Cortisol is not the primary mediator for augmented CXCR4 expression on natural killer cells after acute exercise

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1Faculty of Sport Sciences, Waseda University, Tokorozawa, Saitama, Japan; 2Institute for Biomedical Engineering, Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, Tokyo, Japan; 3Department of Nutrition and Food Science, School of Public Health, Tianjin Medical University, Tianjin, China; 4Graduate School of Natural Sciences, Nagoya City University, Nagoya, Japan

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Okutsu M, Ishii K, Niu K, Nagatomi R. Cortisol is not the primary mediator for augmented CXCR4 expression on natural killer cells after acute exercise. J Appl Physiol 117: 199–204, 2014. First published June 19, 2014; doi:10.1152/japplphysiol.00176.2014.—CXCR4 expression on NK cells and NK cell migration activity. To determine the effect of exercise, we isolated PBMCs from subjects before and after a 90-min exercise at 70% peak O2 uptake (V̇O2peak) and determined the changes in CXCR4 expression on NK cells after exercise. We cultured PBMCs with plasma obtained before and after exercise and with the glucocorticoid antagonist RU-486 to determine NK cell migration activity and the effects of cortisol on CXCR4 expression in vitro. Cortisol treatment increased CXCR4 expression (P < 0.05) and migration activity (P < 0.05) of NK cells. Exercise did not affect CXCR4 expression on NK cells, whereas incubating them with postexercise plasma significantly increased CXCR4 expression (P < 0.05) and migration activity (P < 0.05). RU-486 blocked cortisol-induced CXCR4 upregulation on NK cells, but only partially blocked (7%) CXCR4 upregulation when PBMCs were incubated with postexercise plasma. Thus acute exercise increases CXCR4 expression on NK cells and their migration activity and may contribute to NK cell redistribution after acute exercise; however, cortisol did not appear to be the primary mediator of augmented CXCR4 expression.

cells traffic to lymphoid organs from the peripheral blood and other organs after immunization and infection where they interact with dendritic cells and CD4+ T cells (31). Because the number of circulating NK cells increases during exercise but tends to decrease below the preexercise level after exercise (10, 21), understanding the mechanisms involved would aid in investigating exercise-induced immunomodulation.

The redistribution of NK cells after exercise is primarily caused by catecholamine-induced downregulation of adhesion molecules on NK cells (18, 28). Previous reports showed that exercise had no effect on the redistribution of NK cells in glucocorticoid nonresponders and that glucocorticoid administration reduced the number of circulating NK cells, suggesting that glucocorticoids contributed to regulating NK cell distribution after acute exercise (9, 28). Although glucocorticoid involvement in the redistribution of NK cells after exercise has been suggested, the underlying mechanism for this is not well understood.

To elucidate the molecular mechanism of the redistribution of NK cells after exercise, we focused on chemokine receptors. Chemokines play important roles in both innate and acquired immunity by inducing the directed migration of various leukocyte types through interactions with a group of 7-transmembrane G protein-coupled receptors (33). Among the various chemokine receptors, CXC chemokine receptor 4 (CXCR4) expression on CD4 T lymphocytes and monocytes is augmented by exogenous or endogenous glucocorticoids (6, 22, 32).

CXCR4 is a critical receptor for controlling cell distribution in vivo. CXCR4, a unique receptor for stromal-derived factor 1α (SDF-1α), is constitutively expressed in lymph nodes, lung, liver, and bone marrow (15, 19, 30). CXCR4/CXCL12 interaction is strongly chemoattractive in vivo (15, 19, 30). A distinct breast cancer metastasis pattern that involved the lymph nodes, lung, liver, and bone marrow was characterized by the preferential expression of CXCL12 mRNA in these tissues (17). In vivo, neutralizing CXCL12/CXCR4 interaction significantly impaired breast cancer cell metastasis in regional lymph nodes and lung (17). Importantly, administering a CXCR4 antagonist, AMD-3100, to C57BL/6 mice markedly decreased the number of NK cells in bone marrow and increased them in blood (2), indicating that CXCR4 expression level was critical for the distribution of NK cells. Thus, glucocorticoid-induced augmentation of CXCR4 expression on NK cells may determine the distribution of NK cells.

In this study, we examined the effects of exercise-induced cortisol on CXCR4 expression on circulating NK cells. We...
hypothesized that cortisol released during exercise would increase CXCR4 expression on NK cells and increase their migration activity.

**MATERIALS AND METHODS**

**Subjects.** Seven healthy young men volunteered for this study (Table 1). Five subjects exercised aerobically on a routine basis; two subjects led a sedentary lifestyle. Subjects were informed about the experimental procedures, potential risks, and discomfort associated with their participation, and all provided written informed consent. All experimental procedures were performed with the approval of the ethics committee of Tohoku University.

**Blood sampling.** Subjects refrained from any type of moderate or heavy exercise for at least 12 h before blood sampling. Consuming food or drinks other than water was not allowed for a 10-h period prior to blood sampling. Blood samples were drawn from the antecubital vein between 8:00 A.M. and 10:00 A.M. using a sterile syringe that contained 100 IU of heparin (Novo Nordisk, Copenhagen, Denmark) per 10 ml of blood for both mononuclear cell preparation and plasma separation.

**Cell preparation.** Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood using Ficoll-Paque Plus (Amersham Bioscience, Uppsala, Sweden). Ficoll-Paque Plus (15 ml) was added to a Lymphoprep tube (NYCOMED, Oslo, Norway). These tubes contained a porous membrane that covered the Ficoll-Paque Plus before centrifugation. PBMCs were washed twice with phosphate-buffered saline (PBS; Nissui, Tokyo, Japan) and resuspended in a culture medium of RPMI 1640 (Sigma-Aldrich, St. Louis, MO) with 2 mM glutamine, 10% heat-inactivated fetal calf serum (Sigma-Aldrich), 10 mM HEPES buffer (Wako, Tokyo, Japan), 100 U/ml penicillin (Sigma-Aldrich), and 100 μg/ml streptomycin (Sigma-Aldrich).

**Glucocorticoid treatment of PBMCs.** PBMCs were incubated in 6-well flat-bottom culture plates in culture medium at cell densities ranging from 2 × 10⁶ to 4 × 10⁶ cells/well, with or without cortisol (Sigma-Aldrich) for 24, 48, or 72 h at 37°C in a 5% CO₂ humidified incubator. Cortisol concentrations in the cultures ranged from 10⁻⁶ M to 10⁻¹⁰ M. RU-486 (Sigma-Aldrich), a potent glucocorticoid receptor antagonist, was used at 10⁻⁶ M, as previously described (22, 23).

Because of the transient increase in plasma glucocorticoid levels in vivo, we examined shorter glucocorticoid treatment duration in vitro. PBMCs were incubated with or without 10⁻⁶ M cortisol under the above-mentioned conditions, except that they were washed with PBS for a total of three times at 1, 3, and 6 h of treatment, resuspended in fresh medium, and further incubated in a 6-well plate for a total incubation time of 72 h. We also examined the effects of 10⁻⁶ M RU-486 blockade.

**VO₂peak testing.** VO₂peak was measured during a step-wise incremental exercise on a bicycle ergometer (Monark 828E; Monark Exercise, Vansbro, Sweden). Subjects were instructed to pedal at a constant rate of 60 ± 5 revolutions per minute (rpm). The initial workload was set at 60 W. The workload was subsequently increased by 15 W every minute until either the subjects could not maintain the required pedaling rate (60 rpm) or they were exhausted. Heart rate and perceived exertion rate were monitored throughout this test. VO₂peak and minute ventilation were monitored just before the last VO₂peak test with an AeroMonitor 280 (Minato Medical Science, Osaka, Japan). VO₂peak was recorded as that during the last 30 s of exercise. Acute exercise load and plasma sampling schedule. The subjects performed the bicycle exercise at an intensity that was equal to 70% of their VO₂peak for 90 min because more than 60 min of acute exercise increases NK cells during exercise and decreases NK cells after exercise (27). This exercise was exhausting for all subjects. Each subject began this exercise between 8:00 A.M. and 9:30 A.M. Peripheral blood samples were immediately obtained before and after exercise. Heparinized peripheral blood samples were centrifuged for 10 min at 3,000 g at 4°C. Separated plasma was stored in aliquots at −80°C until cortisol levels were measured or the samples were used for cell cultures.

**Plasma cortisol levels.** Plasma cortisol levels were measured by radioimmunoassay (Immunotech, Marseille, France) in duplicate according to the manufacturer’s instructions. We incubated the cells with plasma obtained either before or immediately after exercise to measure CXCR4 expression and migration activity in the following experiments. Therefore, we did not adjust hemocytocentrifugations for plasma cortisol concentration.

**Effect of exercise on PBMC CXCR4 expression.** To examine the in vivo effects of exercise, PBMCs were collected before and immediately after exercise and immediately analyzed for CXCR4 expression on NK cells by flow cytometry. To examine the in vivo priming effect of exercise on CXCR4 expression on NK cells, cells collected before and immediately after exercise were incubated in culture medium for 72 h at 37°C in a 5% CO₂ humidified incubator.

**Plasma treatment of PBMCs.** To examine the effects of endogenous glucocorticoids induced by acute exercise on PBMCs, we incubated PBMCs in a 24-well flat-bottom plate in culture medium at densities ranging from 1 × 10⁶ to 2 × 10⁶ cells/well, with plasma obtained either before or immediately after exercise. The cells isolated before exercise were incubated with pre- or postexercise plasma from that subject at 25% of the culture volume for 72 h at 37°C in a 5% CO₂ humidified incubator (22, 23). We also examined the effects of 10⁻⁶ M RU-486 blockade. This experiment was necessary to determine whether exercise-induced cortisol had changed CXCR4 expression on NK cells. Because CXCR4-augmented NK cells potentially redistribute to lymphoid organs, we may not have been able to detect CXCR4 augmentation using circulating NK cells.

**Flow cytometry analysis.** PBMCs were washed thrice with PBS prior to staining. To block IgG Fc receptors, freshly isolated or cultured PBMCs were incubated with 4 μl of mouse serum (Sigma) per 4 × 10⁶ cells for 30 min at 4°C. These cells were then washed twice with ice-cold PBS and stained with the following combination of antibodies: FITC-labeled anti-CD56 monoclonal antibody (mAb; BD Biosciences, San Jose, CA), allopregocyanin (APC)-labeled anti-CD3 mAb (DAKO Japan, Kyoto, Japan), and phycoerythrin-labeled anti-CXCR4 mAb (DAKO Japan). Isotype controls (IgG2a, DAKO Japan) were used to account for nonspecific immunoglobulin binding. After incubation with these mAbs at 4°C for 30 min, cells were washed twice with ice-cold PBS and analyzed using a FACSCalibur flow cytometer (BD Biosciences). We evaluated chemokine receptor expression levels on NK cells (CD3⁺/CD56⁻) on the basis of mean fluorescence intensity (MFI).

**Migration assay.** A cell migration assay was performed as previously described (22, 23). We assessed migration of PBMCs using 24-well transwell culture inserts with a 3-μm pore size (BD Biosciences). The lower wells were filled with culture medium with or without human SDF-1α. PBMCs (5 × 10⁶) in culture medium were

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**Table 1. Physical characteristics of subjects**

<table>
<thead>
<tr>
<th>Age, yr</th>
<th>Height, cm</th>
<th>Body Weight, kg</th>
<th>VO₂max, ml·kg⁻¹·min⁻¹</th>
<th>HRmax, beats/min</th>
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<tr>
<td>23.1 ± 0.9</td>
<td>168.7 ± 1.3</td>
<td>59.3 ± 2.4</td>
<td>64.1 ± 3.0</td>
<td>186.9 ± 3.7</td>
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Data are shown as means ± SE. VO₂max, maximal O₂ uptake; HRmax, maximal heart rate.
Cortisol augments CXCR4 expression on NK cells. Incubating PBMCs with 10^{-6} M cortisol increased CXCR4 expression on NK cells in a time-dependent manner \((P < 0.01; \text{Fig. } 1A)\). This increased CXCR4 expression was detectable as early as 24 h after the start of incubation (Fig. 1A). The cortisol-induced CXCR4 expression after 72 h of incubation was approximately 5.3-fold higher (range 2.6–7.6-fold) than the control condition. The glucocorticoid receptor antagonist RU-486 abolished these effects of cortisol on CXCR4 expression (Fig. 1B), indicating that cortisol acted directly to upregulate CXCR4 expression. The cortisol-mediated augmentation of CXCR4 expression on NK cells was dose dependent (Fig. 1C); for example, 1 \times 10^{-6} M of cortisol (comparable to the higher limit of plasma concentration after acute exercise) was significantly higher than 10^{-7} M (comparable to the lower limit of physiological plasma concentrations) \((P < 0.05)\). Therefore, it is reasonable to assume that the cortisol-mediated augmentation of CXCR4 expression on NK cells may explain the increase in CXCR4 on NK cells following exercise. Representative flow cytometry results are shown in Fig. 1D.

Cortisol treatment enhances NK cell migration toward CXCL12. To determine whether this cortisol-induced CXCR4 expression was functional, we determined the migration activity of cortisol-treated NK cells. After 72 h of incubation in the presence of cortisol, NK cells migrated in response to CXCL12 on NK cells. It is well documented that elevated plasma cortisol levels after acute exercise are transient and last for no more than several hours. Thus we examined whether a shorter in vitro exposure period to cortisol could induce CXCR4 expression on NK cells. Cortisol exposure of <3 h was sufficient to induce CXCR4 expression on NK cells at the 72-h time point \((P < 0.05; \text{Fig. } 3)\). Representative flow cytometry results after 6 h of treatment are shown in Fig. 3B.

Changes in plasma cortisol levels after exercise. After exercise, plasma cortisol levels were elevated in six of the seven subjects (Fig. 4A). Because one subject was a cortisol nonresponder, the change in plasma cortisol in the entire group added to the upper wells and incubated at 37°C in a 5% CO2 incubator for 120 min. After incubation, the membrane inserts were removed and migrated cells were stained with an APC-labeled anti-CD3 mAb and an FITC-labeled anti-CD56 mAb, followed by FACSCalibur analysis.

Statistical analyses. Before performing statistical analyses, we tested the residuals for normality with histograms and normality plots. One-way ANOVA was used to assess changes in CXCR4 expression on NK cells after in vitro cortisol and RU-486 treatment, changes in CXCR4 expression on NK cells after exercise, and changes in cell migration activity after cortisol treatment. Changes in CXCR4 expression in response to 1–6 h cortisol and RU-486 treatments were assessed using two-way repeated measures ANOVA. Changes in CXCR4 expression in response to plasma treatments were assessed using two-way ANOVA. All post hoc comparisons were made using Fisher’s protected least significant difference (PLSD) test. The Mann-Whitney U-test was used to assess CXCR4 expression on NK cells after exercise and cell migration activity after treatment with pre- or postexercise plasma. Pearson correlation analysis was used to assess the relationships between percentage increases in postexercise plasma cortisol concentrations and magnitudes of CXCR4 augmentation on NK cells. All statistical analyses were performed using StatView software (SAS Institute, Cary, NC). Results are given as means ± SE. \(P < 0.05\) was considered significant.
A cortisol cortisol+RU486

culture medium

Fig. 3. Effects of cortisol exposure time on CXCR4 augmentation on NK cells. 
A: PBMCs were cultured without or with cortisol (10^-6 M), RU-486 (10^-6 M), or cortisol + RU-486 for 1, 3, or 6 h. PBMCs were then washed twice with PBS and incubated in culture medium for up to 72 h. Cells were harvested and analyzed by flow cytometry for CXCR4 expression on NK cells. B: representative flow cytometry results for time-dependent CXCR4 augmentation on NK cells. Results are expressed as mean-fold changes ± SE of MFI compared with control cells in culture medium alone. *P < 0.05 (two-way repeated-measures ANOVA and Fisher’s PLSD).


did not reach statistical significance. The average increase in plasma cortisol levels of responders was 38% (range 6–98%). Of note, by converting the cortisol concentrations from µg/dl in Fig. 4A into molar concentrations, the concentrations ranged between 2.6 × 10^-7 to 7.5 × 10^-7 molar.

Effects of exercise on CXCR4 expression. CXCR4 expression levels on NK cells collected immediately after exercise did not significantly differ from those on NK cells collected prior to exercise (Fig. 4B). Furthermore, CXCR4 expression levels on NK cells collected immediately after exercise did not significantly change even when these cells were incubated for 72 h (without cortisol or plasma; Fig. 4C). The results for cortisol nonresponders were excluded from Fig. 4, B and C.

Postexercise plasma cortisol levels increase CXCR4 expression levels on NK cells and migration activity but not in an exercise-induced cortisol dependent manner. To examine whether exercise-induced plasma cortisol levels could augment CXCR4 expression, we cocultured PBMCs for 72 h with plasma obtained before and immediately after exercise. There was a strong correlation between percentage increases in the plasma cortisol concentration after exercise and the magnitude of CXCR4 augmentation on NK cells (r = 0.79, P < 0.05; Fig. 5A). The migration activity of postexercise plasma-treated NK cells in response to CXCL12 was apparently greater than that of NK cells treated with control plasma (P < 0.05; Fig. 5B). Incubation with postexercise plasma from subjects with increased postexercise cortisol levels significantly augmented CXCR4 expression on NK cells by 1.32-fold (P < 0.05) compared with that on PBMCs incubated with control plasma obtained before exercise (Fig. 5C). RU-486 treatment only partially blocked (7%) this postexercise plasma augmentation of CXCR4 expression (Fig. 5C). Representative flow cytometry results are shown in Fig. 5D. Results for cortisol nonresponders were excluded from Fig. 5, B and C.

DISCUSSION

Stress-induced glucocorticoids have been shown to bind to immune cells and thus influence immune functions. In particular, glucocorticoids modulate lymphocyte trafficking, including that by NK cells, affecting their immune functions. The aim of this study was to investigate the effects of cortisol released during exercise on CXCR4 expression on NK cells.

Incubating NK cells in vitro with cortisol upregulated CXCR4 expression on NK cells in time- and dose-dependent manners. Short-term cortisol exposure was sufficient to augment CXCR4 expression on NK cells. These effects were clearly blocked by the glucocorticoid receptor antagonist RU-486. Cortisol treatment also enhanced the migration of NK cells toward CXCL12. Exercise did not influence CXCR4 expression, whereas incubating NK cells in vitro with postexercise plasma upregulated CXCR4 expression on these cells and enhanced their migration activity toward CXCL12. RU-486 only partially blocked this postexercise plasma augmentation of CXCR4 expression. These findings suggest that exercise-induced stress mediators alter the migratory activity of NK cells but that cortisol does not seem to be the primary mediator of augmented CXCR4 expression observed on NK cells after acute exercise.

Exercise, a recognized type of stress (12), alters the distribution of lymphocytes (4, 7, 8). The number of circulating NK cells increases during exhaustive exercise, and after exercise, this number tends to decrease to below the preexercise level (10, 21). Acute exercise-induced release of catecholamines

Fig. 4. Effects of exercise on CXCR4 expression on NK cells. A: changes in plasma cortisol levels. By converting the cortisol concentrations from µg/dl into M concentrations, the concentrations ranged between 2.6 × 10^-7 to 7.5 × 10^-7 M. B: PBMCs were collected at the indicated times and analyzed by flow cytometry to determine CXCR4 surface expression on NK cells. C: PBMCs were collected at the indicated times and incubated in culture medium alone for 72 h. Cells were analyzed by flow cytometry to determine CXCR4 surface expression on NK cells. Results are expressed as mean-fold changes ± SE of MFI compared with preexercise PBMCs in culture medium alone. Pre, preexercise; post, postexercise.
such as epinephrine and norepinephrine modulate adhesion molecule expression on NK cells, a phenomenon considered to be the major mechanism contributing to the mobilization of NK cells into circulation. However, the mechanisms that underlie exercise-induced lymphopenia, including that of NK cells, remain elusive. Our study results suggest that physiological cortisol concentrations or postexercise plasma can increase CXCR4 expression on NK cells and enhance their migration activity in response to CXCL12. Of note, in our preliminary experiments indicated that cortisol-induced CXCR4 expression on NK cells was not detectable before 6 h after the start of incubation. A longer exercise duration or a higher intensity of exercise and therefore a longer or stronger response, we tested whether cortisol was involved using the glucocorticoid antagonist RU-486. Although RU-486 seemed to partially block postexercise plasma augmentation of CXCR4 expression, this blockade was not significant and suggested that factors other than cortisol regulated CXCR4 expression on NK cells.

Transforming growth factor-β (TGF-β) is a potent inducer of CXCR4 expression on NK cells (13). However, acute exercise may not induce TGF-β1 synthesis (29). Other cytokines known to be elevated in plasma in response to exercise [e.g., interleukin-6 (IL-6), IL-8, and IL-1 receptor antagonist] do not influence CXCR4 expression on NK cells (29). Further investigations such as differential proteomic or metabolomic analyses of plasma samples obtained before and after exhaustive exercise may be needed to identify the mediator of CXCR4 expression.

Peripheral blood NK cells express the chemokine receptors CCR5, CCR7, CXCR3, and CXCR4, and the ligands for these receptors induce the migration of circulating NK cells in vitro (5, 11, 13). CXCL12, B-lymphocyte chemoattractant (BLC)/CXCL13, EB11-ligand chemokine (ELC)/CCL19, and secondary lymphoid-tissue chemokine (SCL/CCL21) comprise a group of homeostatic chemokines that regulate lymphocyte homing and trafficking in secondary lymphoid tissues. The corresponding receptors are CXCR4, CXCR5, and CCR7 for BLC/CXCL13, ELC/CCL19, and SCL/CCL21 chemokines, respectively. Further research will be needed to examine whether cortisol or postexercise plasma can induce CCR5, CXCR5, and CCR7 expression on NK cells.

Two major NK cell subsets have been identified in humans: CD56dim CD16+ and CD56bright CD16−. The CD56dim CD16+ NK cell subset exhibits higher cytotoxicity, whereas the CD56bright CD16− subset exhibits lower cytotoxicity but has a higher immunoregulatory potential. One month of heavy exercise training resulted in an increase in the number of CD56bright CD16− NK cells but not that of CD56dim CD16+ NK cells (29). Thus these two subsets likely behave differently in response to acute or repeated heavy exercise. Resting CD56dim CD16+ NK cells express CXCR1, CXCR2, CXCR3, CXCR4, and CX3CR1 but do not have detectable levels of CC chemokine receptors on their surfaces (26). In contrast, resting CD56bright CD16− NK cells express little CXCR1, CXCR2, and CX3CR1, but express high CCR5 and CCR7 levels (26). CXCR4 is equally expressed on resting CD56dim CD16+ and CD56bright CD16− human NK cells (26). Further research will be needed to examine whether cortisol or postexercise plasma can induce CXCR4 expression on the CD56bright CD16− and CD56dim CD16+ subsets of NK cells.

CXCR4 expression on NK cells remained unchanged after acute exercise. There is one possible explanation for this finding. Ninety minutes of exercise may have been too short to affect CXCR4 expression. In this study, we showed that incubating PBMCs with 10−8 M cortisol increased CXCR4 expression on NK cells in a time-dependent manner. However, our preliminary experiments indicated that cortisol-induced CXCR4 expression on NK cells was not detectable before 6 h after the start of incubation. A longer exercise duration or a higher intensity of exercise and therefore a longer or stronger exposure of NK cells to cortisol may have had a greater effect on CXCR4 expression.
If CXCL12 expression in any organ is considerably modified by exercise, it may largely affect the distribution of NK cells. Because we lacked the methods needed to quantify the tissue levels of CXCL12 in healthy humans, an animal study will be necessary to further understand NK cell trafficking regulation.

In conclusion, our results demonstrated increased CXCR4 expression and migratory activity by cortisol or exercise plasma-treated NK cells, and these changes may have contributed to the redistribution of NK cells after acute exercise. Exercise plasma-treated NK cells also augmented CXCR4 expression and migration activity, but the contribution of glucocorticoids was limited. Further research will be needed to establish the principal factors responsible for these exercise-induced effects and the clinical significance of the acute exercise-mediated redistribution of NK cells in the circulation.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: M.O. and R.N. conceived and designed the research; M.O. and R.N. performed experiments; M.O. and R.N. analyzed the data; M.O. and R.N. interpreted results of experiments; M.O. and R.N. drafted the manuscript; M.O. and R.N. edited and revised the manuscript; M.O. approved final version of manuscript.

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