Efficacy and Pharmacological Mechanism of Pronase-Enhanced Low-Dose Antibiotics for *Helicobacter pylori* Eradication

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This study examined the efficacy and pharmacological mechanism of pronase-assisted low-dose antibiotics for eradication of *Helicobacter pylori*. Mongolian gerbils infected with *H. pylori* received 7-day treatment (omeprazole, different concentrations of pronase, amoxicillin, and clarithromycin), and the efficacy was assessed using the eradication rate and the colonization of *H. pylori*. In Mongolian gerbils orally administered pronase, the thickness of the gastric mucous layer (GML) was examined using immunohistochemical and alcian blue staining, and the concentrations of amoxicillin in gastric tissue and serum were detected using high-performance liquid chromatography (HPLC). The eradication rates were 80.0% (12/15) in the high-pronase quadruple group (HPQG) and 86.7% (13/15) in the high-antibiotic group (HAG) ($P = 1.000$). The antibiotic dose in the HPQG was only 1/20 that in the HAG. Thirty minutes after oral treatment with pronase, the sticky protein of the GML was hydrolyzed, and the GML became thinner. Higher amoxicillin concentrations in both the gastric tissue and serum were observed in the pronase group than in the Am10 group. The concentration of amoxicillin in the Am10-plus-Pr108 group in gastric tissue was 3.8 times higher than in the Am10 group in 5 min. Together, these data suggest that pronase significantly reduced the dose of antibiotics used in *H. pylori* eradication. The pharmacological mechanism is likely pronase removal of the mucus layer, promoting chemical factor (i.e., gastric acid and pepsinogen) distribution and increasing the antibiotic concentrations in the deep GML, which acted on *H. pylori* collectively. Thus, pronase may enhance the level of antibiotics for eradication of *H. pylori* in the clinic.

Helicobacter pylori is a significant cause of chronic active gastritis, peptic ulcer disease, and gastric cancer (1). Several strategies have been proposed for eradication of *H. pylori*, including the standard first-line treatment (standard triple therapy [STT]) and quadruple, sequential, and concomitant treatments (2). However, the eradication rates for *H. pylori* infection have decreased to less than 80% in many countries (3). One of the reasons for this incomplete eradication is most likely protection of *H. pylori* by the gastric mucous layer (GML), and effective antimicrobial concentrations cannot be achieved in the deep GML or on epithelial cell surfaces, where *H. pylori* is present (4–6). This is mainly due to the barrier of the dense GML, the short residence time of the antimicrobial agents in the stomach, and the degradation of antimicrobial agents, such as amoxicillin and clarithromycin, which are degraded by gastric acid (4).

Approaches to achieve effective antimicrobial concentrations in the deep GML include increasing the administration of high doses of antimicrobial agents on a daily basis, although this is usually accompanied by adverse effects and poor patient compliance (7), and extending the residence time of antimicrobial agents in the stomach by changing the dosage form (4); however, the antibiotic dose is not reduced. A more optimal strategy is not only to reduce the antibiotic dose, but also to improve the local drug concentration in the deep GML.

Pronase, a type of hydrolyzable protease, is known to hydrolyze sticky proteins and to remove the surface gastric mucous layer of the stomach (8) and has also been used as a premedication for endoscopy (9). It also increased the amoxicillin transfer rate from plasma to gastric aspirate by traversing the gastric mucosa (10). Here, pronase alone exhibited no inhibitory effect on *H. pylori*, although it has been reported that pronase has an effect on promoting the eradication of *H. pylori*. However, this effect is not obvious, and the pharmacological mechanism remains unclear

(11, 12). In this work, we report the effect of pronase-enhanced antibiotics on the eradication of *H. pylori* and study its pharmacological mechanism in the Mongolian gerbil model.

MATERIALS AND METHODS

Chemicals and reagents. Pronase samples were a gift from the Beijing Tide Pharmaceutical Co., Ltd. (Beijing, China). Omeprazole was purchased from Wuhan Chufengyuan Technology Co., Ltd. (Wuhan, China). Amoxicillin and clarithromycin were purchased from Southwest Pharmaceutical Co., Ltd. (Chongqing, China). *N*-Acetyl-L-cysteine was purchased from Absin Bioscience Inc. (Shanghai, China). Misoprostol was purchased from Shanghai New Hualian Co., Ltd. (Shanghai, China). Primers for PCR and quantitative reverse transcription (qRT)-PCR were synthesized by BGI-Shenzhen (Shenzhen, China). The gastric mucin primary antibody was purchased from Novus Biologicals (Littleton, CO, USA), and the secondary horseradish peroxidase antibody was purchased from SinoBio Biotech Co., Ltd. (Shanghai, China). Alcian blue stain (pH 2.5) was obtained from Zhuhai Baso Biotechnology Co., Ltd. (Zhuhai, China). The urease test kit was purchased from Zhuhai Kedi Technology Co., Ltd. (Zhuhai, China). High-performance liquid chromatography (HPLC) grade acetonitrile, methanol, and hematoxylin were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). All other reagents were of analytical grade.

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Animals. Six-week-old male specific-pathogen-free Mongolian gerbils (body weight, 50 to 60 g) were purchased from the Center of Experimental Animals (Zhejiang, China) and were maintained under standard laboratory conditions (room temperature, $23 \pm 2^\circ\text{C}$; relative humidity, $55\% \pm 5\%$; 12-h light–12-h dark cycle) with free access to an autoclaved pellet diet and sterile water. After a 1-week equilibration period, the gerbils were used in the experiments. All animal experiments were approved by the Animal Ethical and Experimental Committee of Third Military Medical University, Chongqing, China.

Infection of Mongolian gerbils with *H. pylori*. According to our previous method (13), the *H. pylori* strain M13, which was derived from a Chinese clinical isolate and adapted to colonize the GML of Mongolian gerbils, was used for inoculation in Mongolian gerbils. The bacteria were inoculated in 150 ml of brain heart infusion broth supplemented with 5% fetal bovine serum and 1% glucose with shaking at 220 rpm and 37°C under 5% O_2 and 8% CO_2 . After harvesting, the bacterial suspensions were prepared at a concentration of 2.0×10^9 CFU/ml. All groups of Mongolian gerbils were inoculated with 0.4 ml of *H. pylori* suspension orally by gavage twice a day for only 1 day. The gerbils were housed for 4 weeks under the conditions described above. Four weeks after inoculation, 10 random gerbils from among all of the Mongolian gerbils were sacrificed by cervical dislocation to evaluate the colonization rate of *H. pylori* in the stomach, and the infection rate was 100% for the next experiment.

Detection of *H. pylori*. Infection with *H. pylori* was assessed using bacterial culture, the urease test, and PCR. The stomachs were removed from the gerbils and cut into two parts. One part of the stomach was serial plated on modified Skirrow's medium (15 g peptone, 5 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar, 5 g glucose, 5 mg trimethoprim, 10 mg vancomycin, 2 mg amphotericin, 2,500 U polymyxin, 50 ml rabbit blood), and the agar plates were incubated for 2 days at 37°C under 5% O_2 and 8% CO_2 in three gas incubators (Thermo Scientific). Viable colonies for each stomach were identified as *H. pylori* on the agar plates, which were positive for the gerbils. Another part of the stomach was homogenized with normal saline, and the mixture was identified using the urease test. In addition, the mixture was also centrifuged for 5 min at $13,000 \times g$, and the precipitate was collected for PCR and qRT-PCR. PCR and qRT-PCR analyses of the samples were performed using a previously reported method (14, 15). The DNA was isolated from the precipitate using the Microbial Genomic DNA Extraction Kit (SunShineBio) (13). The PCR primers used to amplify the *ureC* (*glmM*) gene of *H. pylori* were as follows: F, 5'-AAGCTTTAGGGGTGTTAGGGGTTT-3', and R, 5'-AAGCTTACTTTCTACACTAACGC-3'. The assay was run on a C1000 thermal cycler (Bio-Rad). After a denaturation step at 94°C for 3 min, 35 cycles were performed for PCR amplification, where the cycle parameters were 94°C for 45 s, 55°C for 30 s, and 72°C for 30 s, followed by an extension step at 72°C for 5 min. The PCR product was separated by electrophoresis with a 2% gel containing ethidium bromide and visualized with a UV light source. Both the urease test and PCR were positive, which was defined as positive for *H. pylori*. The colonization of *H. pylori* was quantified by amplifying the 16S rRNA gene of *H. pylori*. For the 16S rRNA genes, the primers used were F, 5'-TTTGTAGAGAAGATAATGACGGTATCTAAC-3', and R, 5'-CATAGGATTTACACCTGACTGACTATC-3', and the TaqMan probe was P, 5'-6-carboxyfluorescein (FAM)-CGTGCCAGCAGCCGCGGT-6-carboxytetramethylrhodamine (TAMRA)-3'. The assay was run on a CFX Real-Time System (Bio-Rad). After a denaturation step at 95°C for 2 min, 40 cycles were performed for PCR amplification. The cycle parameters were 95°C for 5 s and 60°C for 30 s.

Eradication of *H. pylori* in vivo. Mongolian gerbils infected with *H. pylori* were assigned to 9 groups (15 animals per group) as follows: (i) nontreated group (NG) (omeprazole, 4 mg/kg of body weight); (ii) low-antibiotics group (LAG) (omeprazole, 4 mg/kg; amoxicillin, 10 mg/kg; and clarithromycin, 5 mg/kg); (iii) high-antibiotics group (HAG) (omeprazole, 4 mg/kg; amoxicillin, 200 mg/kg; and clarithromycin, 100 mg/kg); (iv) *N*-acetyl-L-cysteine group (NACG) (omeprazole, 4 mg/kg;

N-acetyl-L-cysteine, 125 mg/kg); (v) pronase and misoprostol group (PMG) (omeprazole, 4 mg/kg; misoprostol, 0.3 mg/kg; pronase, 108 mg/kg); (vi) high-pronase group (HPG) (omeprazole, 4 mg/kg; pronase, 108 mg/kg); (vii) low-pronase quadruple group (LPQG) (omeprazole, 4 mg/kg; pronase, 27 mg/kg; amoxicillin, 10 mg/kg; and clarithromycin, 5 mg/kg); (viii) medium-pronase quadruple group (MPQG) (omeprazole, 4 mg/kg; pronase, 54 mg/kg; amoxicillin, 10 mg/kg; and clarithromycin, 5 mg/kg); and (ix) high-pronase quadruple group (HPQG) (omeprazole, 4 mg/kg; pronase, 108 mg/kg; amoxicillin, 10 mg/kg; and clarithromycin, 5 mg/kg). The gerbils in these groups were treated twice daily for 7 consecutive days. The gerbils were orally and successively administered omeprazole, pronase, or an *N*-acetyl-L-cysteine, amoxicillin, and clarithromycin mixture, with a time interval of 30 min; misoprostol was orally administered 15 min after taking omeprazole. Four weeks after administration, the eradication of *H. pylori* was examined using bacterial culture, the urease test, and PCR.

Immunohistochemical and alcian blue staining of GML. The healthy gerbils were divided into three groups: the control group ($n = 3$), which received only oral doses of omeprazole (4 mg/kg); the medium-pronase group ($n = 3$), which was successively given oral administration of omeprazole (4 mg/kg) and pronase (54 mg/kg); and the high-pronase group ($n = 3$), which was successively given orally omeprazole (4 mg/kg) and pronase (108 mg/kg), with a time interval of 30 min. Thirty minutes after administration, the gerbils' stomachs were removed and frozen. Frozen tissue sections were obtained, which were stained immunohistochemically with a primary antibody against gastric mucin, according to a method previously reported (16). Other frozen sections were stained using the alcian blue solution (17). The sections were washed with deionized water for 30 min, placed in the alcian blue solution (pH 2.5) for 15 min, and then rinsed briefly with water. Next, the tissue sections were counterstained with hematoxylin staining solution for 15 s and rinsed briefly with water for 10 min. To best observe the GML, the tissue sections were immediately visualized under a microscope after staining. The thickness of the GML was defined as the distance from the outermost edge of the GML to the luminal surface of the surface mucous cells (18). The thickness of the GML was measured, using a microscope, for 60 randomly selected points in each of the control and treatment groups.

Pharmacokinetics of amoxicillin. The gerbils were divided into four groups (36 animals per group) with the following treatment conditions: (i) the Am10 group (omeprazole, 4 mg/kg; amoxicillin, 10 mg/kg; and clarithromycin, 5 mg/kg); (ii) the Am200 group (omeprazole, 4 mg/kg; amoxicillin, 200 mg/kg; and clarithromycin, 100 mg/kg); (iii) the Am10-plus-Pr54 group (omeprazole, 4 mg/kg; pronase, 54 mg/kg; amoxicillin, 10 mg/kg; and clarithromycin, 5 mg/kg); (iv) the Am10-plus-Pr108 group (omeprazole, 4 mg/kg; pronase, 108 mg/kg; amoxicillin, 10 mg/kg; and clarithromycin, 5 mg/kg). The gerbils were orally and successively administered an omeprazole, pronase, amoxicillin, and clarithromycin mixture, with a time interval of 30 min. The amoxicillin concentration was determined in the gastric tissue and serum using HPLC (19, 20). The gastric tissue and blood samples were collected before and 5, 10, 15, 20, 25, 30, 35, 45, 60, 75, and 90 min after oral administration, with three gerbils for each sampling point. The gastric tissue was removed and washed twice in phosphate-buffered saline (PBS). Next, it was homogenized with 0.5 ml PBS and centrifuged for 5 min at $13,000 \times g$. After the supernatants were collected, an equal volume of methanol was added and mixed for 2 min. The mixture was centrifuged for 10 min at $13,000 \times g$, and the supernatant fluid was collected for HPLC analysis. Blood samples were coagulated for 5 h at 4°C and centrifuged for 5 min at $5,000 \times g$. The serum was collected, and subsequent steps were performed as for the gastric tissue operations. The apparatus used for this study was an Agilent 1260 quaternary pump, and the HPLC method included a mobile phase consisting of a mixture of 50 mM phosphate buffer and acetonitrile (97.5:2.5 [vol/vol]). This solution was adjusted to pH 3.0 using concentrated phosphoric acid and pumped at a flow rate of 0.7 ml/min through an Agilent Zorbax

TABLE 1 Different therapies for *H. pylori* eradication in Mongolian gerbils

Group	Eradication rate (%) after 4 wk (n/15)	<i>P</i> value
NG	0 (0)	
LAG	13.3 (2)	0.001 ^a
HAG	86.7 (13)	
NACG	20.0 (3)	
PMG	0 (0)	
HPG	40 (65)	0.022 ^d
LPQG	33.3 (5)	
MPQG	53.3 (8)	0.245 ^c
HPQG	80.0 (12)	1.000 ^b

^a HPQG versus LAG.^b HPQG versus HAG.^c HPQG versus MPQG.^d HPG versus PMG.

Extend C₁₈ reverse-phase column (150 mm by 4.6 mm; 5 μ m) (21, 22). The optimization wavelength of amoxicillin detection was 210 nm.

Statistical analysis. The eradication rates were compared between groups using the χ^2 test or Fisher's exact test. The difference in colonization in the two groups was examined using the independent-sample *t* test. The figures represent the data from three independent experiments, and the data are expressed as means $\pm$ standard deviations (SD). Statistically significant differences between groups were defined as a *P* value of <0.05 . The statistical analyses were performed using SPSS 19.0 statistical software for Windows.

RESULTS

The eradication of *H. pylori* in vivo. Four weeks after administration, the effects of the eradication of *H. pylori* were examined using bacterial cultures (Table 1). The eradication rate in the LAG was 13.3% (2/15), and the HPQG achieved an 80.0% (12/15) eradication rate. There were statistically significant differences in the LAG compared with the HPQG ($P < 0.05$). In contrast, the HPQG showed no significant difference compared with the HAG ($P > 0.05$), which reached an 86.7% (13/15) eradication rate; however, the antibiotic doses in the HPQG were only 1/20 of the dose used in the HAG. In addition, the MPQG achieved a 53.3% (8/15) eradication rate, which showed no significant difference compared with the HPQG ($P = 0.245$). The NACG and HPG showed little eradication effect. The PMG and NG had no eradication effect, with eradication rates of 0% (0/15).

The eradication effects for the different treatments were further confirmed using the qRT-PCR method (Fig. 1). qRT-PCR can demonstrate both the effect of eradication and the differences in the amounts of colonization. Compared with the LAG, the amount of colonization decreased significantly in the MPQG and HPQG. There was no significant difference between the HAG and the MPQG and HPQG, where the *P* values were 0.673 and 0.193, respectively. The PMG had a larger amount of colonization than the other groups.

Hydrolysis of pronase in the GML. To visualize the gerbils' GML, immunohistochemistry and alcian blue staining were performed, as shown in Fig. 2. The GML includes mucins and mucous polysaccharides, which were well preserved in all gerbils that were only orally treated with omeprazole. However, the mucins and mucous polysaccharides from gerbils that were treated with pronase were significantly damaged and were thinner than those from gerbils without pronase premedication. The destructive ef-

fect of the low-pronase group on the GML is not obvious (data not shown).

Amoxicillin concentrations in the gastric tissue and serum. A satisfactory separation of amoxicillin from the endogenous components in the gerbil gastric tissue and serum was obtained (Fig. 3A). As shown in Fig. 3B, the amoxicillin concentration of the Am10-plus-Pr108 group in the gastric tissue achieved a maximum value in 5 min. The value was 12.13 μ g/ml. The amoxicillin concentration of the Am10 group also reached the highest point in 5 min; however, its value was only 3.17 μ g/ml. The value of the Am10-plus-Pr108 group was 3.8 times that of the Am10 group. The amoxicillin concentration in serum in all groups became higher over time. As shown in Fig. 3C, there was a wave in all groups within 15 min, where the amoxicillin was absorbed into the blood by the gastric tissue. The amoxicillin concentration in the gastric tissue and serum in the Am200 group was higher than in the other groups at the same sampling point.

DISCUSSION

To date, there are many methods to enhance the efficacy of eradication of *H. pylori* in the stomach, such as increasing the dose, prolonging the duration, and developing new drugs (23). However, adverse effects and drug waste have become more serious, and the development of new methods will be challenging. Thus, the treatment of *H. pylori* will be a larger issue in the future. A potentially useful method would be to directly improve the local drug concentration in the deep GML. According to Gotoh et al. (11), quadruple therapy with lansoprazole, pronase, amoxicillin, and metronidazole could improve the efficacy of *H. pylori* eradication in humans, which may be due to the increased delivery of antibiotics at the site of infection by virtue of pronase's ability to remove and disrupt the GML. However, the doses of antibiotics were 500 mg of amoxicillin and 250 mg of metronidazole, and the times of therapy were three times daily for 2 weeks, which worsens compliance due to an additional drug being administered at the same time. On this basis, we explored the effect of pronase enhancement of low doses of antibiotics for eradication of *H. pylori* in gerbils.

To comprehensively evaluate the pronase quadruple therapy for the treatment of *H. pylori*, according to the dose of STT (20 mg omeprazole, 1 g amoxicillin, and 500 mg clarithromycin) in humans (24), we performed a preliminary experiment to study the

FIG 1 Colonization of *H. pylori* (Hp) in the stomach using qRT-PCR analysis. Medians (long horizontal lines) and SD (error bars) are shown.

FIG 2 GML of gerbils stained by immunohistochemistry and alcian blue. (1 and 4) The control group. (2 and 5) The medium-pronase group. (3 and 6) The high-pronase group. (A) The GML was immunohistochemically stained. The gray areas are the mucins of the GML, which were labeled by the gastric mucin antibody. (B) The GML was stained using an alcian blue solution. The blue area is the acid mucous polysaccharides, which were stained with alcian blue. The dark-blue areas of panels A and B are the nuclei of the gastric mucous cells, which were stained using hematoxylin staining solution. Original magnification, $\times 200$; bars = 25 μm .

effects of different doses of antibiotics for eradication of *H. pylori* in the gerbil stomach (data not shown). The therapeutic effects of MAG (a drug regimen consisting of 4 mg/kg omeprazole, 20 mg/kg amoxicillin, and 10 mg/kg clarithromycin was administered twice daily for 7 consecutive days) and EAG (extra-low-

antibiotics group; a drug regimen consisting of 4 mg/kg omeprazole, 5 mg/kg amoxicillin, and 2.5 mg/kg clarithromycin was administered twice daily for 7 consecutive days) were 40.0% (6/15) and 6.7% (1/15), respectively. The eradication rates of the HAG and LAG are shown in Table 1. We selected the antibiotic

FIG 3 Detection of amoxicillin in gastric tissue and serum using HPLC. (A) (1) HPLC chromatogram of the amoxicillin standard (45 $\mu\text{g}/\text{ml}$). (2) HPLC chromatogram of drug-free gerbil gastric tissue. (3) HPLC chromatogram of gerbil stomach after oral administration of amoxicillin (10 mg/kg). (4) HPLC chromatogram of drug-free gerbil serum. (5) HPLC chromatogram of gerbil serum after oral administration of amoxicillin (10 mg/kg). (B) Concentration-time profiles of amoxicillin in gastric tissue after coadministration with clarithromycin. (C) Concentration-time profiles of amoxicillin in serum after coadministration with clarithromycin. The error bars indicate SD.

dose of LAG with pronase that was more convincing than other groups, because the antibiotic usage was lower and the cure rate increase was greater.

In addition to the bacterial culture, urease test, and PCR, we used qRT-PCR to examine the amount of colonization of *H. pylori*. qRT-PCR is more sensitive than other methods, and it can detect *H. pylori* even at low concentrations (25). Combined with the eradication rate and the amount of colonization, this further demonstrates that pronase can assist antibiotics in eradicating *H. pylori*.

We studied the pharmacological mechanism underlying pronase-enhanced low-dose antibiotic treatment in *H. pylori* infection. Pronase alone did not exhibit an inhibitory effect on *H. pylori* *in vitro*, even at concentrations of 3.3×10^3 mg/liter, in previous studies (data not shown), which was consistent with previous reports demonstrating that pronase did not inhibit the growth of *H. pylori* (11). Misoprostol is a mucosal protective agent (26). The eradication rate of the PMG was 0% (0/15), and there was a significant difference between the eradication rates of the PMG and the HPG ($P = 0.022$). This suggests that pronase did not play a direct role in eradication of *H. pylori* in the stomach. *N*-Acetyl-L-cysteine, which is a mucolytic agent, has a hydrolysis mechanism similar to that of pronase and has been used in the treatment of *H. pylori* infection (27, 28). In our study, the NACG obtained a 20% (3/15) eradication rate. A combination of the hydrolysis experiment of pronase and the eradication rate in the HPG suggests that the mechanism of pronase is removing the gastric mucus layer and likely promoting chemical factor (i.e., gastric acid and pepsinogen) distribution and increasing the antibiotic concentrations in the deep GML, which acted on *H. pylori* collectively.

H. pylori lives deep in the GML (5, 6), which is formed by two types of mucin layers that are derived from surface mucous cells and gland mucous cells (18). There is sufficient antibiotic concentration in the deep GML to effectively eradicate *H. pylori* (29–31). By detecting the antibiotic concentration in gastric tissue after oral treatment with pronase, the value of the amoxicillin concentration in the Am10-plus-Pr108 group was 3.8 times that of the Am10 group in 5 min. This finding indirectly indicated that pronase promoted the diffusion and absorption of antibiotics in gastric tissue. It showed that pronase enhanced the ability of low doses of antibiotics to achieve an effective concentration that can eradicate *H. pylori*.

In our study, we also detected the concentrations of clarithromycin in gastric tissue and serum according to previous studies (10, 22). However, the peak area is very small, and detection is not sufficiently sensitive. Thus, calculation of the concentrations of clarithromycin is not accurate. We speculate that the reason is that the oral dose is too small. Moreover, amoxicillin also displays a higher crest and larger peak area and exhibits better accuracy. Thus, we chose an amoxicillin concentration to represent the concentration-time profiles of antibiotics in gastric tissue and serum.

In conclusion, we found that 7 days of pronase quadruple treatment with low-dose amoxicillin and clarithromycin exhibited similar eradication rates, with 20 times the dose of antibiotics alone, most likely by the mechanism of reducing the GML thickness to enable the distribution of chemical factors and increase the antibiotic concentration in the deep GML, which acted on *H. pylori* collectively. Thus, this treatment can reduce the use of antibiotics, thereby lowering costs, reducing the emergence of drug-resistant bacteria, and improving compliance. Furthermore, using

pronase-enhanced low-dose antibiotics for the eradication of *H. pylori* is recommended. If pronase is to be applied in the clinic, then additional safety evaluations and further clinical studies are required.

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