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Contributed Paper

Antimicrobial Efficacy of a Combination of Crocodile (*Crocodylus siamensis*) Leukocyte Extract and Hen Egg Lysozyme

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ABSTRACT

The combination of two or more natural antimicrobial substances is extensively used in clinical therapy. In this context, lysozymes represent an interesting group of naturally antimicrobial proteins, since they were found to display synergistic activity in combination with other antimicrobial substances. Furthermore, studies employing *Crocodylus siamensis* leukocyte extract (cLE) recently revealed the potent antimicrobial properties. However, potential synergistic interactions of cLE with other antimicrobials have not been reported to date. In this work, we were thus interested to investigate the synergy of cLE with hen egg lysozyme (hEL) in more detail. Employing a checkerboard technique, the combination of cLE and hEL *in vitro* showed partial synergy against foodborne *V. cholerae* (clinical isolation) with the fractional inhibitory concentration index (ΣFIC) value of 0.75. At the same concentration, a strong synergistic efficacy of the hEL-cLE combination was observed using time-kill assay. SEM images also suggest that the killing mechanism of the combination involves bacterial cell wall lysis and membrane damages. Additionally, *in-vivo* toxicity test of the combination in mice was performed. The results show that the hEL-cLE combination at $5 \times \Sigma$FIC neither induced significant modulation of most biological parameter levels in mice serum, nor affected the histopathological features of mice livers and kidneys. These observations provide clear evidence that the combination of hEL and cLE is essentially non-toxic and represents a promising target for development in clinical therapy from bacterial infection.

Keywords: animal toxicity, antibacterial activity, crocodile leukocyte extract, foodborn pathogen, hen egg lysozyme

1. INTRODUCTION

Recently, the emergence of foodborne illness is one of global concerns in human health. The foodborne illness can be caused by many factors, such as metals, toxins, viruses, parasites and bacteria [1]. Among these causative factors, bacterial pathogen are more concerned about the high risk because the pathogen is usually presented in many foods and vegetables. Examples for the bacteria causing foodborne disease include *Staphylococcus aureus*, *Salmonella enterica*, *Escherichia coli*, *Yersinia enterocolitica* and *Vibrio cholerae* [2]. The contamination of the bacteria can induce the alteration of chemical and physical natures of foods, leading to changes in sensory properties of foods to undesired features and food spoilage. After ingestion of the contaminated or spoiled foods, the bacterial infection or their toxin produced usually cause human illness [3]. Therefore, the prevention of foodborne infection is necessary to be concerned.

Numerous antimicrobial have been increasingly introduced for treating many human diseases caused by the infection of foodborne bacteria [4-5]. Lysozyme, typically found in eggs and milk, is one of many well-known antimicrobial proteins and constitutes an important component of the innate immune system of various animals. Because of its antimicrobial activity and its harmlessness to humans, lysozyme is very attractive as a food preservative and, thus, of great interest for the food industry [6-11]. Especially, this enzyme has been established in combination with other antimicrobial agents. The combination of lysozyme and nisin could inhibit *Lactobacillus curvatus* 845 and induced severe cell damage to *L. curvatus* as well as caused rapid permeabilization of the cytoplasmic membranes of *Staphylococcus aureus* [12]. Moreover, the synergistic activity between histone-derived peptides of coho salmon (*Oncorhynchus kisutch*) and lysozyme was found in killing *Vibrio anguillarum* [13]. More recently, the combination of *Galleria mellonella* lysozyme and anionic peptide 2 showed the synergistic activity against the Gram-negative bacterium *Escherichia coli* [14]. It therefore appears plausible to assume that lysozyme can be combined with other agents to achieve synergistic enhancement of antimicrobial activity.

Additionally, crocodile leukocyte extract (cLE) has been studied extensively with respect to its antimicrobial properties. In a previous report, the antimicrobial properties were found in crude extract from *Alligator mississippiensis* leukocytes [15]. The alligator leukocyte extract was found effective in killing several types of microbes, e.g. bacteria, yeast and viruses, and the active agents were presumed to be cationic peptides. In addition, previous work in our laboratory focusing on leukocyte extracts of *Crocodylus siamensis* revealed a broad spectrum of antimicrobial activity against several bacterial strains, especially foodborne bacterium *V. cholerae*. Also, a novel group of antimicrobial peptides, referred to as Leucrocins and RP9, has been successfully isolated from crocodile leukocyte extract [16-17]. These evidences imply that crocodile leukocyte extract has substantial prospect to be applied as a powerful antimicrobial agent of natural origin. Since synergistic effects between cLE and hen egg lysozyme (hEL) have not been studied before, this work was aimed at determining the synergistic activity *in vitro* against food borne *V. cholerae*, as well as collecting mechanistic information about the mode of antimicrobial action against bacterial cells by SEM. Moreover, in order to assess the *in-vivo* toxicity, the administration of the cLE-hEL combination in mice was performed.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Mice

Bacterial strains, such as Gram-negative *Vibrio cholerae* (clinical isolation) and Gram-positive *Bacillus pumilus* TISTR 905 were used. ICR mice of both sex, each weighing 28-30 mg were used in the experiments. The animals were kept in plastic cages with free access to water and food at the Northeast Laboratory Animal Center, Faculty of Medicine, Khon Kaen University.

2.2 Preparation of Crocodile Leukocyte Extract

Crocodile (*Crocodylus siamensis*) blood samples (10 L) were kindly provided by Sriracha Moda Co., Ltd. (Chon Buri, Thailand). The isolation and extraction of crocodile leukocytes were carried out according to the methodology of Pata et al. (2011) with slight modifications [16]. In the extraction process, crocodile leukocytes were frozen at -70°C for 24 h and then re-suspended with 10% (v/v) acetic acid solution. The re-suspended cells were disrupted using a homogenizer for 5 min on ice and the procedure was repeated 3 times. Any residual acetic acid in the homogenate was eliminated using an acid trap during the drying step.

2.3 Preparation of Hen Egg Lysozyme

Hen eggs were obtained from local markets in Khon Kaen, Thailand. Egg whites were manually separated from the egg yolk and subsequently lyophilized. The resulting egg white powder was mixed gently in 5 L of 0.05 M NaCl for 30 min, followed by precipitation at room temperature for 1 h. The supernatant was collected and adjusted to pH 4.5 with 0.5 M acetic acid in order to eliminate undesirable protein impurities. After keeping the sample solution overnight at room temperature, the supernatant was collected by continuous precipitation with 40% (v/v) ethanol. After precipitation, the supernatant was kept and centrifuged at 8,500 × *g* for 10 min at 4°C. A clear solution containing lysozyme was collected and the remaining ethanol was removed using an evaporator before lyophilization.

2.4 Lysozyme Activity Assay

The lytic activity of lysozyme was assayed using lyophilized cells of *Micrococcus luteus* as the substrate [18]. The cell suspension was prepared freshly at an OD_{540nm} of 0.8-1.0 in 0.05 M Na-phosphate buffer (pH 7.0). Then, 0.1 ml of sample solution were added to 3 ml of the substrate. One enzyme unit (U) was defined as the amount causing a decrease of 0.1 absorbance units at 540 nm within 1 min at 25°C. The activity of lysozyme was calculated as follows:

Activity (U) = (Decrease of OD_{540nm} × (Volume of E + Volume of S)) / (0.1 × volume of E)

where E signifies enzyme (lysozyme) and S signifies substrate. Finally, the specific activity of lysozyme (U/mg) was evaluated from the amount of initial substrate.

2.5 Tricine SDS-PAGE

The protein concentrations of cLE and hEL were estimated according to the Bradford method [19]. Then, protein profiles were acquired by tricine SDS-PAGE employing 16.5% separating gel, 10% spacer gel and 4% stacking gel in an AE-6440 electrophoresis device (ATTO, Japan). Individual proteins from both sources were separated by manipulation at a constant voltage of 90 V for 3 h. The protein bands were visualized by staining with Coomassie Brilliant Blue R-250 for 30 min, followed

by two-fold destaining for 30 min. A Rainbow™ molecular weight marker (General Electric, USA) was used for molecular weight estimation.

2.6 Minimal Inhibitory Concentration (MIC) Determination

The antimicrobial activity was assayed using the liquid growth inhibition technique. Briefly, both cLE and hEL were prepared by two-fold serial dilution in a concentration range of 1-512 µg/ml. Then, 10 µl of each sample were added individually to 100 µl of a bacterial cell suspension of approximately 1×10^6 cfu/ml in nutrient broth and incubated at 37°C for 16-20 h. Bacterial growth of samples was measured at 600 nm using a microliter plate reader and compared to that of the positive (90.9 µg/ml ampicillin) and negative controls (empty samples). The MIC values of samples were defined as the lowest peptide concentration that completely inhibited bacterial growth [20].

2.7 Checkerboard Determination

The antimicrobial activity of a combination of cLE and hEL was determined by the micro-checkerboard technique [21]. The stock solutions and two-fold serial dilutions of each protein to at least 2 × MIC value were prepared according to the recommendations of the National Committee on Clinical Laboratory Standards (NCCLS) immediately prior to testing. All samples were individually added to 100 µl of 10^6 cfu/ml bacterial solutions and then incubated for 16-20 h at 37°C. The interaction for each combination was assessed by calculating the fractional inhibitory concentration (FIC) of each protein and then summarizing their FIC to ΣFIC as follows:

$$\Sigma FIC = FIC_A + FIC_B$$

where FIC_A is the MIC of protein A in the combination divided by the MIC of protein A alone, and FIC_B is the MIC of protein B in combination divided by the MIC of protein B alone. The interaction of each combination is considered as synergism when the ΣFIC is ≤ 0.5, as partial synergism when $0.5 < \Sigma FIC \leq 0.75$, as no effect when $0.75 < \Sigma FIC \leq 2$, and as antagonism when ΣFIC is > 2 [22].

2.8 Bioautography (Gel Overlay Antibacterial Assay)

The protein profile of cLE and commercial hEL were elucidated by SDS-PAGE using 15% separating gel. After electrophoresis, the gel was washed in 2.5% Triton X-100 for 30 min to achieve SDS removal. Then, the gel was washed in 50 mM Tris-HCl, pH 7.5, and subsequently in nutrient broth. To localize the protein bands with antimicrobial activity, the gel was overlaid with soft (0.7%) nutrient agar containing viable *V. cholerae* cells and incubated at 37°C for 12 h. After incubation, the zones of bacterial growth inhibition were identified visually [23].

2.9 Time Kill Assay

V. cholerae cells were inoculated in nutrient broth in the log phase (OD_{600nm} = 0.5-0.8) and then diluted to an OD_{600nm} of 0.001 (~ 10^6 cfu/ml). Briefly, 100 µl of the diluted cells were treated with 10 µl of the individual proteins (cLE or hEL) or their combination, and then incubated at room temperature for 0, 60, 120 and 180 min. After incubation for the specified time, 10 µl of each sample were diluted with nutrient broth and adjusted to a final volume of 1 ml. Subsequently, 10 µl of each dilution was spread separately on nutrient agar plates. The percentage of bacterial growth was determined by assessing colony forming

units (cfu) after 48 h incubation at 37°C. At each incubation time, the growth of untreated bacteria was used as the control and the growth of ampicillin-treated cells was used as the positive control.

2.10 Scanning Electron Microscopy (SEM) Imaging

The morphological alterations of the cell surface of *V. cholerae* after treatment with proteins (cLE or hEL) at 5 × MIC and 10 × MIC, as well as with the combination at 5 × ΣFIC and 10 × ΣFIC for 60 min were also imaged by scanning electron microscopy [24]. The samples were examined under a scanning electron microscope (LEO1450VP, LEO Electron Microscopy, England) operating at 12-20 kV. Again, untreated bacterial cells were used as the control.

2.11 Toxicity Determination in the Vivo-Model

This method section was approved by the animal ethics committee of Khon Kaen University, Khon Kaen, Thailand (NELAC 30/2557). Mice of both sexes were randomly distributed into control and testing groups of 5 animals per cage. In testing groups, the combination of cLE and hLE was administered daily by gavage at 5 × ΣFIC (9.37 mg/kg of body weight), while mice in control groups were supplied with normal saline solution for 30 days. Behavioral abnormalities and other signs of toxicity in the animals were recorded every day after administration and changes of body weight were checked once every week. At the end of the experiment, the animals were anaesthetized and blood samples were taken for determination of serum creatinine, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. Furthermore, mice livers and kidneys were collected for histopathological studies using the haematoxylin-eosin technique. All determinations were carried out by the Veterinary Diagnostic Laboratory, Faculty of Veterinary Medicine, Khon Kaen University.

2.12 Statistical Analysis

The differences between control and testing groups were determined using the statistical software Statistix ver. 8.0. Comparisons among different groups were performed by analysis of variance using ANOVA test. Significant difference calculations between control and testing groups were carried out via the Student's *t*-test, and the significance of all values was considered at *P*-values less than 0.05 ($*P < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Crocodile Leukocyte Extract and Hen Egg Lysozyme

The molecular size of proteins in both cLE and hEL was determined by tricine SDS-PAGE (16.5% separating gel). As shown in Figure 1, the profiles of cLE on the gel comprised several proteins of varying size and many peptides with a molecular weight of less than 10 kDa. The gel profile of hEL demonstrated further that around 40-50% of lysozyme remains in the supernatant after precipitation by 40% ethanol. Additionally, the specific lytic activity of hEL was determined as 3,909 U/mg. These results indicate directly that hEL retained its potential to kill bacteria after partial purification.

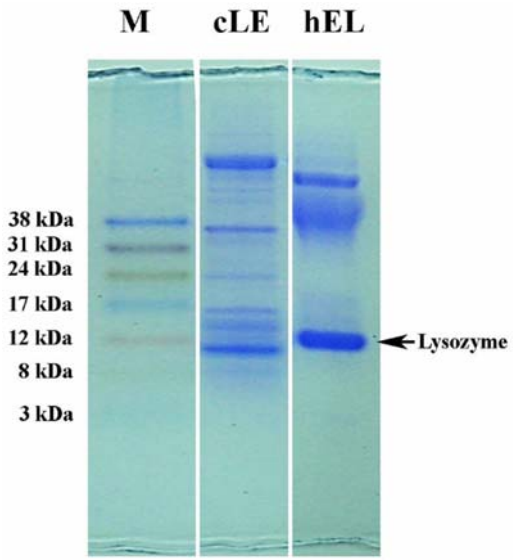

Figure 1. Protein profiles of crocodile leukocyte extract (cLE) and hen egg lysozyme (hEL) determined by tricine SDS-PAGE (16.5 %). Lane M is the standard protein marker; Lane cLE is *Crocodylus siamensis* leukocyte extract; Lane hEL is hen egg lysozyme.

3.2 Antimicrobial Efficacy of a Combination of Crocodile Leukocyte Extract and Hen Egg Lysozyme

The antibacterial activity of cLE and hEL and their synergistic effects were determined (Table 1). First, the MIC values of cLE and hEL were evaluated *in vitro* against Gram-positive *B. pumilus* TISTR 905 and Gram-negative *V. cholerae* (clinical isolation) using the liquid growth inhibition technique. The respective MICs of cLE for *B. pumilus* and *V. cholerae* were determined as 64 µg/ml and 32 µg/ml, while the values for hEL were 16 µg/ml and 2 µg/ml, respectively. To evaluate the synergistic effects of cLE and hEL, the checkerboard assay was employed. The fractional inhibitory concentration (FIC) index of both active compounds was utilized to assess the degree of synergism (total synergism, $\Sigma FIC \leq 0.5$; partial synergism, $0.5 < \Sigma FIC \leq 0.75$; no effect, $0.75 < \Sigma FIC \leq 2$; and antagonism, $\Sigma FIC > 2$). Synergistic effects were investigated in both Gram-strains of bacteria. As shown in Table 1, partial synergism (at $\Sigma FIC = 0.75$) against *V. cholerae* was found between cLE and hEL and their concentration had decreased to 16 µg/ml (0.5 × MIC) and 0.5 µg/ml (0.25 × MIC), respectively. However, no synergism was observed between cLE and hLE against *B. pumilus*. According to the different synergistic activities, these results suggest that both cLE and hEL in the combination may prefer in interacting and acting on Gram-negative bacterial surface more than those on Gram-positive bacterial surface which its cell wall is very rigid and less elastic as it has high proportion of peptidoglycan. Furthermore, our findings are in good agreement with the results of Zdybicka-Barabas et al. (2012), who reported that lysozyme and the anionic defense peptide (GMAP2) from the hemolymph of the greater wax moth *G. mellonella* displays synergistic activity in inhibiting the growth of bacteria, and their combination was specifically active against Gram-negative bacteria [14]. Furthermore, Chung and Hancock (2000) found that lysozyme and nisin appeared to exhibit synergy against Gram-positive bacteria, while their combination had a greater potential to enhance the rapid permeabilization (depolarization) of cytoplasmic membranes and to cause more severe damage to bacterial cells [12]. In both cases of the research, although lysozyme was used as the active substance in the combinations, the antimicrobial efficacy of these combinations was different in killing between Gram-negative and Gram-positive bacteria. These evidences suggest that lysozyme used in the combinations may not be the key factor promoting specific activity against either Gram-negative or Gram-positive bacteria, but the specific property may be given by

another active substance in each combination. In this study, therefore, we suggest that cLE in the combination may play the key factor in leading to the specific synergy on Gram-negative bacteria.

Table 1. MICs and ΣFIC of *Crocodylus siamensis* leukocyte extract (cLE), hen egg lysozyme (hEL) when used either alone or in combination against *B. pumilus* (TISTR 905) and *V. cholerae* (clinical isolation).

Bacteria	MICs (µg/ml)				FICs (µg/ml)		ΣFICs
	Alone		In combination		In combination		
	cLE (A)	hEL (B)	cLE (A)	hEL (B)	FIC_A	FIC_B	
B. pumilus	64	16	64	16	1.0	1.0	2.0
V. cholerae	32	2	16	0.5	0.5	0.25	0.75

ΣFIC is ≤ 0.5 denoting synergism; 0.5 < ΣFIC ≤ 0.75 denoting partial synergism; 0.75 < ΣFIC ≤ 2 denoting no effect; ΣFIC is > 2 denoting antagonism.

In addition, the lysozyme activity of cLE was determined by bioautography using commercial hen egg lysozyme (chEL) for comparison. The bioautographic technique was also used to estimate the molecular size of active substances in each sample. As shown in Figure 2, the SDS-PAGE image comprises a protein band for cLE near 15 kDa, which correlates excellently with the lysozyme band of chEL. A subsequent antibacterial assay on 0.7% nutrient agar featured a chEL band due *V. cholerae* inhibition at exactly the same position, while the activity of cLE is apparently derived from a protein band near 21 kDa, instead. From the previous study, Pata et al. (2007) reported that two protein bands of cLE at 21 and 15 kDa produced clear zones on zymogram refolding gel due to growth inhibition of Gram-positive *Micrococcus lysodeikticus*. Subsequent western immune blotting with anti-hen lysozyme then showed a reaction only for the 15 kDa protein band [25]. However, further elucidation of the antimicrobial activity of cLE in this work by bioautography and comparison to (commercial lysozyme) chEL (C-type lysozyme, ~ 15 kDa) revealed that only one major antimicrobial protein band at approximately 21 kDa effectively inhibited Gram-negative *V. cholerae*.

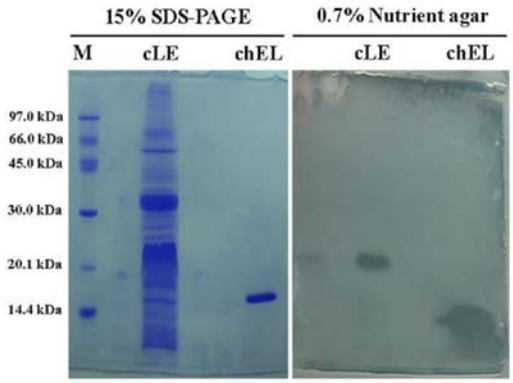

Figure 2. Bioautography of antimicrobial proteins from crocodile leukocyte extract (cLE) and hen egg lysozyme (hEL). Protein profiles of cLE and hEL were acquired by 15% SDS-PAGE. After re-naturation, the antimicrobial activity of the active proteins to inhibit *Vibrio cholerae* (clinical isolation) was detected on 0.7% nutrient agar. Lane M is the standard protein marker; Lane cLE is *Crocodylus siamensis* leukocyte extract; Lane chEL is commercial hen egg lysozyme.

3.3 Time-Killing Plot of the Protein Combination and Its Effects on Bacterial Cells

The cell growth for *V. cholerae* after exposure to antibacterial agents at different intervals is demonstrated in Figure 3. Untreated cells (control) experienced no reduction in viable cell count, grew steadily and at a greatly increased rate after 120 min. However, after treatment with 90.9 µg/ml ampicillin (Amp(90.9)), no significant change of cell growth could be observed. Interestingly, the protein combination, referred to cLE(16) + hEL(0.5), had a high potential to decrease the cell growth of *V. cholerae* by almost 100% within 60 min compared to the capability of 16 µg/ml cLE ((cLE(16)) or 0.5 µg/ml hEL ((hEL(0.5)) alone, which effected less than 50-60% reduction of bacterial growth at the same time. These results suggest that Amp, cLE and hEL may involve different modes of antimicrobial action on bacterial cells, and the antimicrobial capability of cLE does not originate from the lysozyme activity.

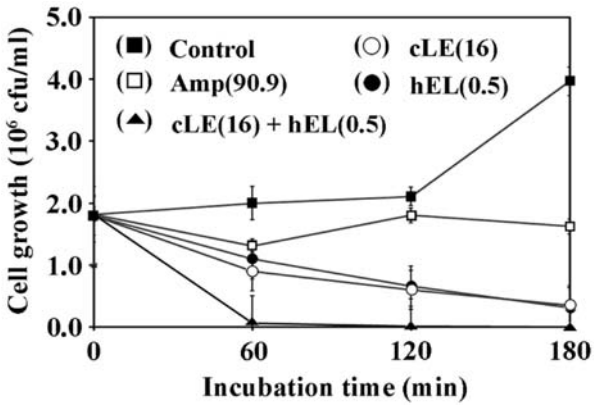

Figure 3. Time-killing curve of *Vibrio cholerae* (clinical isolation) after exposure to crocodile leukocyte extract (cLE), hen egg lysozyme (hEL) either alone or in combination. Control is agents free; Amp(90.9) is ampicillin at 90.9 µg/ml; cLE(16) is *Crocodylus siamensis* leukocyte extract at 16 µg/ml; hEL(0.5) is hen egg lysozyme at 2 µg/ml; cLE(16) + hEL(0.5) is *Crocodylus siamensis* leukocyte extract at 16 µg/ml plus hen egg lysozyme at 0.5 µg/ml. Data are expressed as mean ± standard deviation (n = 3).

In order to determine the effects of cLE and hEL either alone or in combination, morphological changes on *V. cholerae* cells were studied by SEM (Figure 4). Bacterial cells used as control were characterized by a smooth and intact surface (Figure 4a). After incubation with cLE at 5 × MIC, bacterial cells displayed heavy cell swelling at one side of the apical ends (Figure 4b). At 10 × MIC severe membrane damage, including cell shrinking, cell swelling, membrane blebbing, as well as leakage of cytoplasmic contents and micelles were observed (Figure 4c). In contrast, bacterial cells assumed a slightly abnormal shape in the middle region after treatment with hEL at 5 × MIC, without showing comparable magnitudes in cell swelling or membrane blebbing (Figure 4d). Conversely, hEL treatment at 10 × MIC caused an abnormal shape of bacterial cells and membrane shrinking or collapse (Figure 4e). Treatment of bacterial cells with the combination of cLE and hEL induced the same effects of

both cLE and hEL alone (Figure 4f, g). Therefore, these results confirm that cLE and hEL can affect the integrity of the bacterial surface, although the killing mechanism of cLE is presumed to differ from that of hEL, which is known to exhibit its antibacterial action through cell wall lysis. Moreover, from these results, we also suggested that cLE in the combination of cLE and hEL is likely to effect a permeability increase of the outer bacterial cell membrane, thus facilitating the binding of hEL to the peptidoglycan layer and resulting in faster bacteria killing. Furthermore, the occurrence of micelle-like membranes on and around the cells after treatment with cLE and the combination suggests that cLE may elicit its antimicrobial activity on the bacterial membrane via the so-called carpet mode. This model proposed by Pouny et al. (1992) postulates that antimicrobial peptides will aggregate onto the membrane surface while keeping a parallel alignment to the membrane during the process. Then, the antimicrobial peptides bind with the bacterial membrane via their hydrophobic side with the hydrophilic part facing towards the exterior, until clusters of antimicrobial peptides eventually coat the entire bacterial membrane in a carpet-like fashion. As the concentration of antimicrobial peptides increases, the membrane is weakened and the antimicrobial peptides are able to intercalate into the membrane via a detergent-like mechanism. As a result, the membrane is destabilized and broken up into micelles [26]. In this context, the potential to increase cell membrane permeability strongly suggest that the killing mechanism of the cLE-hEL combination may be traced back to the synergy between the carpet mode induced by cLE and the lytic activity of hEL.

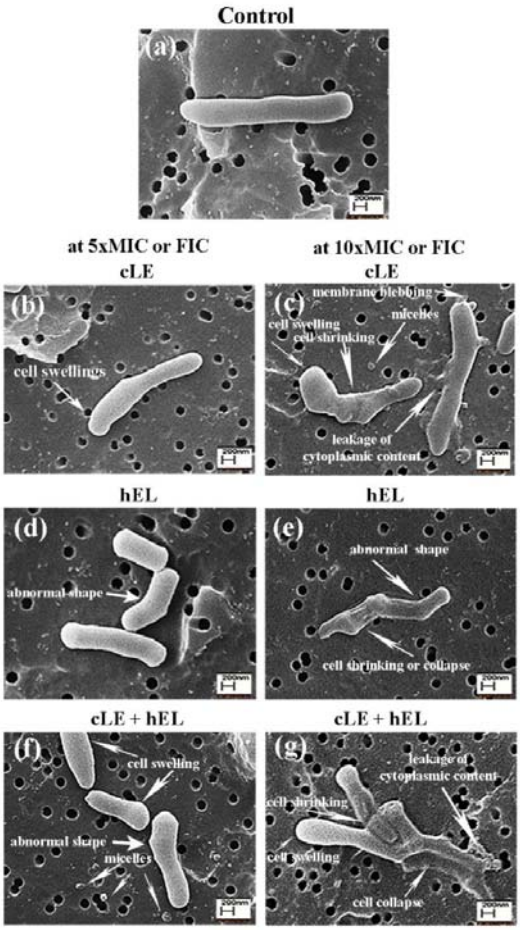

Figure 4. Scanning electron microscopy images of *Vibrio cholerae* cells after treatment with crocodile leukocyte extract (cLE), hen egg lysozyme (hEL) either alone or in combination. The bacteria were incubated without (**a**; control) or in the presence of crocodile leukocyte extract (cLE) (**b** is 5 × MIC and **c** is 10 × MIC), hen egg lysozyme (**d** and **e**; 5 × MIC & 10 × MIC) or the combination of both proteins (cLE + hEL) (**f** and **g**; 5 × ΣFIC & 10 × ΣFIC). Single bar = 200 nm (magnification 15,000 ×).

3.4 Effect of the Protein Combination on Mice

None of the ICR mice showed any behavioral changes or signs indicating toxicity, and no deaths were observed throughout or at the end of the 30-day period of study. The growth rate of the animals was determined via the increase of body weight between control groups and testing groups once every week (Figure 5). As shown in the figure, the mean weights of animals that received the combination of cLE and hEL did not show statistically significant differences compared with animals in the control group at same time intervals. Therefore, it seems likely that the administered combination of cLE and hEL is essentially non-toxic to the animals at the five-fold lethal concentration to bacteria.

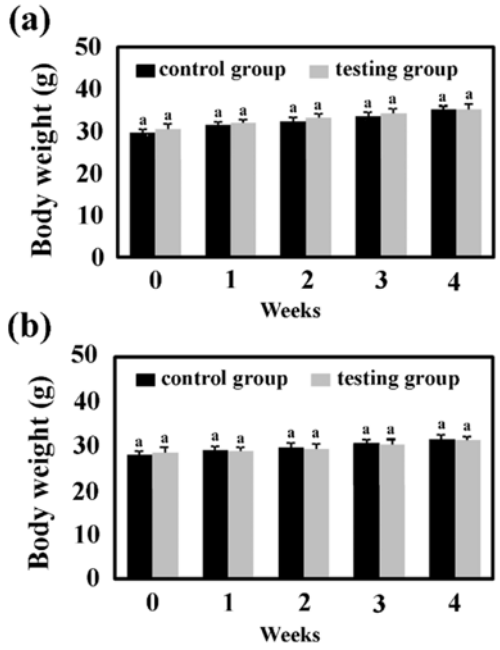

Figure 5. Body weights of ICR mice in control groups and testing groups (n = 5 per group) receiving the combination of crocodile leukocyte extract (cLE) and hen egg lysozyme (hEL) by oral administration for 4 weeks. Panel (**a**): male; Panel (**b**): female. Data are expressed as mean ± standard deviation; *$P > 0.05$.

To further investigate the toxicity profile of the cLE-hEL combination, several biochemical parameters, including creatinine, AST and ALT levels were determined in mice serum (Figure 6). In male mice, no significant changes of parameter levels between control group and testing group ($P > 0.005$) could be detected. Serum creatinine, AST and ALT of male mice in both groups ranged between 0.10-0.12 mg%, 170.5-178.5 U/l and 37.6-39.3 U/l, respectively. In female mice, serum creatinine, AST and ALT levels in both groups did not differ significantly ($P > 0.005$) and ranged from 0.12-0.14 mg%, 181.8-182.6 U/l and 39.0-44.8 U/l, respectively. In the vivo-toxicity tests, aspartate transaminase (AST) and alanine transaminase (ALT) represent biomarker enzymes commonly used for predicting possible toxicity in liver tissue [27]. When the tissue is damaged or injured, elevated quantities of both enzymes are secreted into the blood stream. Our results did not indicate any differences between enzyme serum levels (~175 U/l for AST and ~ 40 U/l for ALT) in control and testing mice groups. These findings are in good correlation with AST and ALT levels reported previously for rodents. A study of the effects of aqueous extracts of the *Herniaria glabra* plant on rats by Rhiouani et al. (2008) documented AST and ALT levels in the control group of ~135 U/l and ~40 U/l, respectively [28]. Furthermore, Liu et al. (2011), investigating effects of mesoporous hollow silica nanoparticles (MHSNs) on rats, showed that ICR mice in the control group had AST and ALT levels of ~180 U/l and 50 U/l, respectively [29]. Similar to AST and ALT for liver toxicity, creatinine serves as an important indicator molecule to assess toxicity in kidneys. Likewise, creatinine levels increase greatly when kidney tissues are damaged by toxic substances. In the present

study, similar creatinine levels at about ~0.12 mg% were determined for all groups including both sexes. This is in accordance to findings of Lasagna-Reeves et al. (2010), who reported creatinine levels ~0.24 mg% for the control group in the investigation of effects of gold nanoparticles with the different doses (40, 200 and 400 µg/kg/day) [30].

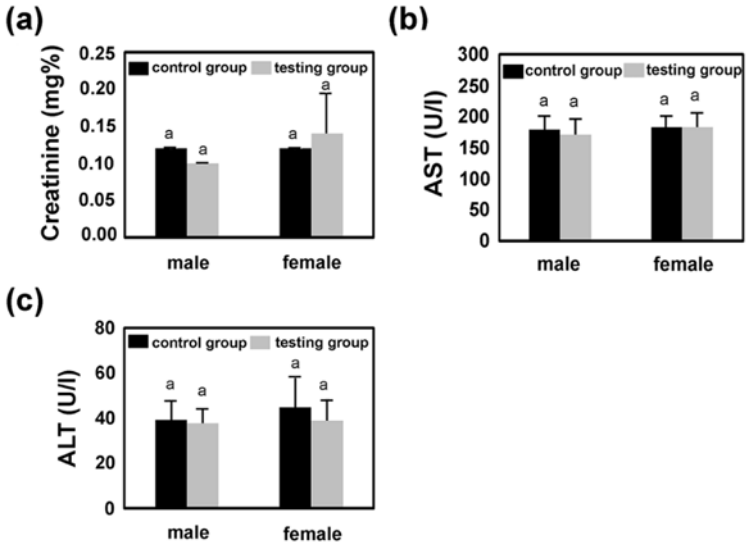

Figure 6. Effect of oral administration of the combination of crocodile leukocyte extract (cLE) and hen egg lysozyme (hEL) on selected biochemical parameters of ICR mice. The combination was given daily by the oral route to testing groups of both sexes of ICR mice (n = 5 per group) for 30 days. Biochemical parameters were measured in mice serum between control groups and testing groups of both sexes after 30 days. Panel (**a**): creatinine; Panel (**b**): aspartate aminotransferase (AST); Panel (**c**): alanine aminotransferase (ALT). Data are expressed as mean ± standard deviation; $^{*}P > 0.05$.

3.5 Effect of the Protein Combination on the Morphology of Mice Livers and Kidneys

In addition to biochemical toxicity determination, a histopathological examination of mice livers and kidneys was conducted to assess potential harmful effects of cLE-hEL combination administration to mice. Figure 7 displays photomicrographs of liver (Figure 7a-d) and kidney specimen (Figure 7e-h); scale enlargement: × 40. Histopathological examination of livers and kidneys of the testing groups (both sexes) showed normal structures without significant differences from the respective control groups. Therefore, it can be concluded that no harmful effects on the livers and kidneys of the animals was induced by administration of the combination of cLE and hEL.

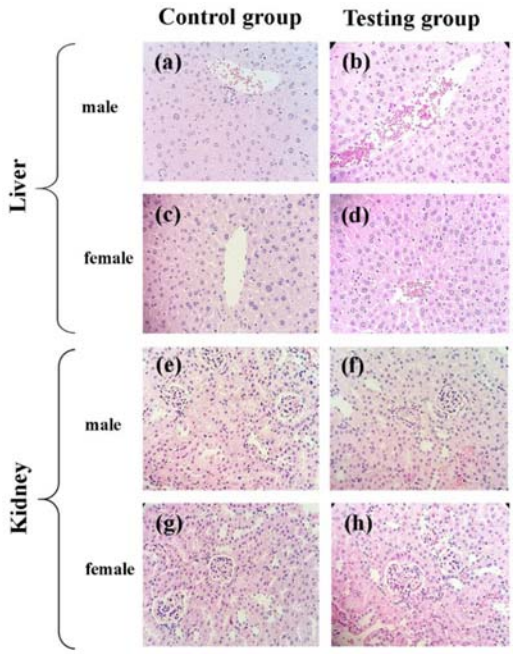

Figure 7. Histopathology of the liver and kidney tissues (× 40) in mice after administration of the combination of crocodile leukocyte extract (cLE) and hen egg lysozyme (hEL) post-exposure for 30 days.

4. CONCLUSIONS

In summary, the presented study provides the first report concerning the mechanism of synergistic action of a cLE-hEL combination against foodborne *V. cholerae*. By SEM, the cell surface of bacteria is identified as a crucial target for the synergistic antibacterial activity by combining cell membrane permeability and cell wall lysis. Moreover, in *in-vivo* toxicity tests, the cLE-hEL combination was not found to be toxic to mice and not affect mice liver and kidney functions via the determination of the levels of blood chemicals (creatinine, AST and ALT) and the tissue histopathology. Consequently, it is anticipated that this combination of antimicrobial agents might be of great benefit for the development of novel food supplements or therapeutic agents of natural origin.

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