Ethanol mediated anti-microbial peptide, cathelicidin/LL-37, 
perturbation in a human pulmonary epithelial cell line

by

Phanuwat Sriyotha¹*

Taylor R. Burns¹

Michael L. McCaskill¹

*Correspondence: psriyoth@tulane.edu
¹Department of Global Environmental Health Sciences, Tulane University School of Public Health and Tropical Medicine, 1440 Canal St. Ste 2100, New Orleans, LA 70112. USA
Abstract

Background: Chronic over-consumption of ethanol increases the prevalence and severity of respiratory infections in human. Increased infection risk to pulmonary tissue by chronic ethanol exposure is partially mediated by reduced levels of vitamin D and the antimicrobial peptide, cathelicidin/LL-37.

Methods: Immortalized pulmonary epithelial cells (Nuli-1) were cultured for three different experiments: CYP2E1 and CYP27B1 quantification, LL-37 quantification and LL-37 localization. For CYP2E1 and CYP27B1 quantification, confluent Nuli-1 cells were treated with +/- 70 mM ethanol, +/- 20 µg/ml Poly (I:C) and +/- 10 µM diallyldisulfide (DADS) for 12 hours. While, LL-37 quantification and localization had two consecutive exposures – chronic-binge exposure (+/- 50 mM ethanol, 72 hours) followed by acute exposure (+/- 70 mM ethanol, +/- 20 µg/ml Poly (I:C)
and +/- 10 µM DADS). After completion of treatments, cells were harvested and analyzed for CYP2E1, CYP27B1 and LL-37 and immunostained to observe using a laser confocal microscope.

**Results:** Excessive exposure to ethanol significantly induced CYP2E1 levels, which might create CYP2E1-mediated reactive oxidative metabolites. Theses toxic metabolites could perturb other metabolism, including that of vitamin D conversion as noticeable reduction of vitamin D converting enzyme CYP27B1 observed in the ethanol treated groups. Excessive exposure to ethanol can also dysregulate the immune response in pulmonary epithelia via perturbation of vitamin D metabolism. Levels of cathelicidin/LL-37 were reduced in ethanol treated groups. This LL-37 reduction was also observed to be comparable in confocal microscopy. However, the impairment of level of CYP27B1 and LL-37 was reversed by treatment with DADS.
Conclusions: Thus, ethanol plays an important role in cathelicidin/LL-37 dysregulation via perturbation of vitamin D metabolism. The CYP2E1 inhibitor, DADS, attenuated effects of ethanol on cathelicidin/LL-37 levels.

**Keywords**

Chronic over-consumption of ethanol, vitamin D perturbation, cathelicidin/LL-37

**Background**

According to the United States Centers for Disease Control and Prevention (CDC), excessive alcohol uses caused over 88,000 population deaths and approximately 2.5 million years of potential life lost (YLL) during 2006-2010. Besides acute causes of death and YLL, alcohol consumption can chronically impair numerous organ and systems, such as cardiovascular,
neurological, hepatic and pulmonary system [1]. People who excessively consume alcohol frequently develop respiratory tracts infection, asthma, pneumonia and chronic obstructive pulmonary disease (COPD) [2]. Chronic exposure to alcohol interferes with metabolism of essential vitamins including vitamin C, D and E, which may or may not consequently have detrimental effects on pulmonary defenses [2-5].

Cathelicidin/LL-37 is an endogenous antimicrobial peptide which plays an essential role in innate defense in mammalian species [6, 7]. Cathelicidin/LL-37 has broad spectrum antimicrobial activity in various tissues and immune responsive cells, such as the epithelia of respiratory system and neutrophils and monocytes respectively [6-9]. Biosynthesis and activation of cathelicidin molecules varies, depending upon type and function of tissue. Cathelicidin expression can be externally triggered by multiple factors, such as infection, inflammation, injury, and UV
radiation, and it is regulated by CAMP gene [7, 10, 11, 12]. Furthermore, the regulation of CAMP gene has a proven association with vitamin D [12, 13].

Vitamin D is a fat-soluble molecule produced endogenously when ultraviolet B (UVB) contacts with human dermal layers and 7-Dehydrocholesterol in skin is converted to vitamin D₃. Vitamin D₃ is then combined with vitamin D-binding protein (DBP) and released into circulatory system [14]. The essential metabolic conversion occurs in liver where vitamin D₃ is bio-transformed into 25-hydroxyvitamin D (25OHD₃) or calcidiol [14, 15]. Calcidiol is an inactive form of vitamin D. Thus, it requires further activation or the second conversion to 1,25-dihydroxyvitamin D (1,25(OH)₂D₃) or calcitriol by catabolizing enzyme CYP27B1 [16]. It had been proved that this conversion occurs not only in the kidneys, but it is also found in different tissues: monocyte, macrophages, and bronchial epithelial cells [6,
Calcitriol or active form of vitamin D can pass through plasma membrane and ligand-activate vitamin D receptors (VDR). The receptors bind VDREs, which are specific DNA sequences. VDREs are found in various locations and regulate hundreds of genes in cell-specific fashion [14]. Since the CAMP gene contain VDREs, this gene is therefore regulated by calcitriol [11, 17]. For example, in viral infection, cathelicidin production is upregulated via toll-like receptor (TLR) activation, which activated CYP27B1 expression and subsequently calcitriol conversion [18]. Alcohol is one of agents that dysregulates calcitriol conversion via CYP27B1 metabolism, leading to reduced expression of VDR targets such as cathelicidin [4, 19].

Despite its adverse effects and carcinogenicity, alcohol is a legacy drug of abuse, and alcohol consumption has been increasingly popular in all ages, races and socioeconomic levels [20]. This in vitro study is significant by aiming to model the
effect of ethanol on the pulmonary epithelial response via vitamin D dysregulation.

**Methods**

**CYP2E1 and CYP27B1 Quantification**

*Cell culture and treatment* – E6/E7 and hTERT Immortalized human epithelial cells (Nuli-1, ATCC CRP-4011) were grown in 25-cm$^2$ culture flasks in Airway Epithelial Cell Basal Medium (ATCC-PCS-300-030) supplemented with Bronchial Epithelial Cell Growth Kit (ATCC-PCS-300-040) under recommended conditions (37 °C and 5% CO$_2$). Cells were split for two different experiments: quantifying level of enzyme CYP2E1 and CYP27B1. For both experiments, the cells were cultured in 25 cm$^2$ culture flasks at density $0.7 \times 10^6$ cells per flask. When the cultured Nuli-1 cells reached 80% confluency, they were given 12-hour acute treatment with these following compounds in
different treatment groups: +/- 70 mM ethanol, +/- 20 µg/ml Polyinosinic-polycytidylic acid sodium salt (Poly (I:C)) and +/- 10 µM diallyldisulfide (DADS). After completion of treatment, cells were scraped, resuspended in 1 ml PBS and stored in micro-centrifuge tubes. The cell samples, then, were lysed by ultrasonication.

*ELISA for CYP2E1 and CYP27B1 quantification* - the cell samples stored in micro-centrifuge tubes were measured for levels of human enzyme CYP27B1 using a commercial ELISA kit (LSBio, F11191), while levels of human enzyme CYP2E1 used the LSBio kit F9041. Moreover, the levels of both enzymes quantified by ELISA assay for each sample were normalized by level of total amount of protein, which quantified by microfluidic content reader (QIAxpert instrument).

**LL-37 Quantification and Localization Experiment**
Cell culture – E6/E7 and hTERT immortalized human epithelial cells (Nuli-1, ATCC CRP-4011) were grown in 25-cm² culture flasks in Airway Epithelial Cell Basal Medium (ATCC-PCS-300-030) supplemented with Bronchial Epithelial Cell Growth Kit (ATCC-PCS-300-040) and 50 ng/ml 25-hydroxycholecalciferol or calcidiol dissolved in 0.1% DMSO, a vehicle control. The culturing process were maintained under recommended conditions (37 °C and 5% CO₂). Cells were split into two flasks for two different experiments: quantifying level of antimicrobial peptide, cathelicidin/LL-37 and localization using an immunofluorescence confocal microscope. Cells for the cathelicidin/LL-37 quantification were cultured in 25 cm² culture flasks at density 0.7x10⁶ cells per flask, while cells prepared for a confocal analysis were grown in 6-well glass bottomed cell culture plates, with a cover glass thickness 0.15 mm, at density 0.3x10⁶ cells per well.
Cell treatment – after Nuli-1 cells were plated in designated plates, they were sustained in recommended culturing media and condition for 72 hours. For both experiments, cells were categorized into two treatment groups: chronic-binge exposure to ethanol and control. The chronic-binge exposure group was pre-treated with 50 mM ethanol, while the control group remained in culture medium alone. After 60 hours of pre-treatment, all groups were given a respective acute treatment for 12 hours with all these following compounds in different treatment groups: +/- 70 mM ethanol, +/- 20 µg/ml (Poly (I:C)) and +/- 10 µM DADS.

Cell harvesting – upon completion of treatment, treated cells in 25-cm² flasks were scraped and resuspended in 1 ml phosphate buffered saline (PBS) and stored in micro-centrifuge tubes. To agitate and lyse cells structures, the samples were disrupted using ultrasonic frequency with cooling to avoid protein degradation.
ELISA for LL-37 quantification – the cell samples stored in micro-centrifuge tubes were measured for levels of antimicrobial peptide, cathelicidin/LL-37 using a commercial ELISA kit (Hycult Biotech – HK321). Moreover, the levels of cathelicidin/LL-37 quantified by ELISA assay for each sample were normalized by level of total amount of protein, which quantified by microfluidic content reader (QIAxpert instrument).

Immunofluorescence staining and confocal microscopy for localization of LL-37 – upon completion of treatment, treated cells in the 6-well plated were fixed with 4% formalin solution and incubated 10-15 minutes at 37 °C. To bind glycoprotein of plasma membrane, the cells were stained with 5.0 µg/ml wheat germ agglutinin (WGA, Alexa Fluor 488) and incubated for 10 minutes at room temperature. Then the cells were overlaid with 0.1% Triton X-100 in PBS and incubated for 10 minutes at 37 °C for permeabilization. To reduce non-specific binding, the
blocking step was performed using 10% fetal bovine serum (FBS) dissolved in PBS and incubated for 30 minutes at room temperature after adding blocking solution. To tag cathelicidin/LL37, the cells were subsequently stained with 2% anti-cathelicidin (Alexa Fluor647) and incubated overnight at 4°C. Nuclear elements? which ones? in cell samples were also stained using 4’, 6-diamidino-2-phenylindole (DAPI, Alexa Fluor 350). All described immunofluorescence-staining process was performed in a dark room. In order to localize cathelicidin/LL-37 molecules, stained-cell slides were observed through Nikon A1 confocal microscope system.

**Results**

**Alcohol metabolizing enzyme CYP2E1 levels in Nuli-1 pulmonary epithelial cells**
CYP2E1 levels in Nuli-1 cells after treatment of EtOH and DADS were shown in Fig. 1. Ethanol considerably stimulated increasing CYP2E1 levels based on unexposed ethanol in control groups (Table 1). Increasing of CYP2E1 was noticeably attenuated by treatment of DADS according comparison between EtOH group and EtOH/DADS (Table 2).

**Table 1.** $t$-test results comparing CYP2E1 levels between Control and EtOH

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Mean</th>
<th>SEM</th>
<th>95% CI</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.5388</td>
<td>0.05382</td>
<td>1.156 to 1.644</td>
<td>&lt; 0.05</td>
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<tr>
<td>EtOH</td>
<td>1.939</td>
<td>0.01754</td>
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<td></td>
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</table>

**Table 2.** $t$-test results comparing CYP2E1 levels between EtOH and EtOH/DADS

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Mean</th>
<th>SEM</th>
<th>95% CI</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>1.939</td>
<td>0.01754</td>
<td>-1.606 to -1.387</td>
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<td>EtOH/DADS</td>
<td>0.4421</td>
<td>0.0184</td>
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</table>
**Vitamin D activating enzyme CYP27B1 level in Nuli-1 pulmonary epithelial cells**

CYP2E1 levels in Nuli-1 cells after treatment of EtOH and DADS were shown in Fig. 2 and Fig 3. Ethanol exposure significantly reduced level of CYP27B1 based on normal level of this enzyme in the control group (Table 3). Reduction of CYP27B1 level in ethanol exposed group was reversed nearly back to normal as observed in significantly higher CYP27B1 in EtOH/DADS (Table 4).

**Table 3. t-test Results Comparing CYP27B1 Levels between Control and EtOH**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Mean</th>
<th>SEM</th>
<th>95% CI</th>
<th>p-value</th>
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<tr>
<td>Control</td>
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<td>EtOH</td>
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<td>0.0185</td>
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<td></td>
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</table>

**Table 4. t-test Results Comparing CYP27B1 Levels between EtOH and EtOH/DADS**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Mean</th>
<th>SEM</th>
<th>95% CI</th>
<th>p-value</th>
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</table>
Anti-microbial peptide, cathelicidin/LL-37 in Nuli-1 pulmonary epithelial cells

Cathelicidin/LL-37 levels in Nuli-1 cells after treatment of EtOH, DADS and Poly (I:C) were shown in Fig. 2. Exposure to ethanol in chronic-binge and acute manner noticeably reduced cathelicidin/LL-37 (Table 5). Chronic-binge exposure to ethanol more significantly attenuated level of cathelicidin/LL-37 than the exposure in acute fashion, in spite of immune stimulation by Poly (I:C) (Table 6).

Table 5. Bonferroni’s Multiple Comparisons Test on Cathelicidin/LL-37 Levels among Ethanol-treated Groups

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Mean Difference</th>
<th>Standard Error of Difference</th>
<th>95% CI of Difference</th>
<th>Adjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>0.0325</td>
<td>0.0185</td>
<td>0.1364 to 0.3616</td>
<td>&lt; 0.05</td>
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<td>EtOH/DADS</td>
<td>0.2815</td>
<td>0.0185</td>
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<tr>
<td>Experiment Group</td>
<td>Mean</td>
<td>SEM</td>
<td>95% CI</td>
<td>p-value</td>
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<td>------------------</td>
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</tr>
<tr>
<td>72hr Ctrl+12hr</td>
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<td>-13.41 to -0.4457</td>
<td>0.04</td>
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<tr>
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<td>10.28</td>
<td>1.334</td>
<td>3.801 to 16.76</td>
<td>0.01</td>
</tr>
<tr>
<td>EtOH+12 hr Ctrl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72hr Ctrl+12hr</td>
<td>17.21</td>
<td>1.334</td>
<td>10.73 to 23.69</td>
<td>&lt; 0.05</td>
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<tr>
<td>Ctrl vs.</td>
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<tr>
<td>72hr EtOH+12 hr</td>
<td>33.56</td>
<td>1.094</td>
<td>-45.43 to 34.47</td>
<td>0.61</td>
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<tr>
<td>Ctrl Pic</td>
<td></td>
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<tr>
<td>72hr Ctrl+12hr</td>
<td>39.04</td>
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<tr>
<td>EtOH/Pic</td>
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</table>

**Table 6.** *t*-test Results Comparing Cathelicidin/LL-37 Levels between Chronic-binge Alcohol Exposure and Acute Exposure, with Immune-stimulant Treatment

**Discussion**
Alcohol can be metabolized to acetaldehyde by three enzymes. Alcohol dehydrogenase(s) or ADH play a primary role in alcohol converting pathway; while microsomal cytochrome P450 enzyme CYP2E1 takes part in metabolizing activities when blood alcohol concentration increases – a secondary pathway for ethanol metabolism [21]. Since our study emphasized in chronic over-consumption of ethanol, the relevant metabolizing enzyme was CYP2E1. Based on a previous study, mean blood ethanol concentration (BAC) of alcohol users diagnosed with alcohol intoxication in the emergency room was above 65 mM and could be higher among alcoholic abusers [22]. This level, therefore, was used to justify level of ethanol treatments in our study, which were 50 mM and 70 mM for chronic-binge manner and acute exposure, respectively. As shown in our result, CYP2E1 levels was considerably increased by ethanol treatment (Fig. 1). CYP2E1 induction by ethanol can also generate reactive oxygen
species (ROS) causing toxicity or dysregulations of other metabolism, e.g. liver toxicity, nutrients and vitamin metabolisms [21]. However, this disturbance could be attenuated by the CYP2E1 inhibitor diallyl disulfide (DADS) as shown our experiment (Table 2). The protective role of DADS is also found other published studies. CYP2E1-mediated reactive oxidative metabolite; malondiadehyde (MDA) and MDA deoxyguanosine (M1dG) was observed to be significantly reduced by DADS treatment [23]. Moreover, McCaskill et al. found that dietary supplementation of DADS could reverse pulmonary metabolic disturbances in chronic ethanol treated mice [24].

The level of vitamin D activating enzyme CYP27B1 was reduced among ethanol treated groups when compared with the untreated controls (Table 3). This is probably due to adverse effects of excessive exposure to ethanol on the vitamin D metabolism. This phenomenon was also observed in other
published studies found that ethanol intake decreased renal CYP27b1 gene expression and this enzyme was reduced by approximately 60% in the upper broncho-alveolar lavage fluid (BALF) collected from chronic-alcoholic consumers [4, 5, 25].

Chronic alcohol exposure causes immune dysfunction through effects on multiple organs. In the lungs, persistent inflammation impedes immune defense via reduction of Toll-like receptor (TLR) responses and other proinflammatory cytokines [26]. Cathelicidin/LL-37 is antimicrobial peptide, which has an important role in the innate immune response to respiratory infection [9]. As observed in our experiment, cathelicidin/LL-37 responded to a surrogate molecule of mimicking viral infection (Poly (I:C)). This induction noticeably increased level of cathelicidicin/LL37 in Poly (I:C) treated groups when compared to non-Poly (I:C) matched groups or untreated controls [6]. Production of cathelicidin/LL-37 is associated with multiple
factors. The production is expressed only at low, constitutive levels without pathogen-induced elements, but it can be upregulated by vitamin D via activation of vitamin D receptor (VDR) located in promoter region of CAMP gene [11]. With this association, excessive alcohol exposure results in ethanol-mediated cathelicidin/LL-37 dysregulation. According to Fig.3 and Fig.4, cathelicidin/LL-37 levels were reduced in every ethanol-treated group with compared to their matched controls. These data provide evidence that chronic over-consumption of ethanol could attenuate pulmonary immune defense.

Our experiment has several limitations. First, this experiment was unable to include treatment of DADS into the LL-37 quantification. However, based on CYP2E1 and CYP27B1 levels, response of cathelicidin/LL-37 with treatment of DADS could be predicted. Secondly, our model was performed based on a single cell line (Nuli-1). This could result in over- or under-
estimate the response of the co-culturing model or intact organisms. Treatment with ethanol and other compounds mimicked human exposure by considering physiological and behavioral relevance. The actual responses in humans could be more or less different based on physiological, metabolic and polymorphism of individuals. Finally, since alcohol is a volatile compound, it can evaporate from the cell culture plates and ethanol levels in the medium are consequently reduced. However, the declining of alcohol levels *in vitro* can be mimic the alcohol elimination observed in *in vivo*.

**Conclusions**

In summary, chronic over-consumption of ethanol can perturb vitamin D conversion and can further lead to reduction of immune response (cathelicidin/LL-37) of pulmonary epithelia. However,
this adverse effect can be reversed by treatment of CYP2E1 inhibitor, DADS.

**Competing interests**

The authors declare that they have no competing interests.

**Authors contributions**

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**Acknowledgements**

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**References**

   Alcohol related disease impact (ADRI) application.


9. Patricia M´endez-Samperio, Elena Miranda, Artemisa Trejo Departamento de Inmunolog´ia, Escuela Nacional de


antimicrobial peptide gene by 1α,25-dihydroxyvitamin D3 in primary immune cells
doi://dx.doi.org/10.1016/j.jsbmb.2014.02.004


CYP2E1 levels in Nuli-1 cells treated with EtOH and DADS

**Fig. 1** Represents levels of CYP2E1 enzyme in Nuli-1 cells treated with 80 mM ethanol (EtOH) and 10 µM diallyl disulfide (DADS) for 12 hours. Exposure to ethanol induced highest level of CYP2E1. Levels of CYP2E1 in the other groups were significantly lower than ethanol exposed group and had relatively comparable levels among themselves.
CYP27B1 levels in Nuli-1 cells treated with EtOH and DADS

**Fig. 2** Represents levels of CYP27B1 enzyme in Nuli-1 cells treated with 80 mM ethanol (EtOH) and 10 µM diallyl disulfide (DADS) for 12 hours. Lowest level of CYP27B1 was observed in EtOH treated group. The CYP27B1 levels were found to be relatively similar among the other treatment groups.
**Cathelicidin/LL-37 Levels in Nuli-1 Cells treated with**

**EtOH, DADS and Poly (I:C)**

![Graph showing cathelicidin/LL-37 levels in Nuli-1 cells treated with EtOH, DADS and Poly (I:C).](image)

**Fig. 3** Represent levels of LL-37 in Nuli-1 cells treated with 50 mM ethanol (EtOH) for the first 72 hours followed by 70 mM EtOH, 10 µM diallyl disulfide (DADS) and 10 µg/ml Poly (I:C) for 12 hours. Both chronic-binge and acute exposure to ethanol reduced levels of cathelicidin/LL-37, but the chronic-binge exposure suppressed cathelicidin/LL-37 more significantly than the acute exposure.
Anti-microbial peptide, cathelicidin/LL-37 in Nuli-1 cells treated with EtOH, DADS and Poly (I:C)

Fig. 4 Anti-microbial peptide, cathelicidin/LL-37 (pink) was most fluorescent in Poly (I:C), followed by Control, EtOH/DADS and EtOH, respectively. Plasma membrane was stained with wheat germ agglutinin (green) and nucleus was stained in blue fluorescence of DAPI.