Enhanced trabecular bone resorption and microstructural bone changes in rats after removal of the cecum

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Enhanced trabecular bone resorption and microstructural bone changes in rats after removal of the cecum. Am J Physiol Endocrinol Metab 303: E1069–E1075, 2012. First published August 21, 2012; doi:10.1152/ajpendo.00242.2012.—The cecum, the proximal part of the large intestine, has the highest rate of calcium absorption compared with other intestinal segments. Previously, we showed that rats with the cecum surgically removed (cecectomized rats) had severe negative calcium balance, low bone mineral density (BMD), and a compensatory increase in colonic calcium absorption. Herein, we used the computer-assisted bone histomorphometric technique and microcomputed tomography (μCT) to analyze bone microstructural defects in cecectomized rats at 1 and 3 mo postsurgery compared with age-matched sham-operated control rats. Relatively low BMD as determined by dual energy X-ray absorptiometry was observed in the femora, tibiae, and lumbar vertebrae of the 3-mo cecectomized rats. μCT analysis revealed decreases in the tibial cortical thickness, periosteal and endosteal perimeters, and moment of inertia in cecectomized rats. The histomorphometric results further showed that trabecular bone volume and number were markedly decreased, whereas trabecular separation was increased in the proximal tibial metaphysis of cecectomized rats, thus leading to a decrease in trabecular volumetric BMD. Since osteoclast surface and eroded surface were increased after cecectomy, such bone loss in cecectomized rats appeared to result from an enhanced bone resorption. Moreover, decreases in bone formation rate and osteoblast surface indicated a suppression of osteoblast-mediated bone formation. In conclusion, cecectomy induced widespread osteopenia in rats presumably by enhancing the osteoclast-mediated bone resorption and suppressing bone formation. The present results underline the important role of cecum in the body calcium homeostasis.

bone histomorphometry; bone loss; cecectomy; osteoblast; osteoclast

THE CECUM, the proximal part of the large intestine, is known to be the site where the microfloral fermentation produces acidic molecules, such as succinic and short-chain fatty acids, that in turn release free-ionized calcium from insoluble complexes (7, 17, 19). The cecum has also been reported to have the highest rate of calcium absorption compared with the small intestine and colon (13, 21). Our recent investigation further demonstrated that surgical removal of the rat cecum (cecectomy) led to impaired calcium metabolism due to a huge fecal calcium wasting (11). It was noteworthy that compensatory increases in colonic calcium transporter expression and calcium absorption were evident to regain the otherwise lost calcium, thereby palliating calcium imbalance in cecectomized rats (11).

Nevertheless, this colonic compensation in cecectomized rats was not adequate, and calcium release from bone was also enhanced to maintain normocalcemia, thus resulting in low bone mineral density (BMD) and bone mineral content (BMC) observed as early as 1 mo postsurgery (11). Although osteopenia was evident in the 1-mo cecectomized rats, as determined by bone densitometric technique, the detailed histological and microstructural bone changes in these rats and whether such changes were long-lasting are not known. At the cellular level, bone loss usually results from enhanced osteoclast-mediated bone resorption, suppressed osteoblast-mediated bone formation, or a combination of both (31, 36). In animals with prolonged calcium wasting and negative calcium balance, as in the cecectomized rats, the number and activity of osteoclasts should be increased by elevated levels of calciotropic hormones, such as parathyroid hormone (PTH), thereby stimulating osteoclast-mediated bone resorption and calcium release (2, 36). Generally, the severity of bone microstructural defects could be inferred from the pattern of bone loss. Specifically, in mild negative calcium balance, a hyperresorptive event may occur only in the trabecular structure, e.g., metaphysis of the long bone and vertebrae, which has greater surface area than the cortical structure, and diaphysis of the long bone (6). However, in more severe cases, bone loss may also be observed in the cortical structure (30).

Therefore, the main objectives of the present study were to 1) investigate the histological and microstructural bone changes in the trabecular and cortical sites of cecectomized rats by bone histomorphometric technique and microcomputed tomography (μCT) and 2) determine whether the cecectomy-induced bone loss was long-lasting and persisted up to 3 mo postsurgery. Since the cecum was found to be an important site of calcium absorption (11), it was hypothesized that cecectomy induced a severe trabecular microstructural defect that lasted for 3 mo postsurgery.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats (8 wk old, weighing 180–200 g) were obtained from the National Laboratory Animal Centre, Salaya, Nakhon Pathom, Thailand. They were placed in hanging stainless-steel cages, fed standard chow containing 1.0% wt/wt calcium, 0.9% wt/wt phosphorus, and 4,000 IU/kg vitamin D (CP, Bangkok, Thailand), and given reverse osmosis water ad libitum under a 12:12-h light-dark cycle. Room temperature was controlled at 25 ± 2°C, and relative humidity was 50–60%. Room average illuminance was 150–200 lux in the daytime. Rats were acclimatized for ≥7 days before surgery and pair-fed to ensure equal calcium absorption.

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intake in both cecectomized and sham-operated groups. To avoid a bias due to age-dependent bone changes, the data of cecectomized rats were compared with that of the corresponding “age-matched” sham-operated rats. The study was approved by the Institutional Animal Care and Use Committee of the Faculty of Science, Mahidol University, Thailand. All animals were cared for in accordance with the principles and guidelines of the American Physiological Society’s Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training.

Experimental design. Age-matched rats were randomly divided into two groups, i.e., cecectomized and sham-operated (control) groups. At the end of 1 and 3 mo postsurgery, arterial blood (5 ml from the left ventricle), femora,ibiae and L5–6 vertebrae were collected from each rat. Serum specimens were kept frozen at −80°C for later determination of PTH levels. Bone specimens from 3-mo cecectomized rats and their corresponding controls were subjected to BMD and BMC analyses by dual energy X-ray absorptiometry (DEXA). Histological and microstructural bone changes were determined in ex vivo tibiae from 1- and 3-mo cecectomized rats by bone histomorphometry. The tibial specimens (n = 5/group) were also analyzed by μCT.

Surgery and postoperative care. General anesthesia (40 mg/kg ip pentobarbitone sodium; Ceva Santé Animale, Libourne, France) and cecectomy were performed according to the methods of Kurosawa et al. (15) and Jongwattanapisan et al. (11). The 2 x 2 in. abdominal skin was shaved and cleaned with povidone iodine. Then, a 1.5-cm median laparotomy was performed with a pair of sterile surgical scissors. Muscles, omentum, and other connective tissues were retracted for a clear operative field. The cecum located in the right lower quadrant was lifted from the abdominal cavity. Blood vessels supplying the cecum were ligated with polyglactin 5/0 (W9501T Ethicon; Johnson & Johnson, St. Stevens-Wolwe, Belgium) before the cecum was gently ligated with silk 2/0 near the vessels supplying the cecum were ligated with polyglactin 5/0. In sham-operated rats, a 3-mm incision was made on the cecal sac in an area devoid of major blood vessels, and the wound on the intestine was closed by simple continuous suturing with 5/0 polyglactin (DuPont). After the operation, rats were warmed under an overhead heating lamp at 37°C (skin temperature) [35°C] and monitored intensively for respiratory rate, color of mucous membrane, and recovery of corneal and withdrawal reflex before being returned to their cages, when they regained consciousness without signs of bleeding or respiratory failure. All rats were administered once daily for 3 days with an analgesic drug (10 mg/kg sc enrofloxacin; Bayer Healthcare, Leverkusen, Belgium) and a systemic antibiotic (10 mg/kg sc enrofloxacin; Bayer Healthcare, Leverkusen, Germany). At the end of the experiments, rats were subjected to median thoracotomy and laparotomy under anesthesia for autopsy and tissue collection. None of rats had gut obstruction, abdominal fluid, leakage of thoracotomy, and femoral shaft (middle third and distal one-third of the femur), and femoral shaft (middle third and distal one-third of the femur) were dissected from the 3-mo cecectomized and age-matched sham-operated rats (control) were cleared of adhering muscles and connective tissues. As described previously by Suntornsaraton and colleagues (27, 28), two-dimensional areal BMD (aBMD) and BMC were determined in whole femora, whole tibiae, L5–6 vertebrae, femoral metaphyses (proximal one-third and distal one-third of the femur), and femoral shaft (middle one-third of the femur) by a DEXA system (model Lunar PIXImus2; GE Medical Systems, Madison, WI) operated with software version 2.10. The dual-energy supply was 80/35 kVp at 500 μA. The DEXA system was calibrated daily by a standard material with known BMD and BMC of 0.0690 g/cm² and 0.697 g, respectively. The interassay coefficient of variation was <0.3%.

Cortical microstructural analysis by μCT. Ex vivo tibiae were analyzed by a μCT system (model SkyScan 1178; SkyScan, Kontich, Belgium) with an X-ray tube voltage of 65 kV and current of 615 μA, with a 0.5-mm aluminum filter. The scanning angular rotation was 180°, and the angular increment was 0.54°. The voxel size was 85 μm² isotropically. The volume of interest was between 1.4276 and 1.8576 mm distal to the proximal tibial growth plate (50 slices). All images were reconstructed and analyzed by a computer cluster running the SkyScan CT-analyzer software package (version 1.11.10). Regarding three-dimensional (3D) reconstruction, 8-bit images were used with a ring artifact correction of 10 and a beam hardening correction of 30%. Histogram limits were between 0 and the right end of the high-density tail. Morphometric indices of cortical bone region measured in the present study were cortical thickness (mm), cortical bone area (mm²), cortical perioisteal perimeter (mm), and cortical endosteal perimeter (mm). Moment of inertia (mm⁴), which represents the resistance to bending around an axis that lies in the cross-sectional plane, was determined for both x- and y-axes (i.e., a bone cross-section showed eccentricity). In some experiments, 3D volumetric bone mineral density (vBMD) was also determined in the trabecular region of tibial metaphysis.

Trabecular microstructural analysis by bone histomorphometry. As described previously by Thongchote et al. (32), tibiae from cecectomized and age-matched sham-operated rats were cleaned of adhering muscles and connective tissues and then dehydrated in 70, 95, and 100% vol/vol ethanol for 3, 3, and 2 days, respectively. Thereafter, dehydrated bone specimens were embedded in methyl methacrylate resin at 42°C for 48 h. The resin-embedded tibiae were first adjused to obtain the same orientation and then longitudinally cut at 7- and 12-μm thickness by a rotary microtome equipped with a tungsten carbide blade (model RM2255; Leica, Nussloch, Germany). For the staining histomorphometric technique, 7-μm longitudinal sections were mounted on standard microscope slides, deplastinated, dehydrated, and processed for the Goldner’s trichrome staining (34) and later visualized under a light microscope. In addition, 12-μm unstained sections were examined for the double lines of calcine labeling (10 mg/kg sc, injected at 6 days interval; Sigma) under a fluorescent microscope. Image capture and analysis were performed under a fluorescence/light microscope (model BX51TRF; Olympus, Tokyo, Japan), using the computer-assisted Osteomasure system with software version 4.1 (Osteometric, Atlanta, GA). The region of interest covered the trabecular region of proximal tibial metaphysis at 1–2 mm distal to the growth plate (i.e., secondary spongosia). The static histomorphometric parameters obtained from the Goldner’s trichrome-stained sections consisted of trabecular bone volume normalized by tissue volume (%), trabecular number (mm⁻¹), trabecular separation (μm), trabecular thickness (μm), osteoblast surface normalized by bone surface (%), osteoid thickness (μm), osteoid surface (%), osteoclast surface (%), and eroded surface (%). Dynamic histomorphometric parameters obtained from the unstained sections included double-labeled surface (%), mineral apposition rate (μm/day), and bone formation rate (μm³/μm²·day⁻¹). Osteocyte lacunae area (μm²) of the tibial cortical envelope in each stained section was measured by Image J 1.46 (http://rsbweb.nih.gov/ij/index.html). Osteocyte lacunae that were not in the focal plane (i.e., having ill-defined borders) were excluded from analysis. The nomenclature, symbols,
and units complied with the report of the American Society for Bone and Mineral Research Nomenclature Committee (22).

Statistical analysis. Results are expressed as means ± SE. Two sets of data (cecectomized group vs. age-matched sham-operated group) were compared by unpaired Student’s t-test. The level of significance was P < 0.05. Data were analyzed by GraphPad Prism 5.0 for Mac OS X (GraphPad Software, San Diego, CA).

RESULTS

Cecectomy-induced bone loss was long-lasting and persisted up to 3 mo postsurgery. A massive bone loss after 3-mo cecectomy could be observed by DEXA. As depicted in Fig. 1, A–C, there were significant decreases in aBMD values in whole femur, whole tibia, and L5–6 vertebrae of the 3-mo cecectomized rats compared with the age-matched sham-operated rats. BMC values in whole femur and whole tibia, but not in L5–6 vertebrae of the 3-mo cecectomized rats, were also lower than those of sham-operated rats (Fig. 1, D–F). Similar results were observed in three femoral subregions since both aBMD and BMC of proximal and distal metaphyses and diaphysis (femoral shaft) were lower in the cecectomized rats compared with the sham-operated rats (data not shown).

Deterioration of cortical structure was evident in cecectomized rats. A 3D μCT analysis also demonstrated decreases in cortical thickness, cortical bone area, cortical periosteal perimeter, and cortical endosteal perimeter in the tibial diaphysis (midshaft) of 1-mo cecectomized rats compared with those of sham-operated rats (Fig. 2, A–D). In the 3-mo groups, cortical bone area (P = 0.078), cortical periosteal perimeter (P = 0.062), and cortical endosteal perimeter (P = 0.056), but not cortical thickness (P = 0.213), tended to be lower in cecectomized rats than in sham-operated rats (Fig. 2, A–D). Cecectomized rats also exhibited lower moments of inertia compared with sham-operated rats (Fig. 2, E and F).

The cecectomized rats manifested overt trabecular bone loss. The present histomorphometric analysis further revealed that cecectomized rats had reductions in calcified trabeculae (green color in Goldner’s trichrome-stained sections; Fig. 3) and trabecular bone volume (Fig. 4A) in the proximal tibial metaphysis at 1 and 3 mo postsurgery compared with their age-matched sham-operated rats. Relatively low trabecular bone volume appeared to result from a decrease in trabecular number and an increase in trabecular separation, with no change in trabecular thickness (Fig. 4, B–D), leading to expansion of the marrow cavity (Fig. 3). Thus, trabecular vBMD as determined by μCT was also decreased in both 1- and 3-mo cecectomized rats (Fig. 4E).

The osteoblast-mediated bone formation was impaired after cecectomy. Several histomorphometric parameters revealed a deleterious effect of cecectomy on the osteoblast-mediated
bone formation. Specifically, the osteoblast surface was decreased significantly in the 3-mo but not 1-mo cecectomized groups (Fig. 5A), whereas osteoid thickness was increased in both cecectomized groups (Fig. 5B) compared with the sham-operated controls. However, osteoid surface was not changed after cecectomy (Fig. 5C). In the unstained sections, double lines of calcein labeling, a representative of newly mineralized areas, could be observed in the tibial trabeculae of both cecectomized and sham-operated rats (data not shown), suggesting that matrix calcification did occur during this 6-day period. However, further quantitative dynamic histomorphometric analysis in tibial trabeculae showed that double-labeled surface and bone formation rate, but not mineral apposition rate, were decreased significantly in the 1-mo cecectomized rats compared with the sham-operated rats (Fig. 6). None of the three dynamic parameters was altered in the 3-mo group (Fig. 6).

Osteoclast-mediated bone resorption was enhanced after cecectomy. Regarding bone resorption-related parameters, osteoclast surface tended to increase at 1 mo postcecectomy (P = 0.051) and later increased significantly at 3 mo compared with the corresponding sham-operated rats (Fig. 7A). Eroded surface, a representative of microscopic bone erosion by osteoclast-produced acids and proteolytic enzymes (22, 31, 33), was also increased in the tibial metaphyseal trabeculae of 1- and 3-mo cecectomized rats (Fig. 7B). However, osteocyte lacunar area of the tibial cortical envelope remained unchanged after cecectomy (Fig. 7C). Circulating levels of PTH, which is

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**Fig. 3.** A–D: representative photomicrographs of the proximal tibial metaphyses in Sham and Cecec rats at 1 and 3 mo postsurgery. Bone sections were processed for Goldner’s trichrome staining, in which mineralized bone matrix, erythrocytes, and cytoplasm were stained green, orange, and red, respectively. Nos. of bone trabeculae (arrows) in Cecec rats were conspicuously less than those in Sham rats, thus expanding the marrow space (Ma). Ep, epiphyseal plate (growth plate); Ct, cortical envelope. Scale bars, 500 μm.

**Fig. 4.** A: trabecular bone volume normalized by tissue volume (BV/TV). B: trabecular number (Tb.N). C: trabecular separation (Tb.Sp). D: trabecular thickness (Tb.Th). E: trabecular volumetric bone mineral density (vBMD) in the proximal tibial metaphysis of Sham and Cecec rats at 1 and 3 mo postsurgery. BV/TV, Tb.N, Tb.Sp, and Tb.Th were obtained from Goldner’s trichrome-stained sections by bone histomorphometry, whereas vBMD was determined by μCT. Nos. in parentheses are the nos. of animals. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the corresponding Sham group.
responsible for activating osteoclast maturation and activity (2, 36), were elevated in the 3-mo cecctomized rats (Fig. 7D), whereas 25-(OH)-D₃ levels in the 3-mo cecctomized rats (52.23 ± 2.56 ng/ml; n = 10) were comparable with those in sham-operated rats (50.48 ± 1.79 ng/ml; n = 10, P = 0.291).

DISCUSSION

In herbivorous and omnivorous hindgut fermenters (e.g., rodents), the cecum harbors a number of microfloral species that break down indigestible fiber and produce several nutrients, such as short-chain fatty acids, thiamine, folate, and vitamin K (16, 20, 23). The cecal epithelial cells also absorb essential minerals, including zinc and calcium (10, 14). Despite being reported to have the highest calcium absorption rate compared with other intestinal segments, the cecal calcium absorption was previously believed to contribute less than 10% of total calcium absorbed by the whole intestine (3, 4, 13, 21). Thus, the physiological role of cecum in body calcium homeostasis was elusive. However, we demonstrated recently that the cecum was indeed crucial for body calcium homeostasis, since 1-mo cecctomized rats manifested a negative calcium balance, fecal calcium wasting, and pervasive bone loss, as determined by DEXA (11). In the present study, cecctomy has been shown to profoundly affect trabecular microstructure, the deterioration of which could be observed for as long as 3 mo postsurgery. In other words, the compensatory colonic calcium hyperabsorption in cecctomized rats could not adequately meet the body calcium demand (11); therefore, calcium was continuously drawn from bone, thereby culminating in severe osteopenia. Uncoupling of trabecular bone formation and resorption did occur in cecctomized rats since bone histomorphometric analysis revealed an increase in osteoclast activity with decreased osteoblast activity. The cecctomy-induced trabecular and cortical osteopenia may have resulted in part from elevation of circulating PTH, which was released from the parathyroid gland presumably in response to negative calcium balance (5, 11, 29).

A decrease in osteoblast-mediated bone formation and/or an increase in osteoclast-mediated bone resorption are the principal causes of low BMD (osteopenia) and osteoporosis in humans and rodents (1, 24, 27, 31). The observed increases in osteoclast surface and eroded surface in the tibial metaphysial trabeculae of cecctomized rats suggested that the osteoclast activity and osteoclast-mediated bone resorption were enhanced, possibly as a result of elevated PTH levels (5, 18, 29). Indeed, activation of osteoclasts by PTH is indirect through osteoblasts (for reviews, please see Refs. 2 and 36). After binding to its receptors in osteoblasts, PTH stimulates the production and secretion of the receptor activator of nuclear factor-κB ligand that directly augments osteoclastogenesis and osteoclast activity, thereby leading to bone resorption and calcium release from bone (2, 31, 36). Thus, the cecctomy-induced bone resorption was part of the compensatory mechanism that helped to maintain body calcium homeostasis in the face of a large fecal calcium wasting.

Besides the enhanced bone resorption, the cecctomy-induced trabecular bone loss was also caused by suppression of osteoblast activity and bone formation. However, since osteoblast surface was not decreased significantly in the first month postcecectomy, an early phase of bone loss in cecctomized rats may result primarily from the enhanced bone resorption and low calcium supply to bone rather than a decrease in osteoblast proliferation. Similarly, in pigs fed low-calcium diets (≤0.4% wt/wt), a decrease in trabecular bone volume appeared to be a direct consequence of the enhanced osteoclast activity and defective mineralization (9). Despite no significant reduction in the mineral apposition rate at either 1 or 3 mo postcecectomy, the actual amount of calcium accretion was inadequate for the complete mineralization of the osteoblast-produced osteoid. Therefore, the osteoid thickness was increased significantly, and the areas with newly mineralized osteoid, as represented by double-labeled surface (Fig. 6A), were decreased in cecctomized rats. It was apparent that fecal calcium wasting after cecctomy was the salient cause of insufficient
calcium supply for ostoid mineralization (11). Since exposure to low extracellular calcium concentration could decrease osteoblast proliferation (8), a long-term shortage of intestinal calcium supply might eventually dampen the osteoblast number and activity, consistent with a decrease in osteoblast surface at 3 mo postcecectomy (Fig. 5A). Therefore, the impaired bone formation in cecectomized rats resulted initially from inadequate calcium supply (1 mo), followed by decreased osteoblast activity (3 mo).

The cecectomy-induced bone resorption and suppression of bone formation eventually culminated in dramatic trabecular microstructural defects, such as decreased trabecular number and increased trabecular separation in the trabecular part of the long bone (e.g., tibial metaphysis). Although bone mechanical property was not determined directly in the present study, this massive deterioration of the trabecular microstructure and decreased moment of inertia could lead to a decrease in trabecular bone strength and increased fracture risk. Previously, Shiga et al. (25) reported a decrease in femoral bone strength in cecocolonectomized rats on day 20 postoperation. Furthermore, the present μCT and DEXA studies suggested that cecectomy not only induced trabecular microstructural defects but also worsened the cortical structure by decreasing the thickness of the cortical envelope. Since both cortical periosteal and endosteal perimeters were decreased, the impaired radial growth of the tibial shaft was the principal cause of low diaphyseal BMD observed in the cecectomized rats. The osteoclast-mediated bone resorption in cortical envelope was not determined directly in the present study, but it should occur due to elevated PTH levels and may contribute to low diaphyseal BMD. The enhanced cortical bone resorption, if present, suggested that negative calcium balance in cecectomized rats was very severe; otherwise, bone loss would have occurred predominantly in the trabecular sites, which have larger surface areas for calcium release than the cortical sites (6). On the other hand, osteocytic osteolysis should not contribute to the cecectomy-induced cortical bone loss since cecectomized rats did not exhibit an increase in osteocyte lacunar area. It was likely that the defects of cortical structure as determined by μCT, but not trabecular structure, became less severe at 3 mo postcecectomy, although the exact cellular mechanism of this cortical adaptation is not known. However, the persistent trabecular deterioration explained why some measurements, which included trabecular structure (e.g., whole tibial BMC), revealed significant changes at 3 mo postcecectomy.

Although further investigation is required to demonstrate the underlying cellular mechanism of the cecectomy-induced suppression of osteoblast function and bone formation, it is noteworthy that the cecum is capable of producing several humoral factors, such as glucagon-like peptide-1 and serotonin (12, 26), which in turn modulate the functions of osteoblasts and osteoclasts (35). Thus, besides inadequate intestinal calcium absorption (11), removal of the cecum might affect the circulating levels of these mediators, thereby disrupting the bone remodeling process of cecectomized rats.

In conclusion, the cecectomy-induced structural deteriorations were observed at both trabecular and cortical sites, leading to low BMD in the affected areas. It was possible that PTH release in response to cecectomy-induced fecal calcium wasting and negative calcium balance contributed to the observed microstructural defects by enhancing osteoclast-mediated bone resorption. As a result, the tibial metaphysis showed trabecular separation and decreases in trabecular bone volume and trabecular number. The long-lasting and widespread osteopenia in cecectomized rats thus corroborated the hypothesis that the cecum was an important site for intestinal calcium absorption, and its absence impaired body calcium homeostasis dramatically.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

N.C. and N.K. did the conception and design of the research; N.C., P.S., and P.J. performed the experiments; N.C., P.S., P.J., K.W., and N.K. analyzed the data; N.C., K.W., and N.K. interpreted the results of the experiments; N.C., P.S., P.J., and K.W. prepared the figures; N.C., K.W., and N.K. drafted the manuscript; N.C., K.W., and N.K. edited and revised the manuscript; N.C., P.S., P.J., and K.W. approved the final version of the manuscript.

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