Archives of Microbiology (2020) 202:555–565
https://doi.org/10.1007/s00203-019-01771-4

ORIGINAL PAPER

An innovative role for tenoxicam as a quorum sensing inhibitor in *Pseudomonas aeruginosa*

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Received: 4 October 2019 / Revised: 30 October 2019 / Accepted: 7 November 2019 / Published online: 15 November 2019

Abstract

Pseudomonas aeruginosa is an opportunistic pathogen exhibiting higher resistance to commonly used antibiotics. Microbial resistance to antibiotics is a major problem that hinders attempts to control microbial infections. Quorum sensing inhibitors could help us solve such problem by repressing quorum sensing that controls the production of virulence factors in many pathogens including *P. aeruginosa*. In this study, the influence of tenoxicam, a non-steroidal anti-inflammatory drug, on quorum sensing in *P. aeruginosa* was characterized. Treatment of *P. aeruginosa* with tenoxicam decreased production of many virulence factors such as pyoverdin, rhamnolipids, pyocyanin, elastase, proteases, and hemolysins. Moreover, qRT-PCR revealed a significant reduction in expression of quorum sensing genes in tenoxicam-treated *P. aeruginosa* in comparison with untreated bacteria. Tenoxicam markedly reduced the capacity of *P. aeruginosa* to kill mice infection model. Mice injected with tenoxicam-treated *P. aeruginosa* exhibited higher survival rate as compared with those inoculated with untreated bacteria. Current data clearly demonstrate that tenoxicam has quorum sensing inhibitory effect on *P. aeruginosa.* Tenoxicam could play a role in reduction of *Pseudomonas* quorum sensing-dependant virulence factors production, and therefore affect its pathogenesis in the host. In summary, the current study suggests that tenoxicam could be used as adjuvant to antibiotics in the management of diseases caused by *P. aeruginosa*.

Keywords Tenoxicam · *Pseudomonas aeruginosa* · Quorum sensing inhibitors · Virulence

Introduction

Pseudomonas aeruginosa is a prevalent opportunistic human pathogen and considered a major cause of many hospital-acquired infections (Delden and Iglewski 1998). *P. aeruginosa* could give rise to serious diseases such as urinary tract infections (UTIs), bacteremia, burn-wound infections, and cystic fibrosis (Hidron et al. 2008; Elbossaty 2017). *P. aeruginosa* virulence potential is essentially attributed to secretion of various virulence determinants such as proteases, elastase, pyocyanin, pyoverdin, exotoxin A, rhamnolipids, and hemolysins (Veesenmeyer et al. 2009). Antibiotics abuse besides their improper usage resulted in a considerable increment in microbial resistance to the commonly prescribed antibiotics (Fischbach and Walsh 2009; Elbossaty 2017). Therefore, novel non-antibiotic therapies, as an alternative approach are substantially required to help in treating microbial infections (El-Mowafy et al. 2014).

Quorum sensing inhibitors (QSIs) are drugs that interfere with bacterial cells communication system known as quorum sensing (QS) which controls the expression of many virulence factors (Antunes et al. 2010; Rasko and Sperandio 2010; Marshall 2013). Quorum sensing in *P. aeruginosa* is controlled mainly by secretion of different autoinducers such as oxododecanoyl-homoserine lactone (C12-HSL), butyryl-homoserine lactone (C4-HSL), and *Pseudomonas* quinolone-based intracellular signal (PQS) (Pesci et al. 1999; Deep et al. 2011). Once the bacterial population reached a certain density (quorum), enough

Communicated by Erko Stackebrandt.

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amounts of autoinducers permeate in the medium, thus, the expression of virulence genes takes place (Pesci et al. 1999; Jimenez et al. 2012).

Numerous natural compounds were tested for their QS inhibitory activity, and some of them showed reasonable activity such as penicillic acid and patulin extracted from *Penicillium* species, and furanone compounds extracted from algae *Delisea pulchra* (Givskov et al. 1996; Rasmussen et al. 2005). Moreover, many chemically synthesized compounds showed potent QS inhibitory activity such as phenylpropionyl-homoserine lactones, phenyloxyacetyl-homoserine lactones, and halogenated furanones (Hentzer et al. 2002; Geske et al. 2008; Ni et al. 2009). Unluckily, the toxicity of many chemically synthesized QSIs hinders their use in humans. To overcome this problem, many FDA approved drugs are being evaluated for their potential QS inhibitory effect (El-Mowafy et al. 2014).

Tenoxicam is a member of non-steroidal anti-inflammatory drugs (NSAIDs) that are widely used as analgesics and anti-inflammatory. It has been proven that some NSAIDs, like aspirin and diclofenac sodium, exert a QS inhibitory activity in *P. aeruginosa* (El-Mowafy et al. 2014; Abbas 2015). Similarly, diflunisal, another NSAID, showed a promising inhibitory effect on virulence factors production in *Staphylococcus aureus* (Khodaverdian et al. 2013).

Based on the previously proved QS inhibitory effect of some NSAIDs in different pathogens, the present study aims to investigate the possible QS inhibitory effect of tenoxicam in *P. aeruginosa*. Current study could provide a valuable way to control the problem of antibiotic resistance in *P. aeruginosa*.

Materials and methods

Bacterial strains, culture conditions and identification

Five *P. aeruginosa* clinical isolates from UTI patients at Port Said General Hospital in addition to the standard strain *P. aeruginosa* PAO1 were included in the current study. For convenience, clinical isolates were referred as isolate P1–P5. Urine specimens were collected from clean-catch midstream urine in sterile jars. The specimens were streaked on MacConkey medium (Oxoid, UK) and incubated aerobically at 37 °C for 24 h. Non-lactose fermenting pale colonies from MacConkey were identified depending on standard microbiological technique for *P. aeruginosa* including Gram-staining, motility, pigment production on cetrimide agar (Oxoid, UK), growth at 42 °C, and citrate utilization test (Koneman et al. 2006).

Minimal inhibitory concentration determination and the effect of sub-inhibitory concentration of tenoxicam on bacterial growth

The minimal inhibitory concentration (MIC) of tenoxicam (Global Napi, Egypt) was determined using agar dilution method following the Clinical Laboratory and Standards Institute (CLSI) guidelines (2012). Briefly, overnight bacterial cultures of tested isolates were diluted in Mueller–Hinton (MH) broth (Lab M, UK) to achieve a final concentration of 10^7 CFU/ml which corresponds to 0.5 Mcfarland standard. Tenoxicam was added to sterile nutrient agar in Petri plates before agar hardening to reach final concentrations of 1.25, 2.5 5, 10, 20, 40 and 80 mg/ml. The plates' surfaces were inoculated with 1 µl of the tested isolates and incubated overnight at 37 °C. The MIC was considered as the lowest concentration of tenoxicam that prevented the apparent growth of bacteria. Furthermore, the viability of *P. aeruginosa* isolates was examined in the presence of sub-inhibitory concentrations of tenoxicam described by Nalca et al. (2006). Bacterial isolates with and without 1/4 MIC of tenoxicam were grown overnight in Luria–Bertani (LB) broth (Lab M, UK) at 37 °C. The optical densities (OD_{600}) representing growth of tenoxicam-treated and untreated isolates were measured using Biotek spectrofluorometer.

Characterization of the influence of tenoxicam on *P. aeruginosa* virulence factors production

Pyoverdin estimation

Pyoverdin determination was performed according to the method described by Gupta et al. (2011). The reduction in the fluorescence produced by pyoverdin was assessed following bacterial treatment with tenoxicam. Cultures of *Pseudomonas* in LB broth were grown overnight, in presence and absence of 1/4 MIC of tenoxicam, and then centrifuged at 10,000*g* for 15 min. The pyoverdin fluorescence in cell-free supernatants (diluted to 1/10 with 50 mM Tris–HCl and pH adjusted to 7.4) was measured at 460 nm while the samples were excited at 400 nm in Biotek spectrofluorometer.

Rhamnolipids determination

Oil spreading technique was implemented for rhamnolipids determination following the protocol by Kalyani et al. (2014). The ability of rhamnolipids to disperse oil by its surfactant activity was determined. Crude oil (20 µl) was added to 40 ml of distilled H_2O in Petri plates, to form a thin oil layer. Then 20 µl of cell-free supernatants of tested *Pseudomonas* isolates in presence and absence of 1/4 MIC

of tenoxicam were carefully dropped into the middle of the oil layer. The diameters of clear zones appeared on the water surface are directly proportional to the rhamnolipid amounts produced by tested isolates.

Pyocyanin estimation

The ability of tenoxicam to reduce *Pseudomonas* pyocyanin production was assessed by measuring the red color produced after treating pyocyanin in the culture supernatants with chloroform and HCl. Pyocyanin determination was performed according to Essar et al. (1990), using King A broth (peptone 2 g, MgCl2 0.14 g and 1 g K2SO4 in 100 ml distilled H2O). *Pseudomonas* isolates were grown in King A broth with and without 1/4 MIC of tenoxicam for 48 h at 37 °C. A volume of 2.5 ml of *Pseudomonas* culture supernantant was added to 3 ml chloroform followed by 1 ml of 0.2 N HCl to extract pyocyanin. The color of pyocyanin in chloroform layer was measured at 520 nm using spectrofluorometer.

Total proteases determination

Skim milk technique with modifications was utilized to determine the total proteolytic activity of tested isolates (Skindersoe et al. 2008). The test examines the proteolytic activity according to change in the turbidity of skim milk. Cell-free supernatants of *Pseudomonas* isolates (0.5 ml) in presence and absence of 1/4 MIC of tenoxicam were added to 1 ml of sterile skim milk solution (1.25 g skim milk in 100 ml distilled H_2O) and incubated at 37 °C for 1/2 h. The optical density of the mixtures was measured at OD_{600} which reflects the proteolytic activity of proteases.

Hemolysins estimation

To determine the inhibitory effect of tenoxicam on the hemolytic activity of *Pseudomonas* isolates, the protocols by Gupta et al. (2011) and Abbas (2015) were followed. The ability of tenoxicam to inhibit hemolysin activity was measured based on reducing hemoglobin release from erythrocytes following addition of bacterial cultures supernatants treated with tenoxicam. An aliquot of 1 ml of cell-free supernatants, in presence and absence of 1/4 MIC of tenoxicam, was added to 1 ml of 2% sheep RBCs in saline and incubated at 37 °C for 2 h. The absorbance of released hemoglobin was measured at OD_{540} nm after centrifugation at 5000*g* for 5 min. Both a negative control (*B*); RBCs in LB broth, and a positive control (*T*); totally lysed RBCs with SDS (0.1%) were prepared. The percentage of hemoglobin released due to erythrocytes lysis was calculated from the equation: $[X-B/T-B]\times 100$, where *X* is the OD values of lysed RBCs by tenoxicam-treated or untreated bacterial isolates. The hemolytic activity of tenoxicam-treated *Pseudomonas* isolates was compared to that of untreated isolates as a percentage.

Elastolytic activity estimation

The elastolytic activity of tenoxicam-treated and untreated *Pseudomonas* isolates was evaluated following Ohman et al. (1980) using Elastin Congo Red (ECR). Elastase produced by the bacteria can hydrolyse elastin congo red substrate and the released congo red dye can be estimated spectrophotometrically. Briefly, 0.5 ml ECR solution (10 mg/ml) (Sigma, USA) in Tris buffer (pH 7.0) was added to 0.25 ml cell-free supernatants of bacterial cultures, and then the mixtures were incubated at 37 °C for 3 h. The absorbance of released ECR in supernatants was measured at OD_{495} nm using spectrofluorometer.

RNA extraction and relative gene expression measurement using qRT-PCR

Pseudomonas isolates were grown overnight in LB broth at 37 °C in presence and absence of 1/4 MIC of tenoxicam. Bacterial RNA was extracted at the mid-log phase (OD_{600} 0.5–0.6) according to the manufacturer protocol using GeneJET RNA Purification Kit (Thermoscientific, USA). Reverse transcription followed by qRT-PCR of QS genes *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA*, and *pqsR* was performed using SensiFAST™ SYBR® Hi-ROX One-Step Kit (Bioline, UK). RT-qPCR analysis was set up via StepOne Real-Time PCR thermal cycler (Applied Biosystem, USA) using primers described in (Table 1). The QS genes relative expression values obtained were normalized to the housekeeping gene

Table 1 Primers used in RT-PCR (El-Mowafy et al. 2014)

Gene name	Primer sequence	Amplicon size (bp)
ropD (F)	5′-CGAACTGCTTGCCGACTT-3′	131
ropD (R)	5′-GCGAGAGCCTCAAGGATAC-3′	
lasI (F)	5′-CGCACATCTGGGAACTCA-3′	176
lasI (R)	5′-CGGCACGGATCATCATCT-3′	
lasR (F)	5′-CTGTGGATGCTCAAGGACTAC-3′	133
lasR (R)	5′-AACTGGTCTTGCCGATGG-3′	
rhlI (F)	5′-GTAGCGGGTTTGCGGATG-3′	101
rhlI (R)	5′-CGGCATCAGGTCTTCATCG-3′	
rhlR (F)	5′-GCCAGCGTCTTGTTCGG-3′	160
rhlR (R)	5′-CGGTCTGCCTGAGCCATC-3′	
pqsA (F)	5′-GACCGGCTGTATTCGATTC-3′	74
pqsA (R)	5′-GCTGAACCAGGGAAAGAAC-3′	
pqsR (F)	5′-CTGATCTGCCGGTAATTGG-3′	142
pqsR (R)	5′-ATCGACGAGGAACTGAAGA-3′	

F forward, *R* reverse

rpoD. Specific PCR amplification was confirmed by agarose gel electrophoresis. The relative gene expression in tenoxicam-treated isolates was compared to their expression levels in the untreated cultures according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Mice survival test

The effect of tenoxicam on *P. aeruginosa* pathogenesis was investigated using in vivo mice survival experiment following the protocol by Kim et al. (2015) with modifications. The animal study was performed according to the ethical standards of Medical Research Center, Ain Shams University, Cairo, Egypt. Briefly, phosphate-buffered saline (PBS) containing approximately (2.5×10^7 CFU/ml) of *P. aeruginosa* PAO1 was prepared from overnight bacterial cultures in LB broth in presence and absence of 1/4 MIC of tenoxicam. Three-weeks-old healthy female albino mice (*Mus musculus*) of equal weight were divided randomly into four groups, 10 mice each. In group 1, bacterial infection was initiated by inoculating mice intraperitoneally with 100 µl of PBS containing tenoxicam-treated bacteria, while group 2 was inoculated with 100 µl of PBS containing untreated bacteria. Two negative control groups were included; mice in group 3 were inoculated with 100 µl of sterile PBS while mice in group 4 were left uninoculated. All groups were kept with normal aeration, and feeding at room temperature. Mice survival in each group was monitored every day for 3 successive days, plotted using Kaplan–Meier method and calculated using Log-rank test, GraphPad Prism 5 at $P < 0.001$ for significance.

Statistical analysis

The influence of tenoxicam on *P. aeruginosa* QS-dependant virulence factors production was analyzed using GraphPad Prism 5 software package with One Way ANOVA according to Dunnet's or Tukey's Multiple Comparison Tests at $P < 0.05$ and $P < 0.001$ for significance. Results were calculated as the means ± standard errors of three biological experiments with three technical replicates each.

Results

Clinical isolates identification

Clinical isolates were confirmed as *P. aeruginosa* as they were Gram-negative short rods, motile, able to grow at 42 °C with green pigmentation on cetrimide agar, and gave positive citrate utilization test.

Determination of MIC and growth inhibition activity of tenoxicam

First, the MIC of tenoxicam against tested *Pseudomonas* isolates was determined and was found to be 20 mg/ml. Next, the QSI effect of tenoxicam and virulence factors inhibition in *P. aeruginosa* was estimated using a concentration of 1/4 MIC (5 mg/ml), as other sub-inhibitory concentrations (2.5 and 1.25 mg/ml) did not exhibit any inhibitory effect on virulence factors production phenotypically. The inhibitory effect on *Pseudomonas* isolates virulence factors secretion by tenoxicam may be attributed to a bactericidal action of tenoxicam on bacteria. To exclude this possibility, the effect of 1/4 MIC of tenoxicam on bacterial growth was determined. No significant change was observed in the growth of both tenoxicam-treated and untreated isolates (Fig. 1), proposing that a concentration of 1/4 MIC of tenoxicam has no bactericidal effect on bacterial viability.

Influence of tenoxicam on the production of *P. aeruginosa* QS-dependant virulence factors

The effect of tenoxicam on the production of QS-dependant virulence factors by *Pseudomonas* isolates was evaluated. Importantly at 1/4 MIC of tenoxicam, pyoverdin, rhamnolipids, and pyocyanin production were significantly reduced in tenoxicam-treated isolates compared to untreated isolates (Fig. 2a–c), respectively. There was a significant reduction observed in pyoverdin production from 100% in untreated isolates to 7%, 17%, 7%, 2%, 3%, and 5% in PAO-1, P1, P2, P3, P4, P5 tenoxicam-treated isolates, respectively.

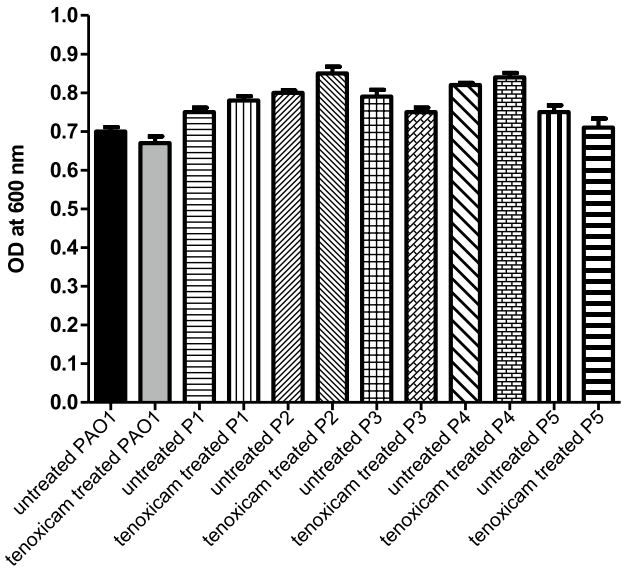

Fig. 1 Tenoxicam has no effect on *Pseudomonas aeruginosa* growth. The OD_{600} of bacterial overnight cultures in LB was measured both with and without 1/4 MIC of tenoxicam

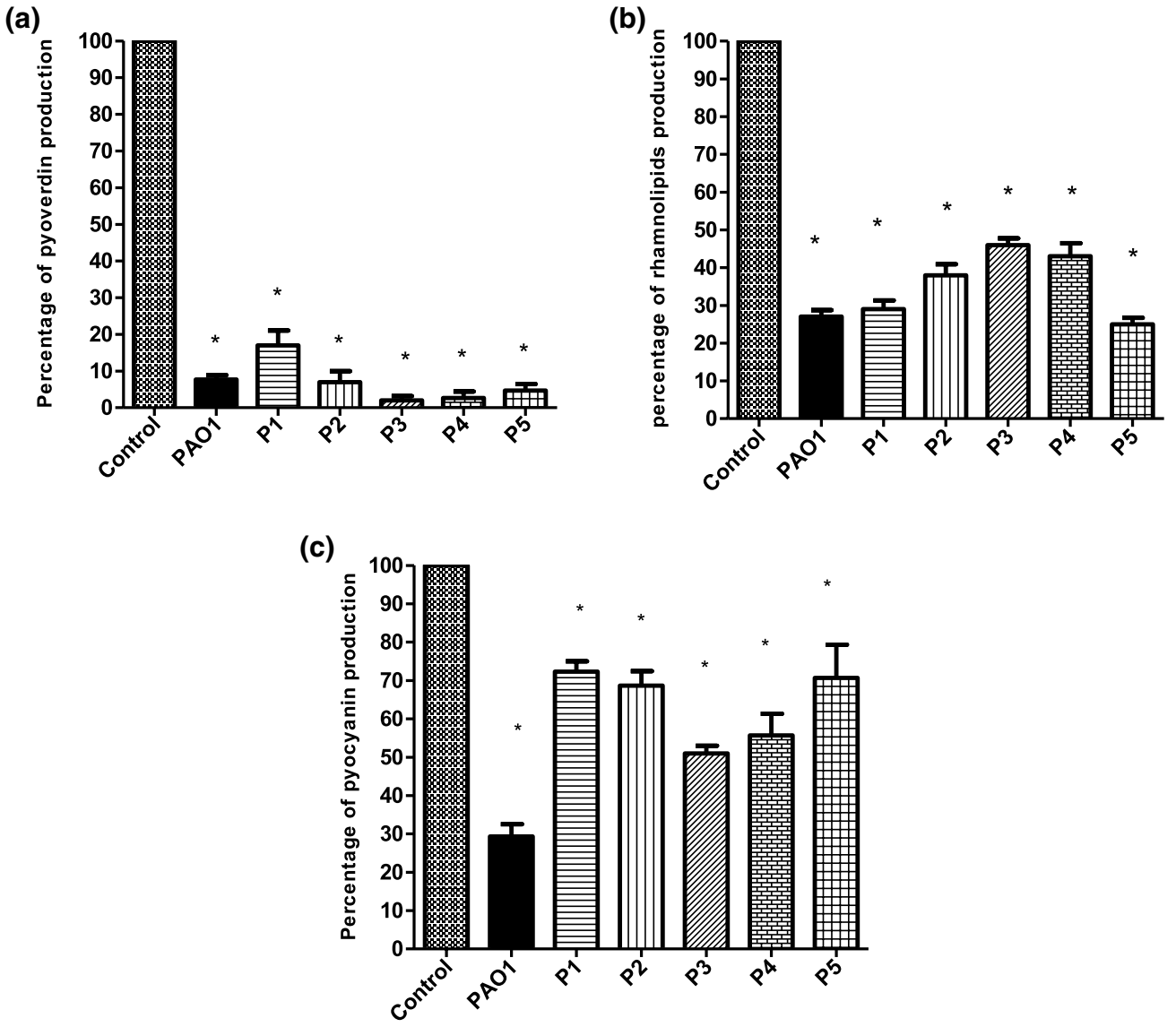

Fig. 2 Virulence factors production was significantly reduced in tenoxicam-treated bacteria in comparison with untreated bacteria; **a** pyoverdin production was reduced in tenoxicam-treated bacteria. Pyoverdin fluorescence was measured at 460 nm while the samples were excited at 400 nm after overnight culturing of bacteria in LB broth with and without 1/4 MIC of tenoxicam, **b** rhamnolipids production was decreased in tenoxicam-treated bacteria. The diameter of oil circles was measured after culturing in LB broth with presence and without 1/4 MIC of tenoxicam, **c** absorbance of pyocyanin was measured at 520 nm following 48 h culturing of bacteria in LB broth and chloroform extraction with and without 1/4 MIC of tenoxicam. The data shown are the means ± standard errors of three biological experiments with three technical replicates each. $P < 0.05$ (following One Way ANOVA) was considered significant

Moreover, rhamnolipids production was significantly reduced from 100% in untreated isolates to 27%, 29%, 38%, 46%, 43%, and 25% in PAO-1, P1, P2, P3, P4, P5 tenoxicam-treated isolates, respectively. In addition, pyocyanin production was also reduced from 100% in untreated isolates to 29%, 72%, 69%, 51%, 56%, and 71% in PAO-1, P1, P2, P3, P4, P5 tenoxicam-treated isolates, respectively.

In addition, the effect of tenoxicam on the production of hydrolytic enzymes, proteases, hemolysin, and elastase was also determined. There was a significant change observed in the production of proteases, hemolysins, and elastase in tenoxicam-treated isolates compared to untreated isolates (Fig. 3a–c), respectively. At 1/4MIC of tenoxicam, proteases production was reduced from 100% in untreated isolates to 34%, 24%, 46%, 21%, 22%, and 14% in PAO-1, P1, P2, P3, P4, P5 tenoxicam-treated isolates, respectively. Furthermore, the hemolysins production was reduced to 11%, 31%, 50%, 6%, 9%, and 70% in PAO-1, P1, P2, P3, P4, P5 tenoxicam-treated isolates, respectively. Also, elastase production was reduced from

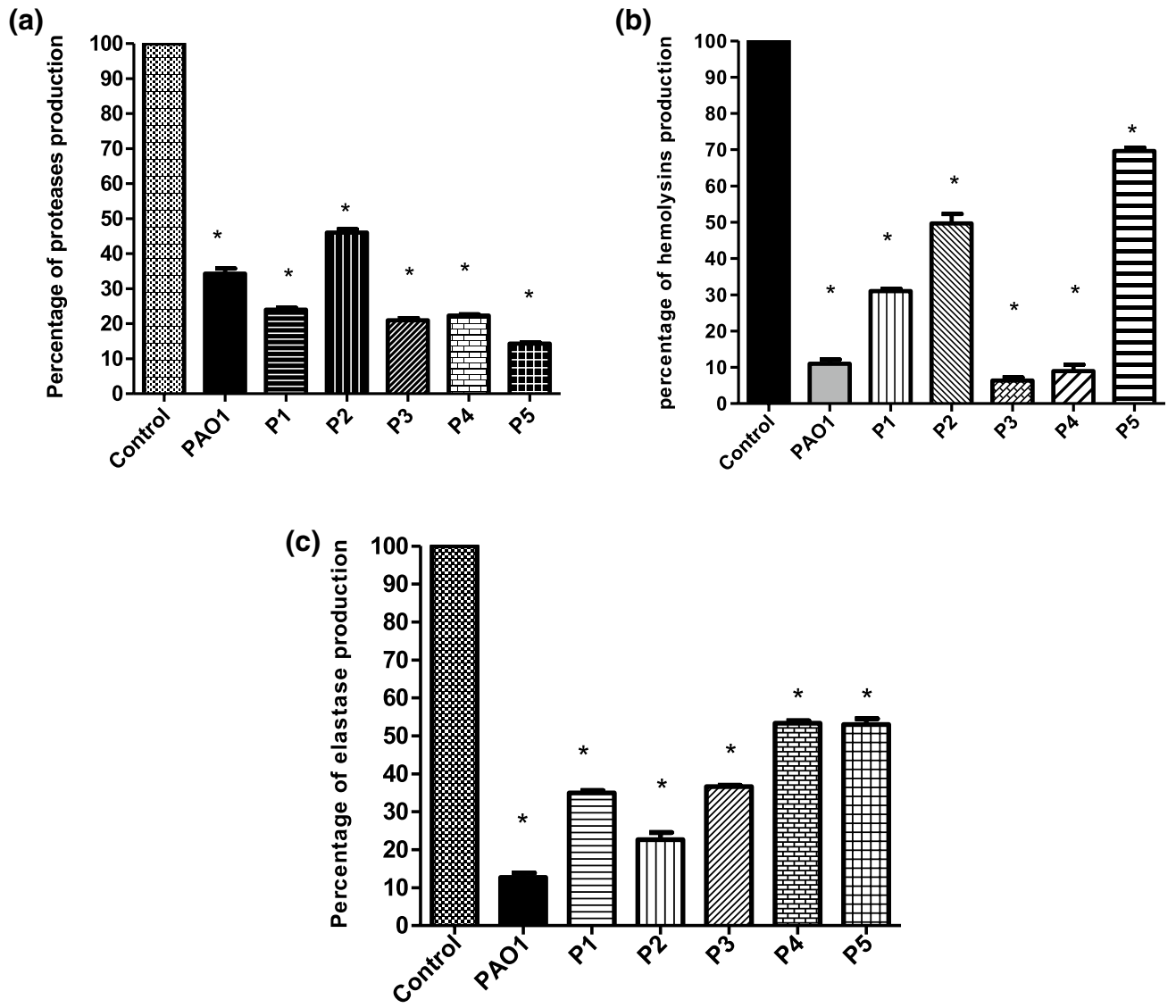

Fig. 3 Hydrolytic enzymes virulence factors production was significantly decreased in tenoxicam-treated bacteria as compared to control untreated bacteria; **a** Proteases production was reduced in tenoxicam-treated bacteria. OD_{600} was measured after overnight culturing of bacteria in LB broth with and without 1/4 MIC of tenoxicam followed by incubation of supernatants with skim milk for 1/2 h at 37 °C, **b** Hemolysin production was reduced in tenoxicam-treated bacteria. Absorbance of hemoglobin color released by the effect of hemolysins on erythrocytes was measured at 540 nm, **c** Elastase production was reduced in tenoxicam-treated bacteria. Absorbance of color formed by the effect of elastase on ECR was measured at 495 nm after culturing of bacteria in LB media with and without 1/4 MIC of tenoxicam for 3 h. The data shown are the means ± standard errors of three biological experiments with three technical replicates each. $P < 0.05$ (following One Way ANOVA) was considered significant

100% in untreated isolates to % in PAO-1, P1, P2, P3, P4, P5 tenoxicam-treated isolates, respectively.

Estimation of tenoxicam effect on expressions of *Pseudomonas* QS genes using RT-qPCR

Tenoxicam effect on the relative expressions of *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA*, and *pqsR* was estimated from the experimental *Ct* values (Livak and Schmittgen 2001). The expression of *lasI* was reduced from 100% in untreated bacterial isolates to 57%, 66%, 71%, 43%, 49%, and 59% in PAO-1, P1, P2, P3, P4, P5 tenoxicam-treated isolates, respectively. Furthermore, the expression of *lasR* was also reduced from 100% in untreated isolates to 6%, 17%, 28%, 11%, 27%, and 15% in PAO-1, P1, P2, P3, P4, P5 tenoxicam-treated isolates, respectively (Fig. 4a and b). Moreover, expression of *rhlI*- and *rhlR* genes was significantly decreased in tenoxicam-treated isolates in comparison with untreated cells (Fig. 5a and b). The expression of *rhlI* was reduced from 100% in untreated isolates to 18%, 34%, 33%, 43%, 27%

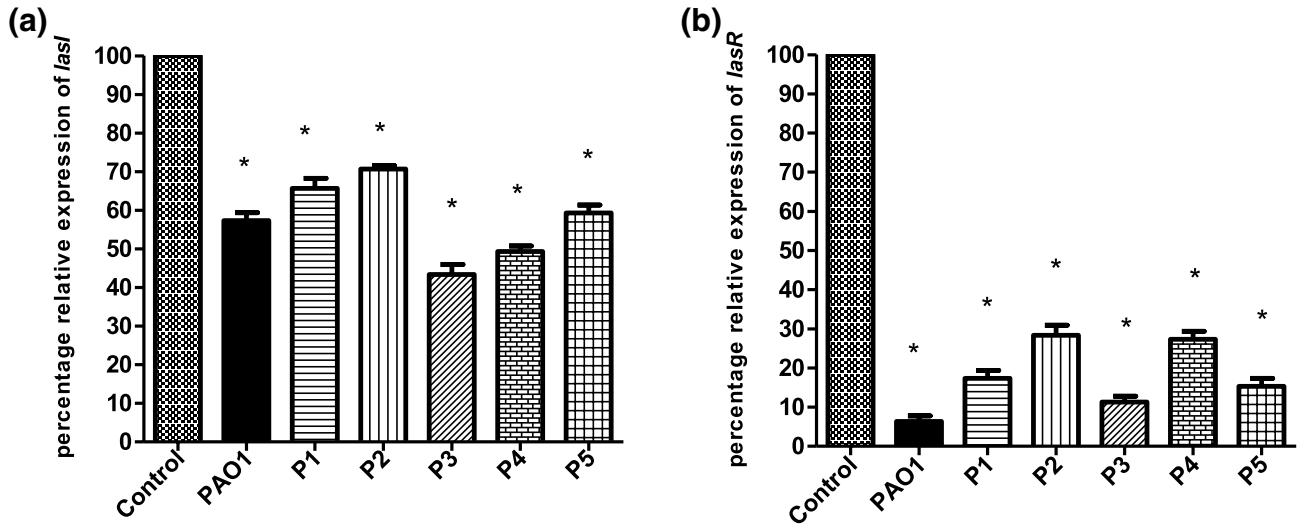

Fig. 4 RT-qPCR exhibited reduced expression of **a** *lasI* and **b** *lasR* in tenoxicam-treated isolates in comparison with untreated isolates. The data shown are the means ± standard errors of three biological experiments with three technical replicates. $P < 0.05$ (One Way ANOVA) was considered significant

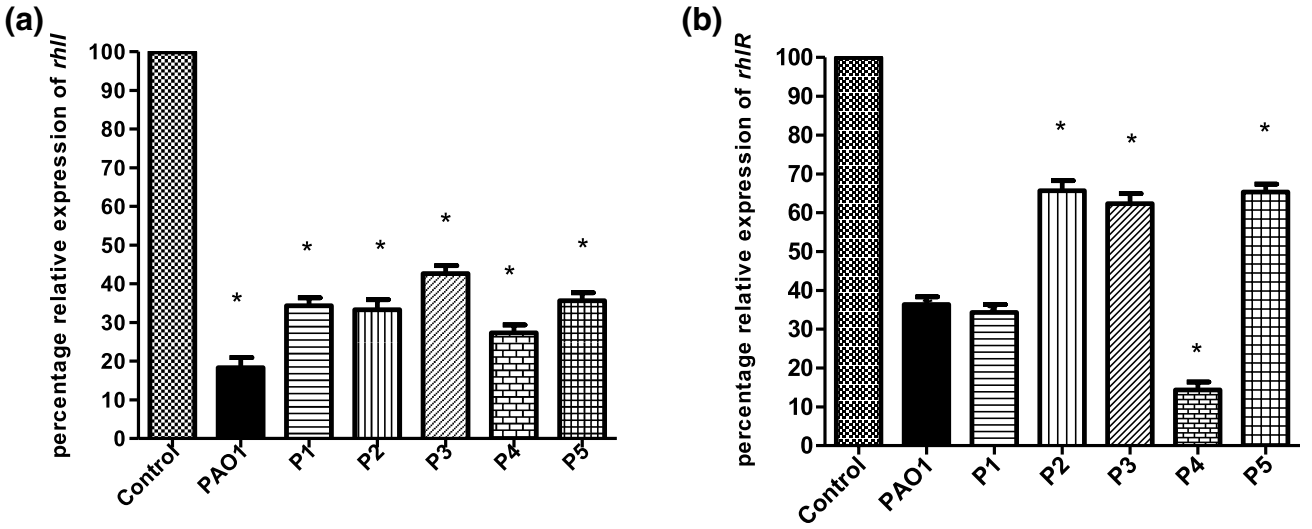

Fig. 5 RT-qPCR showed reduced expression of **a** *rhlI* and **b** *rhlR* in tenoxicam-treated isolates in comparison with untreated isolates. The data shown are the means ± standard errors of three biological experiments with three technical replicates. $P < 0.05$ (One Way ANOVA) was considered significant

and 36% in PAO-1, P1, P2, P3, P4, P5 tenoxicam-treated isolates, respectively. Similarly, the expression of *rhlR* was reduced from 100% in untreated isolates to 36%, 34%, 66%, 62%, 14% and 65% in PAO-1, P1, P2, P3, P4, P5 tenoxicam-treated isolates, respectively. Finally, the expression of *pqsA* and *pqsR* was significantly decreased following treatment with tenoxicam (Fig. 6a and b). The expression of *pqsA* was reduced from 100% in untreated isolates to 31%, 20%, 61%, 45%, 51%, and 61% in PAO-1, P1, P2, P3, P4, P5 tenoxicam-treated isolates, respectively. In addition, expression of *pqsR* was reduced from 100% in untreated isolates to 5%, 23%, 15%, 30%, 5%, and 35% in PAO-1, P1, P2, P3, P4, P5 tenoxicam-treated isolates, respectively.

Mice survival was increased in mice injected with tenoxicam-treated *P. aeruginosa*

Studying the effect of tenoxicam on *P. aeruginosa* virulence was evaluated using mice survival test. Interestingly, at an infection dose of 2.5×10^7 CFU, mice injected with tenoxicam-treated *P. aeruginosa* showed a significant increase in survival rate as compared to those

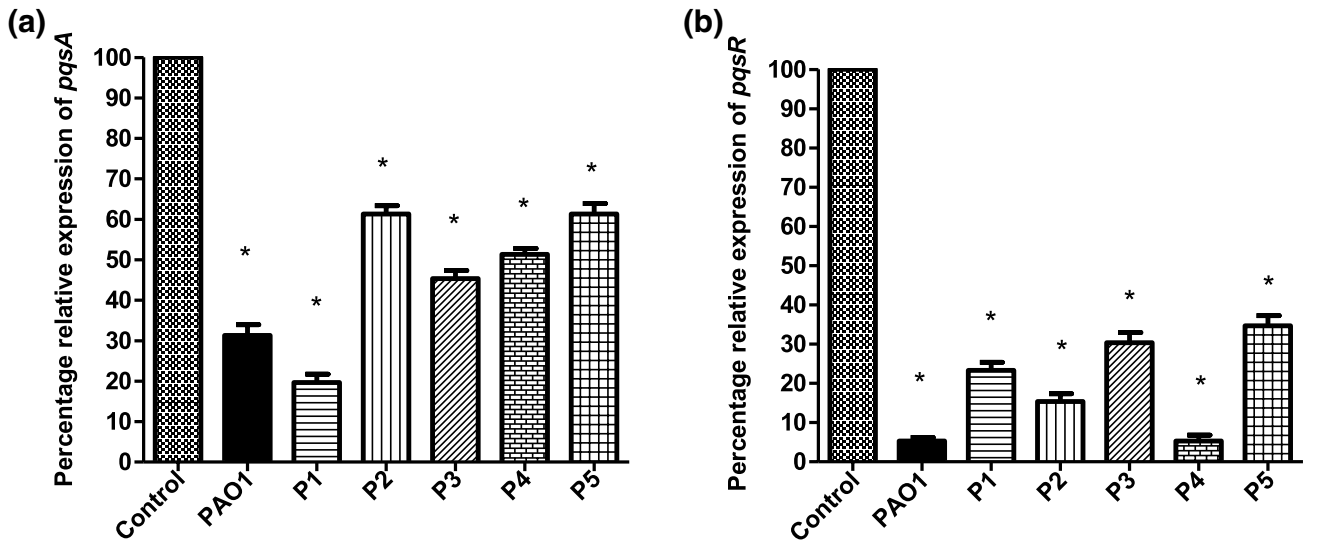

Fig. 6 RT-qPCR exhibited reduced expression of **a** *pqsA* and **b** *pqsR* in tenoxicam-treated isolates in comparison with untreated isolates. The data shown are the means ± standard errors of three biological experiments with three technical replicates. $P < 0.05$ (One Way ANOVA) was considered significant

mice injected with untreated bacteria. Only 20% of mice injected with untreated *P. aeruginosa* remained alive at the end of the experiment (Fig. 7). On the other hand, 80% of mice injected with tenoxicam-treated *P. aeruginosa* remained alive. Importantly, mice in control groups (both uninfected and PBS-injected mice) did not show any death at the end of the experiment. The in vivo experiment results indicate that treatment of *P. aeruginosa* with tenoxicam remarkably reduced its pathogenesis in mice.

Discussion

Pseudomonas aeruginosa is a notorious opportunistic pathogen that infects mostly patients with a suppressed immune system (El-Shaer et al. 2016). It has been shown that QS plays an important role in *P. aeruginosa* virulence and pathogenesis (Whitehead et al. 2001). In QS process, bacteria secrete and detect small signal molecules (autoinducers) with a specificity that allows them to identify their own autoinducers (Marshall 2013). Upon exposure to sufficient amount of a particular autoinducer, bacterial cells stimulate

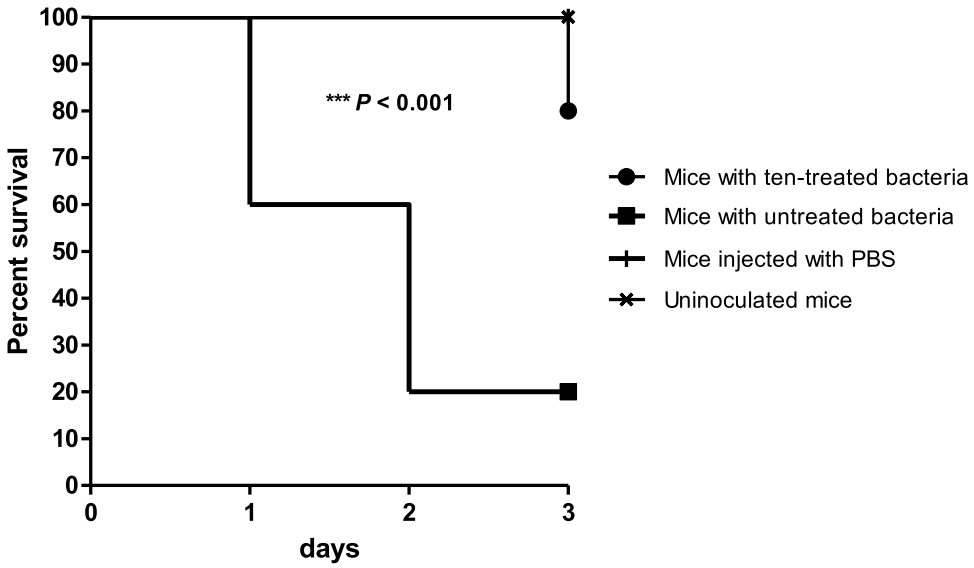

Fig. 7 Survival rate was significantly increased in mice injected with tenoxicam-treated bacteria in comparison with untreated bacteria. Mice survival was monitored every day for 3 days and plotted using Kaplan–Meier method. Tenoxicam-treated *Pseudomonas aeruginosa* killed significantly fewer mice as compared to untreated bacteria ($P < 0.0001$ log-rank test) and ($n = 10$ mice in each group), *ten* tenoxicam

a response cascade turning on a number of genes associated with the regulation of different bacterial processes including expression of virulence factors (Lazdunski et al. 2004; Marshall 2013; Moradali et al. 2017). It has been suggested that QSIs could be the next generation for treatment of microbial infections, especially in multi-drug resistant infections (Rasko and Sperandio 2010; Marshall 2013). In contrast to traditional antibiotics, QSIs markedly repress bacterial virulence without affecting bacterial growth and hence, minimizing the probability of generating bacterial resistance to these drugs (Bjarnsholt et al. 2010).

Pseudomonas aeruginosa pathogenesis depends on the presence of a plenty of virulence factors such as pyoverdin, rhamnolipids, pyocyanin, elastase, proteases, and hemolysins. These virulence factors are responsible for *Pseudomonas* host-colonization, and the establishment of infections (Khalifa et al. 2011). These virulence factors not only play a role in *Pseudomonas* survival but also are important for host tissue necrosis, invasion of host cells in addition to interruption of host defense mechanisms through degradation of host immunoglobulins A and G (Khalifa et al. 2011; El-Mowafy et al. 2014).

The influence of some NSAIDs such as salicylic acid, aspirin, and diclofenac sodium on *P. aeruginosa* QS-dependent virulence factors expression has been previously investigated. These drugs have a potent inhibitory effect on QS-dependent virulence factors production in *P. aeruginosa* through reducing the levels of secreted autoinducers and blocking their binding with receptors (Chow et al. 2011; El-Mowafy et al. 2014; Abbas 2015).

In the current study, tenoxicam-treated *P. aeruginosa* exhibited a significant reduction in virulence factors production as compared to untreated bacteria, suggesting that tenoxicam has a potential QS inhibitory effect. Tenoxicam could decrease the levels of C12-HSL and C4-HSL autoinducers and block their binding to receptors. This leads to inhibition of QS and its dependent-virulence factors production in *P. aeruginosa*, which is similar to other NSAIDs that have QS inhibitory effect (Chow et al. 2011; El-Mowafy et al. 2014; Abbas 2015).

QS genes are key regulators of virulence factors production as well as the antibiotic resistance of *P. aeruginosa* (Jimenez et al. 2012). In *P. aeruginosa*, QS consists of three hierarchically arranged systems, namely *las*, *rhl* and *pqs* that work in coordination under the control of *las* system (El-Shaer et al. 2016). It was found that suppression of *las* system will be correlated with inhibition of other QS systems and subsequent virulence reduction (Rasmussen and Givskov 2006; El-Shaer et al. 2016; Ouyang et al. 2016). It has been reported that inhibition of *las* system significantly affect protease and elastase production, while down-regulation of the *rhl* system reduces production of pyocyanin, rhamnolipid, elastase and proteases (Singh et al. 2017).

Considering the master role of QS in controlling virulence factor production, we anticipated that the tenoxicam-mediated inhibition of QS-dependant virulence factors production could occur through affecting QS genes. Importantly, qRT-PCR revealed that tenoxicam significantly reduced the expression levels of QS genes *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA,* and *pqsR*. The current results of qRT-PCR augment the phenotypic results as the production of *P. aeruginosa* virulence factors are largely under the control of *las*, *rhl* and *pqs* systems (Gupta et al. 2011). Similar to our findings, previous studies demonstrated that some approved NSAIDs such as salicylic acid (Prithiviraj et al. 2005; Chow et al. 2011) and aspirin (El-Mowafy et al. 2014) are efficient QSIs at the molecular level in *P. aeruginosa*. In addition, many other compounds were found to act as QSIs by inhibiting QS circuits at the molecular level such as linolenic acid and tobramycin combination (Chanda et al. 2017), gall extracts (Mohabi et al. 2017), and *Delftia tsuruhatensis* plant extract (Singh et al. 2017).

Based on the above mentioned, tenoxicam inhibitory effect could be similar to that of other QSIs through interference with *las* system that controls other QS systems resulting in an interruption of *rhl* and *pqs* and reduction of their dependent-virulence factors production. However, more future work, such as RNA-sequencing and western-blot analysis of virulence proteins expression, is required to prove this hypothesis.

Considering the current data revealing that tenoxicam remarkably decreased production of virulence factors in *P. aeruginosa*, it was pivotal to assess the impact of tenoxicam on bacterial virulence using mice survival test. Importantly, mice injected with untreated *P. aeruginosa* showed higher mortality in comparison with mice injected with tenoxicam-treated bacteria. Present findings exhibit that treating *P. aeruginosa* with tenoxicam largely influences QS-dependant virulence factors production leading to decreases bacterial pathogenesis as a result of QS interruption. In agreement with our study, Hentzer et al. (2003) reported that mice survival was enhanced in mice injected with *P. aeruginosa* treated with synthetic furanone derivatives which act as QSIs as compared to those injected with untreated bacteria. Similarly, Lesic et al. (2007) and Prateeksha and Shoeb (2017) reported that mice survival was increased when injected with *P. aeruginosa* treated with the QSIs, halogenated anthranilic acid analogs and polyphenolic compounds of honey, respectively, when compared with mice injected with untreated bacteria.

In conclusion, the development of antibiotic resistance by many bacteria makes it mandatory to find out new therapeutic options for infection control. Tenoxicam may be a useful agent in this approach due to its inhibitory action on *Pseudomonas* quorum sensing and pathogenesis. Targeting quorum sensing-controlled virulence has

no growth pressure on bacteria, so the resistance is less likely to occur.

Acknowledgements We would like to thank the members of Medical Research Center, Cairo, Egypt for their help in mice survival experiment.

Compliance of ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The animal study was approved by The Institutional Animal Care and Use Committee, Zagazig University (ZU-IACUC). All animals were handled according to guidelines from the Animal Ethics Board (Zagazig University). All participants provided written informed consent prior to enrolment in the study.

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