a scintillation cocktail, and [^{3}H] radioactivity incorporated into lymphocytes was counted by using a liquid scintillation counter. The incorporation of [^{3}H] into lymphocytes was expressed as mean count per min (cpm) for triplicate cultures.

The significance of difference between means of groups and of a group at various sampling occasion was tested by the paired Student's *t*-test. A P value <0.05 was considered significant.

RESULTS

In phase 1, immediately after grouping, all pigs in Group B violently attacked all pigs in Group A and bit their ears, shoulder and necks for 9 hr. The numbers of wounds in these sites was more than 20 per pig in Group A. However, counterattacks by pigs in Group A were scarcely seen and the pigs in Group B had no injuries at all. After fighting ended, the agonistic behavior was not observed throughout phase 1. On the other hand, any attack by the pigs in Group B against the pigs in Group A or aggressive interaction among pigs after regrouping was not seen in phase 2.

The results of plasma cortisol concentration in phase 1 are shown in Table 1. There was no significant difference in the plasma cortisol concentration among the three groups 1 hr before grouping. The plasma cortisol concentrations of pigs in Groups A and B 1 hr after grouping were significantly higher compared to those 1 hr before grouping and at this time in control pigs. The cortisol concentrations had dropped to base-line levels 24 hr after grouping, and there was no difference in plasma cortisol concentration 24 hr after grouping among three groups. The plasma cortisol concentrations in phase 2 are shown in Table 2. A significant change of plasma cortisol concentration was not observed from 1 hr before to 24 hr after regrouping in pigs of Groups A and B or control pigs.

The results of the lymphocyte blastogenesis of PBMC induced by PWM, Con A and PHA in phase 1 are shown in Figs. 1, 2 and 3, respectively. The incorporation of [^{3}H] into lymphocytes stimulated with PWM, Con A and PHA on Days 3, 8 and 19 in pigs in Groups A and B was significantly lower than those at the same days in control pigs. A significant difference of lymphocyte blastogenesis in pigs between Groups A and B was not found throughout

Table 1. Concentration of plasma cortisol before and after grouping in phase 1

Experimental groups	Before grouping	After grouping	
	1 hr	1 hr	24 hr
Grouping			
Unmoved (Group B)	92.9 ± 15.4	216.5 ± 19.6*	94.5 ± 17.2
Moved (Group A)	91.2 ± 14.5	224.3 ± 11.8*	94.6 ± 18.8
Control	94.8 ± 12.8	96.4 ± 18.3	93.1 ± 18.4

Mean ± SD (nM/*l*). *P<0.01 significantly different from control, 1 hr before grouping and 24 hr after grouping.

Table 2. Concentration of plasma cortisol before and after grouping in phase 2

Experimental groups	Before grouping	After grouping	
	1 hr	1 hr	24 hr
Grouping			
Unmoved (Group B)	89.6 ± 21.5	93.5 ± 19.5	92.1 ± 14.3
Moved (Group A)	87.3 ± 16.5	92.3 ± 18.6	88.6 ± 12.6
Control	86.5 ± 14.3	91.5 ± 19.5	90.5 ± 14.6

Mean ± SD (nM/*l*).

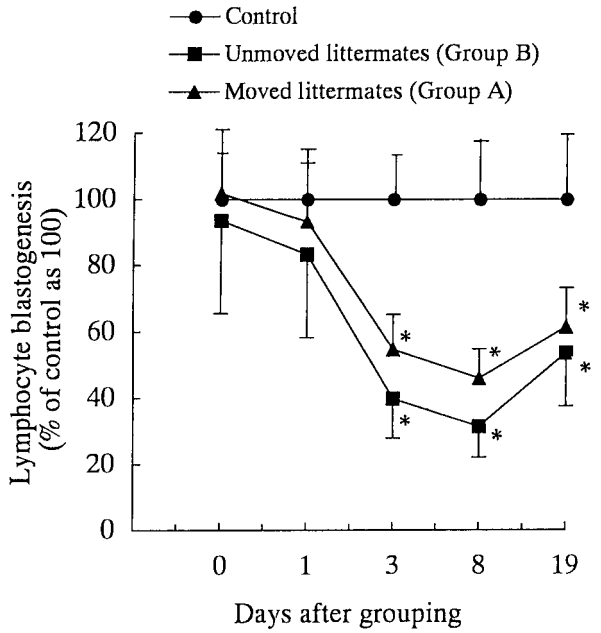

Fig. 1. Lymphocyte blastogenesis induced by pokeweed mitogen in phase 1 (mean ± SD). *P<0.01 significantly lower than those in control littermates.

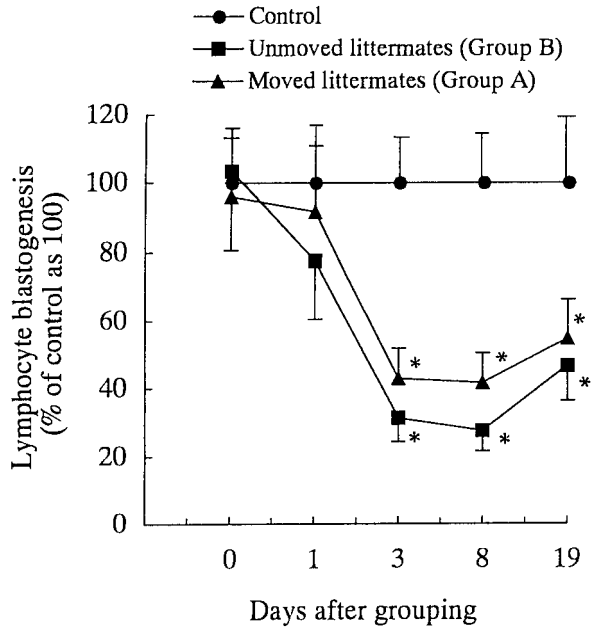

Fig. 2. Lymphocyte blastogenesis induced by concanavalin A in phase 1 (mean ± SD). *P<0.01 significantly lower than those in control littermates.

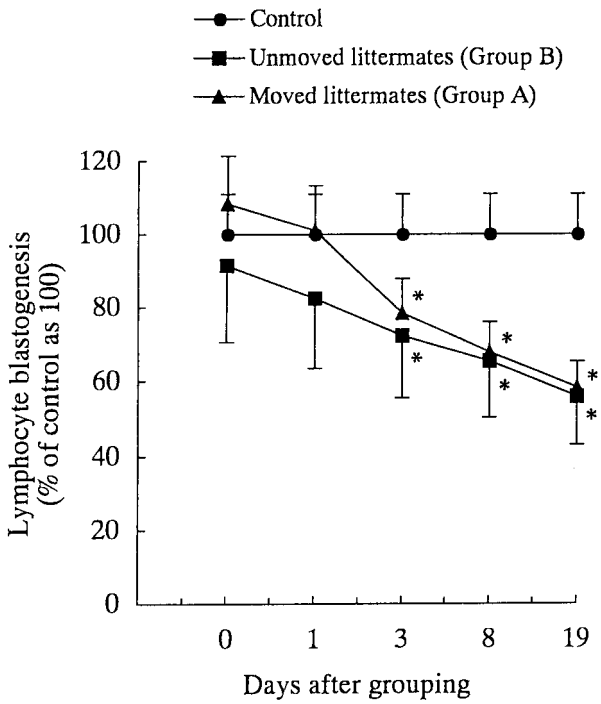

Fig. 3. Lymphocyte blastogenesis induced by phytohemagglutinin in phase 1 (mean ± SD). *P<0.01 significantly lower than those in control littermates.

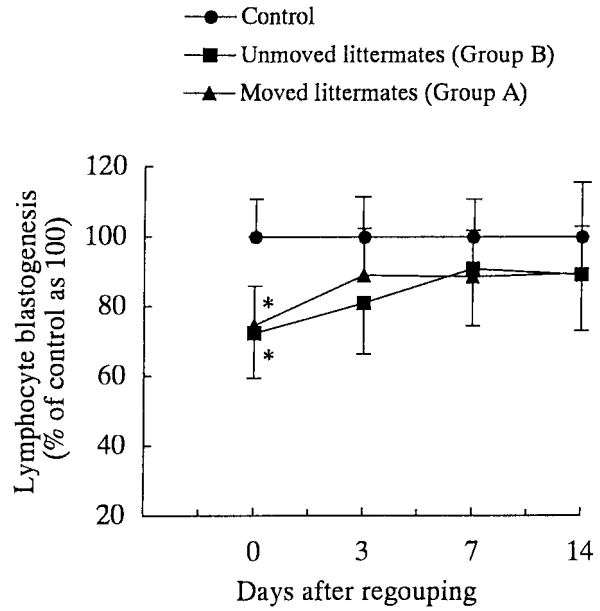

Fig. 4. Lymphocyte blastogenesis induced by pokeweed mitogen in phase 2 (mean ± SD). *P<0.01 significantly lower than those in control littermates.

phase 1.

Figures 4, 5 and 6 show the lymphocyte blastogenesis in phase 2. The incorporation of [^{3}H] into lymphocytes stimulated with PWM, Con A and PHA on Day 0 and PHA on Day 3 in pigs of Groups A and B was significantly lower than those in control pigs. However, there was no significant difference in the lymphocyte blastogenesis on Days 7 and 14 among the three groups.

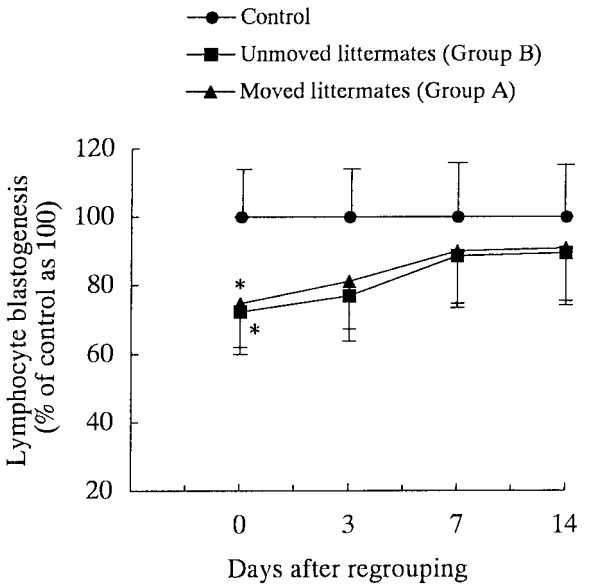

Fig. 5. Lymphocyte blastogenesis induced by concanavalin A in phase 2 (mean ± SD). *P<0.01 significantly lower than those in control littermates.

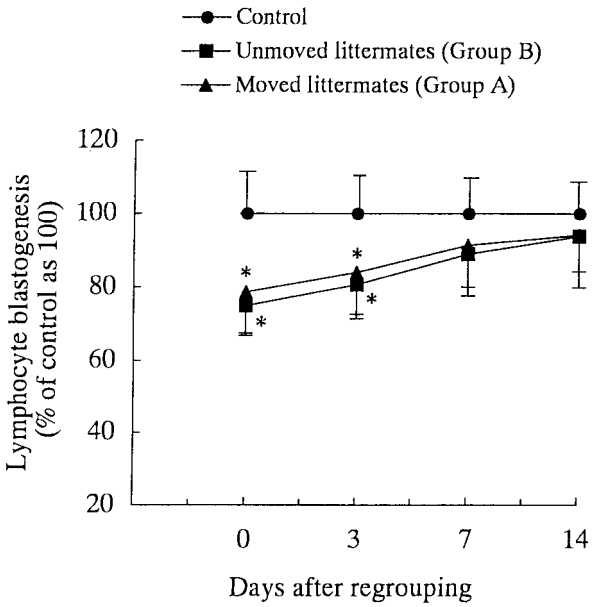

Fig. 6. Lymphocyte blastogenesis induced by phytohemagglutinin in phase 2 (mean ± SD). *P<0.01 significantly lower than those in control littermates.

DISCUSSION

It is believed that grouping is a stressor for pigs, since when two groups of pigs from different social origins are placed together in a new pen, agonistic behavior occurs [5–11, 14] and the concentration of plasma cortisol rises [2, 6, 12]. A similar result was observed in phase 1. However, the increase of plasma cortisol was also observed in pigs only exposed to a new pen but not grouped [4]. Therefore, a possibility arises that two factors might affect the increase of plasma cortisol in pigs after grouping: one is exposure to a new pen and the other is agonistic behavior or fighting.

The concentrations of plasma cortisol 1 hr before grouping in phases 1 and 2 were not different among pigs in three groups and were the same as that in normal pigs [2, 4, 6, 12]. In this experiment, the pigs in Group B were not exposed to a new pen and only fought in phase 1, since they were housed in their own pen throughout the experiment. The increase of plasma cortisol in pigs in Group B was observed 1 hr after grouping in phase 1. On the other hand, neither aggressive encounters among pigs nor an increase of plasma cortisol was observed in pigs of Groups A and B after regrouping in pase 2. These findings suggest that fighting after grouping two groups of unacquainted pigs activates the hypothalamic-pituitary-adrenal axis and leads to an increase of plasma cortisol, and the fighting after grouping is the actual stressor for pigs.

Dantzer and Mormede [4] observed that the higher concentration of plasma cortisol was found in subordinate rather than in dominant pigs when two groups of pigs were put in a new pen. In phase 1, it is obvious that the dominants were the pigs in Group B and the pigs in Group A were subordinate from the observations of agonistic behavior and wounds after grouping. However, there was no difference in the increase of plasma cortisol concentration between subordinate and dominant pigs after grouping in phase 1. Aggressive interaction among pigs to establish a new ranking order was shown when unfamiliar pigs were placed in a new pen in many studies [7–11]. However, only one-sided attacks by pigs in Group B against pigs in Group A was observed in phase 1. Therefore, the difference of behavior after grouping pigs might result in the difference in plasma cotisol concentrations between dominant and subordinate pigs. Dantzer and Mormede [4] thought that the increase of plasma cortisol in subordinate pigs after grouping causes the activation of the hypothalamic-pituitary-adrenal axis by a psychological stressor [4], but the other reasons were not fully obvious.

Blecha *et al.* [2] reported that when two unacquainted piglets were placed in a new pen, the decrease of lymphocyte blastogenesis induced by PWM, ConA and PHA in peripheral blood was not observed one day after grouping, while an increase of plasma cortisol was. There were some differences between a study reported by Blecha *et al.* [2] and the author's studies, including a previous report [5], in the method for grouping pigs and the blood collecting time to determine the lymphocyte blastogenesis after grouping. Blecha *et al.* [2] determined the lymphocyte blastogenesis only one day after grouping. In phase 1 of this experiment, the suppression of lymphocyte blastogenesis of PBMC induced by mitogens was not seen one day after grouping. This agrees with the results in Blecha *et al.* [2].

However, in a previous study [5], when two unacquainted piglets were grouped for 9 days as the same method in

phase 1, the suppression of the lymphocyte blastogenesis of PBMC induced by PWM, Con A and PHA was observed throughout the experiment. Therefore, phase 1 was carried out to clarify the effect of fighting on the period of suppression of lymphocyte blastogenesis after grouping. The suppression of lymphocyte blastogenesis of PBMC induced by PWM, Con A and PHA was observed in pigs of both Groups A and B for 19 days after grouping in phase 1. Furthermore, lymphocyte blastogenesis induced by mitogens was lower in the pigs of Groups A and B than those in control pigs on Day 0 in phase 2. However, the suppression of lymphocyte blastogenesis was not seen in pigs of Groups A and B on Days 7 and 14 after regrouping in phase 2. These results suggest that the lymphocyte blastogenesis of PBMC induced by mitogens is suppressed at least for 26 days after grouping when fighting occurs once among two unacquainted pigs after grouping.

In pigs, suppression of lymphocyte blastogenesis of PBMC or peripheral whole blood induced by the mitogens such as PWM, ConA or PHA accompanied by an increase in plasma cortisol were reported in early weaning [18], restraint [3], and the administration of ACTH [16] or ACTH-releasing hormone [19]. The coculture of cortisol and lymphocytes from thymocytes, splenocytes and PBMC in pigs also suppressed the lymphocyte blastogenesis induced by mitogens [17]. Therefore, it seems reasonable to suppose that the higher-than-normal cortisol released from the adrenal cortex by the activation of the hypothalamic-pituitary-adrenal axis suppresses the lymphocyte blastogenesis of PBMC induced by mitogens.

In a previous study [5], it was shown that the restriction of a pig's nose for 20 sec to collect blood samples did not affect the lymphocyte blastogenesis of PBMC induced by PWM and ConA. From the results of control pigs in this experiment, it is clear that the nose restriction during 12 sec in pigs did not change either plasma cortisol or lymphocyte blastogenesis of PBMC induced by mitogens. However, it was reported that holding suckling piglets for 1 min resulted in the increase of plasma cortisol and the suppression of lymphocyte blastogenesis of peripheral whole blood induced by PWM, Con A and PHA [3]. Therefore, it is necessary to be careful and quick when piglets are restricted for management, i.e., castration, cutting the teeth or tail, and injection of vaccines, iron or antibiotics.

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