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Abstract: Amoxicillin is a commonly prescribed drug for anti- bacterial infection. In this study, we are interested in the effect of the drug on the cellular DNA integrity. Amoxicillin was added to the human or hamster cells in culture, and the DNA adducts induced by the drug were accessed by a comet assay with nuclear extract incubation (Wang et al., 2005 Anal Biochem 337: 70-75). Amoxicillin at 5 mM rapidly induced DNA adducts in human AGS cells. The level of DNA adducts attained a maximum at about 1 h, and then declined steadily and reached almost the basal level at 6 h following the drug treatment. Similar induction pattern of DNA adducts was found with amoxicillin-related antibiotics such as ampicillin but not with the unrelated antibiotics such as kanamycin. For studying the repair kinetics, the cells was treated with amoxicillin for only 1 h and continued cultured in the absence of the drug for a certain period of time before subsequent analysis. Repair of the amoxicillin-induced DNA adducts was essentially completed within 4 h. Such repair may not involve nucleotide excision repair (NER) pathway because the repair was completed with similar kinetics in both NER proficient Chinese hamster CHO-K1 cells and its isogenic NER deficient UV24 cells. Instead, the repair may involve base excision repair (BER) pathway because immunodepletion

of OGG1, a glycosylase involved in BER rendered the nuclear extract unable to excise DNA adducts induced by amoxicillin in the modified comet assay. Furthermore, amoxicillin induced intracellular reactive oxygen species (ROS) at the tempo similar to that of DNA adducts induction. Thus, we hypothesize that amoxicillin causes oxidative DNA damage in mammalian cells via ROS.

2006/11/15

Editor of *Mutation Research-Genetic Toxicology and Environmental Mutagenesis*
Dear Editor:

We would like to submit the manuscript **Antibiotic amoxicillin induces DNA adducts in mammalian cells possibly via the reactive oxygen species** for publication in *Mutation Research-Genetic Toxicology and Environmental Mutagenesis*. The paper describes the study on the genetic toxicity of amoxicillin, which is commonly prescribed for antibacterial infection. Our study suggests that amoxicillin and its like such as ampicillin can cause oxidative DNA damages. Though the repair of such DNA lesions may be completed within hours following drug administration, the DNA lesions may be a potential risk for people whose repair capacity is deficient or compromised under certain circumstances. This work has not been considered published elsewhere, and the submission is under the consensus of all its contributors.
We thank you for consideration and appreciate your comments.

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Antibiotic amoxicillin induces DNA adducts in mammalian cells possibly via the reactive oxygen species

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Abstract

Amoxicillin is a commonly prescribed drug for anti- bacterial infection. In this study, we are interested in the effect of the drug on the cellular DNA integrity. Amoxicillin was added to the human or hamster cells in culture, and the DNA adducts induced by the drug were accessed by a comet assay with nuclear extract incubation (Wang et al., 2005 Anal Biochem 337: 70-75). Amoxicillin at 5 mM rapidly induced DNA adducts in human AGS cells. The level of DNA adducts attained a maximum at about 1 h, and then declined steadily and reached almost the basal level at 6 h following the drug treatment. Similar induction pattern of DNA adducts was found with amoxicillin-related antibiotics such as ampicillin but not with the unrelated antibiotics such as kanamycin. For studying the repair kinetics, the cells was treated with amoxicillin for only 1 h and continued cultured in the absence of the drug for a certain period of time before subsequent analysis. Repair of the amoxicillin-induced DNA adducts was essentially completed within 4 h. Such repair may not involve nucleotide excision repair (NER) pathway because the repair was completed with similar kinetics in both NER proficient Chinese hamster CHO-K1 cells and its isogenic NER deficient UV24 cells. Instead, the repair may involve base excision repair (BER) pathway because immunodepletion of OGG1, a glycosylase involved in BER rendered the nuclear extract unable to excise DNA adducts induced by amoxicillin in the modified comet assay. Furthermore, amoxicillin induced intracellular reactive oxygen species (ROS) at the tempo similar to that of DNA adducts induction. Thus, we hypothesize that amoxicillin causes oxidative DNA damage in mammalian cells via ROS.
Keywords: amoxicillin; DNA adducts; comet-nuclear extract assay; base excision repair; reactive oxygen species

1. Introduction

Amoxicillin, a derivative of penicillin, is commonly-used to treat the bacterial infection [1]. Amoxicillin and the like of antibiotics act by inhibiting the formation of cell wall [2,3]. Mammalian cells do not contain cell wall and so are unsusceptible to the similar effect. However, a recent study indicates that the drug may have potential to interact with DNA and cause DNA damage in human cells [4,5]. In this study, we re-examined the effect of amoxicillin on DNA by using a modified comet assay to access the total DNA adducts [6]. The conventional alkaline comet assay (or single cell gel electrophoresis) is suitable for accessing DNA strand breaks; DNA adducts without strand breaks are often undetected by the conventional comet assay. Though combined with specific enzyme digestion the comet assay may be useful to evaluate the specific DNA adducts induced by damaging agents [7], the usefulness is limited by the prerequisite knowledge about the DNA adducts caused by the DNA damaging agent of interest. The modified comet assay used in the present study contains a nuclear extract (NE) digestion step (see the schematic illustration in Fig 1A) and because NE, in principle, contains all the repair enzymes, the comet-NE assay appear to by-pass the limitation aforementioned. Thus, the comet-NE assay seems to be more suitable to study the DNA damages un-investigated or unclear such as those induced by amoxicillin. Besides, excision repair mechanisms such as base excision repair or nucleotide excision repair can be divided to four steps: recognition of damage site excision, DNA synthesis, and ligation [8,9]. Since all the DNA adducts and the intermediate products during the repair process can be detected by comet-NE assay, unless the DNA adduct has been excised and the gap refilled and resealed, they will be detected as un-repair. Therefore, the method allows the kinetic study of complete repair not just the detection of the removal of DNA adducts such as the protocols for

detecting the specific damage sites. Our study suggests that amoxicillin and the similar antibiotics induce DNA damage possibly via reactive oxygen species. Repair of the amoxicillin- induced DNA adducts was completed within several hours following the treatment of the drug.

2. Materials and Methods

2.1 Cell cultures

Human cell lines AGS and NB4 as well as the Chinese hamster cell lines CHO-K1, and UV24 cells were originally obtained from the American Type Culture Collection (Manassas, VA, USA). AGS and NB4 cells were derived from gastric adenocarcinoma and acute promyelocytic leukemia, respectively. The CHO-K1 and UV24 cells were maintained in 1X McCoy's 5A medium (Sigma). The AGS and NB4 cells were maintained in 1X DMEM medium (Gibco, Grand Island, NY, USA) and 1X RPMI medium (Gibco, Grand Island, NY, USA), respectively. The culture media were supplemented with 10% fetal calf serum, and 0.03% glutamine. The cells were cultured at 37^{o}C in a water-saturated atmosphere containing 5% CO_2.

2.2 Treatment of antibiotic

The cultured cells at logarithmic phase were treated with amoxicillin, ampicillin or kanamycin (Sigma, St. Louis, MO, USA; dissolved in DMSO) at the indicated concentration for the indicated period of time before DNA damage analysis.

2.3 UV irradiation

The treatment was done as described previously [10]. Cells, after being washed

with 1×PBS, were exposed in uncovered dishes to UV using germicidal lamps (254 nm, Sankyo Denki Co., Tokyo, Japan) in a UV box at different doses calibrated with a UV radiometer (UVP Inc., San Gabriel, CA, USA).

2.4 Lymphocyte isolation and nuclear extract (NE) preparation

Lymphocytes from peripheral blood of a healthy donor were isolated by centrifugation in a density gradient of Ficoll-Paque PLUS (Amersham Biosciences AB, Uppsala, Sweden). The nuclear extract (NE) was prepared by the method described by Challberg and Kelly with some modification [11]. Briefly, the lymphocytes were washed once with hypotonic buffer (20 mM Hepes, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol and containing 0.2 M sucrose). The pellets were resuspended in cold hypotonic buffer without sucrose, allowed to swell for 10 min on ice, and then lysed with 10 strokes of Dounce homogenizer. The homogenate was pass through a 22G needle for 10 times. The lysate was centrifuged at 2000 × g for 5 min. The nuclear pellet was resuspended in the buffer (20 mM Hepes, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, and 0.5 mM dithiothreitol containing 10% sucrose), and the resulting nuclei were stored in -70^{o}C until use. The nuclei were thawed on ice and allowed to swell in 100 mM NaCl on ice for 1 h. The mixture was centrifuged at 15000 × g for 20 min at 4^{o}C. The supernatant was applied to a YM-10 Microcon filter (Millipore, Bedford, MA, USA) to remove the deoxynucleotide substrates. Protein concentration was determined by the BCA Protein Assay Kit (Pierce, USA) using bovine serum albumin as a standard.

2.5 Comet- NE assay

The assay was performed as described previously [6]. For preparing the gels on

each slide, 100 µl of 1.4% agarose in phosphate-buffered saline (PBS) at 65°C was placed onto a glass microscope slide pre-warmed at 60°C. The gel was covered with coverslip immediately, and the slide was chilled on ice. The coverslip was removed and an aliquot of 100 µl of cells containing agarose was then added. This was done by mixing equal volume of 1.2% low-melting agarose and cell suspension (1x 10^6 cells /ml in PBS); the mixture was kept at 40 °C. The coverslip was added and removed as described above. Then, 100 µl of 1.2% low-melting agarose was applied as the third layer of agarose. After the third layer of gel was made, the slide was immersed in ice-cold cell lysis solution and stored at 4 °C for at least 2 h. Cell lysis solution contained 2.5 M NaCl, 100 mM EDTA, and 10 mM Tris (pH adjusted to 10 with NaOH), and 1% N-laurylsarcosine, 1% Triton X-100, and 10% DMSO were added immediately before use.

After cell lysis, the slides were washed three times with deionized water. The NE digestion was done by adding a total of 20 µl of excision mixture containing 0.6 µg of NE, 50 mM Hepes-KOH (pH 7.9), 70 mM KCl, 5 mM $MgCl_2$, and 0.4 mM EDTA, 2 mM ATP, 40 mM phosphocreatine, and 2.5 mM creatine phosphokinase onto each slide. A coverslip was applied, and the slides were incubated at 37°C for 2 h in a sealed box containing a piece of wet tissue paper. After the incubation, the slides were denatured in 0.3 N NaOH, 1 mM EDTA for 20 min. Electrophoresis was carried out in the same denaturation solution at 25 V, 300 mA for 25 min. The slide was washed briefly in deionized water, blotted, and then transferred to 0.4 M Tris-HCl, pH 7.5. DNA was stained by adding 40 µl of 50 µg/ml propidium iodide onto the slide. A coverslip was applied, and the slide was examined under a fluorescence microscope (Axioplan 2, Zeiss Co, Thornwood, NY, USA). The image of 50 cells per treatment was recorded with close-circuit display camera (CoolSNAP). The migration of DNA from the nucleus of each cell was measured with a computer program

(http://tritekcorp.com) using the parameter of comet moment.

2.6 Immunodepletion

Two µg of nuclear extract mixed with 0.2 µg of antibody was gently shaken in a rotator for more than 12 h at 4 ^{o}C. The mixture was centrifuged at 6500 × g for 10 min and the supernatant was transferred to a new tube. Antibodies for OGG1 (sc-12075; Santa Cruz), XPB (sc-293, Santa Cruz), XPF (sc-10164, Santa Cruz), and actin (sc-1616; Santa Cruz) were purchased from Santa Cruz Biotechnology. The immunodepleted NE was used in comet-NE assay shown in Fig 6.

2.7 Measurement of intracellular ROS

Intracellular oxidant levels were determined using 2,7-dichlorodihydrofluorescein (H_2DCF), which is oxidized in cells to the fluorescent dichlorofluorescein [12]. Cells were incubated with H_2DCF-diacetate (DCF-DA) (Molecular Probes, Eugene, Oregon, USA) at 25 µM for 30 min at 37^{o}C and were then washed with ice cold PBS. Measurements were performed by flow cytometric analysis with excitation at 506 nm and emission at 526 nm.

2.8 Statistics

Data are expressed as mean ± standard derivation throughout this paper. All experiments were performed independently at least twice. Statistical analyses were performed with Student's t-test.

3. Results

To examine the DNA adducts induced by amoxicillin, we used the modified comet assay recently reported [6]. The method includes a nuclear extract digestion step in the conventional comet assay following the cell lysis in detergent (illustrated in Fig 1A). Since repair proteins are present in nuclear extracts, the inclusion of nuclear extract digestion will generate strand breaks and the breaks are detected by comet assay as illustrated by the example where UV-induced adducts were analyzed (Fig 1B). The example illustrates that comet-NE is more quantitative than the conventional comet assay in studying the DNA adducts without strand breaks.

3.1 Amoxicillin induces DNA adducts in AGS cells

When cultured AGS cells were treated continuously with amoxicillin at 5 mM and the DNA adducts were accessed by comet-NE assay, we found the DNA adducts appeared as early as 20 min after drug treatment. The DNA adducts increased to a peak level at about 60 min following drug treatment, then, the level of DNA adducts gradually declined and became almost the basal level after 6 h following drug treatment (Fig 2). In a separate experiment, AGS cells were treated with amoxicillin of various concentrations for 1 h; the levels of DNA adducts showed amoxicillin dose dependence if the drug concentration is ≤ 5 mM. The levels of amoxicillin-induced DNA adducts did not increase when the antibiotic concentrations were greater than 5 mM (Fig 3). For studying the repair of amoxicillin-induced DNA adducts, the cells were treated with amoxicillin for only 1 h and then the change of DNA adducts was monitored following the time course after the removal of the antibiotic. The repair was essentially completed at about 4 h as shown in Fig 4, indicating that amoxicillin-induced DNA adducts can be repaired fairly rapidly in cells.

3.2 Repair of amoxicillin-induced DNA adducts was NER independent

Since it is unclear whether amoxicillin would form bulky covalent complex with

DNA, which usually requires nucleotide excision repair (NER), it is of interest to know if NER is involved to remove the amoxicillin-induced DNA adducts. Thus, we conducted the repair experiment with NER proficient hamster CHO-K1 cells and the isogenic, NER deficient UV24 cells [13]. Results of the experiment shown in Fig 5 indicate that the CHO-K1 cells and UV24 cells had similar repair capacity. Thus, the repair of the amoxicillin-induced DNA adducts does not involve NER.

3.3 Excision of amoxicillin-induced DNA adducts requires OGG1

Since drug concentration greater than 5 mM did not increase the level of DNA adducts and since the amoxicillin-induced DNA adducts were repaired relatively fast, we consider the DNA adducts induced by amoxicillin might be of oxidative damages. The oxidative DNA damages usually require base excision repair (BER). To examine if BER is essential to remove amoxicillin-induced DNA adducts, we conducted the comet-NE assay using nuclear extracts immunodepleted of OGG1, one of the glycosylases to remove 8-oxo-guanine adducts in BER [14]. The results, shown in Fig 6, indicate that OGG1 is a critical component for the nuclear extracts to excise the amoxicillin-induced DNA adducts. In contrast, XPB or XPF, which are essential for NER, were not essential to excise amoxicillin-induced DNA adducts. This is consistent with the previous experiment with hamster cell lines that NER was not involved in removing amoxicillin-induced DNA adducts.

3.4 Amoxicillin induced reactive oxygen species

As the results of the previous experiment suggest that the amoxicillin-induced DNA adducts were of oxidative stress, we examined the effect of amoxicillin on the cellular ROS. The data show that amoxicillin caused a rapid increase of ROS at 30 min following the drug treatment (Fig 7). The level of ROS at 1h dropped significantly.

The temporal change of ROS matches the kinetic study of DNA adducts induced by the antibiotic (Fig 2).

3.5 *Ampicillin, an amoxicillin-related antibiotic but not kanamycin, an amoxicillin-unrelated antibiotic induced DNA adducts*

To test if the induction of the DNA adducts is amoxicillin specific, we checked if the similar induction occurred with amoxicillin-related antibiotics. We found that ampicillin but not kanamycin had similar induction pattern of DNA adducts (Fig 8); the levels of the DNA adducts induced by kanamycin were apparently lower. Thus, the induction of DNA adducts by amoxicillin was specific.

4. Discussion

In this report, we used a modified version of comet assay to examine the effect of amoxicillin on DNA. The method, termed comet-nuclear extract (NE) assay, was quantitative as illustrated in the experiment using UV as DNA damaging agent (Fig 1). Unless in special conditions, the comet-NE assay does not require nuclear extracts or the DNA substrates from specific cells. In this study, nuclear extracts from NB4 cells or human lymphocytes were used simply because of the availability.

Our results indicate that amoxicillin induced DNA adducts at the concentrations similar to those used in the previous study [4]. The induction of the DNA adducts was detected within tens of minutes following the addition of the antibiotic to the cells; the induction reached the plateau and declined gradually to the basal level several hours after the treatment (Fig 2). The decline of DNA adducts was interpreted as the result of repair, and the interpretation is consistent with the kinetic change of the DNA adducts observed following the drug was removed (Fig 4). We showed that the repair was not dependent on nucleotide excision repair (NER) for it occurred equally

effectively regardless the proficiency of NER of the cells (Fig 5). The DNA adducts induced by amoxicillin were initially considered as the covalent complex of amoxicillin-DNA, which might require NER to repair. The independence of NER of the repair of amoxicillin-induced DNA adducts was further supported by the comet-NE assay with nuclear extracts immunodepleted of specific repair proteins (Fig 6). The excision of amoxicillin-induced DNA adducts did not require XPA or XPF, the repair proteins of NER, on contrary, the excision need the OGG1, a typical repair protein in BER to remove the oxidized purines such as 8-oxo-guanine. This observation is supported that amoxicillin induced elevation of intracellular ROS, which occurred within ten of minutes following addition of the drug (Fig 7). Our study support the conclusion derived from the previous study that amoxicillin induced oxidative DNA adducts [4]. However, our data disagree with their description that amoxicillin-induced DNA adducts were completely repaired after 60 min- a study primarily based on the conventional comet assay. Regardless the differences in evaluating the DNA adducts, both studies indicate that amoxicillin-induced DNA adducts can be repaired quite effectively within a short period of time after administration of drug. Nevertheless, amoxicillin -induced DNA adducts may pose potential genotoxic problem to those who are genetically or physiologically deficient in the capacity to remove the oxidative DNA damage. We are currently testing this suspicion. Another investigation of interest is the role of the β-lactam ring in the induction of DNA adducts. Ampicillin but not kanamycin at the same concentration showed similar induction pattern of DNA adducts as that of amoxicillin (Fig 8). Amoxicillin and ampicllin but not kanamycin contain the β-lactam ring.

Conclusion- Although many antibiotics have been known to cause DNA damages [15,16], amoxicillin or similar antibiotics which was usually considered non-genotoxic have potential to injure genomic DNA possibly via the induction of

intracellular reactive oxygen species.

Acknowledgement

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Figure legends

Fig 1. (A) Schematic illustration of comet –NE assay; (B) comet –NE assay but not conventional comet assay show the dependence of UVC-induced DNA adducts on UV dose. NB4 cells were UV-irradiated at the indicated dose (0-10 J/m^2) and harvested right after the irradiation for analysis. The level of DNA damage was expressed by comet moment.

Fig 2. Induction of DNA adducts by amoxicillin. AGS cells were treated with amoxicillin at 5 mM for the indicated period of time (0 - 360 min) and harvested for comet-NE assay. (untreat: cells without drug treatment. DMSO: cells treated with DMSO (solvent for amoxicillin)).

Fig 3. Amoxicillin dose effect on the induction of DNA adducts. AGS cells were treated with amoxicillin at the indicated concentration (0-10 mM) for 1 h and harvested for comet-NE assay. (untreat: cells without drug treatment.)

Fig 4. Repair kinetic of DNA adducts induced by amoxicillin. AGS cells were treated with amoxicillin at 5 mM for 1 h, and then cultured in fresh media (without amoxicillin) for the indicated period of time (0-4 h) and harvested for comet-NE assay. (untreat: cells without drug treatment.)

Fig 5. Repair of DNA adducts induced by amoxicillin is NER independent. Both NER proficient CHO-K1 cells (A) and NER deficient UV24 cells (B) were treated with amoxicillin at 5 mM for 1 h, and then cultured in fresh media (without

amoxicillin) for the indicated period of time (0-4 h) and harvested for comet-NE assay. (untreat: cells without drug treatment.)

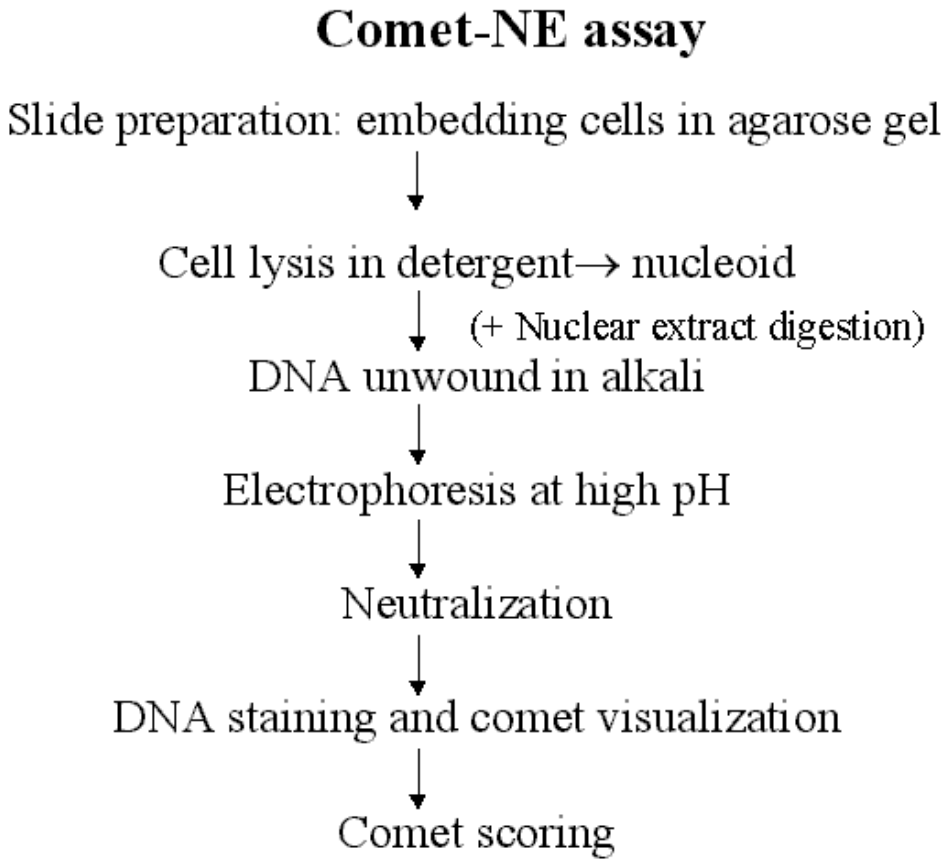
Fig 6. OGG1 but not XPB or XPF is essential for nuclear extracts to excise amoxicillin-induced DNA adducts. AGS cells were treated with 5 mM amoxicillin for 1 h; the cells were harvested for comet-NE assay with the nuclear extract or nuclear extracts immunodepleted of OGG1, XPB, XPF or actin, respectively. (untreat: cells without drug treatment.)

Fig 7. Induction of reactive oxygen species by amoxicillin. AGS cells were treated with amoxicillin at 10 mM for 0-1 h and harvested for ROS analysis. The mean values of fluorescence intensity are 151 ± 3 and 126 ± 4 at 30 min and 1 h, respectively following drug treatment, while the cells without drug treatment is 108 ± 7. The cells without treatment of antibiotic otherwise subjected to the similar process were used as control. (untreat: cells without drug treatment.)

Fig 8. Patterns of DNA adducts induced by the amoxicillin-related or -unrelated antibiotics. AGS cells were treated with ampicillin and kanamycin, respectively at 5 mM for the indicated period of time (0-360 min) and harvested for comet-NE assay.

Fig. 1.

(A)


Comet-NE assay
Slide preparation: embedding cells in agarose gel
Cell lysis in detergent→ nucleoid
(+ Nuclear extract digestion)
DNA unwound in alkali
Electrophoresis at high pH
Neutralization
DNA staining and comet visualization
Comet scoring

(B)

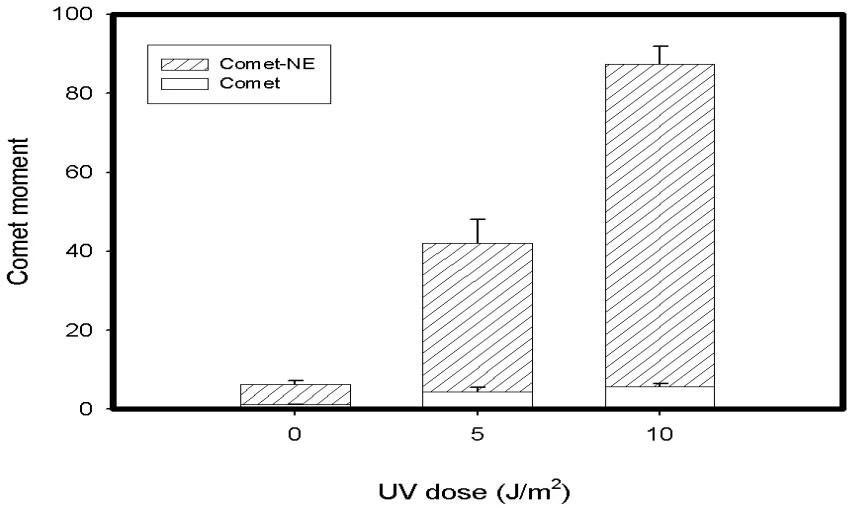

Comet-NE
Comet
Comet moment
100
80
60
40
20
0
0
5
10
UV dose (J/m2)

Figure 2 (amox)

Fig. 2.

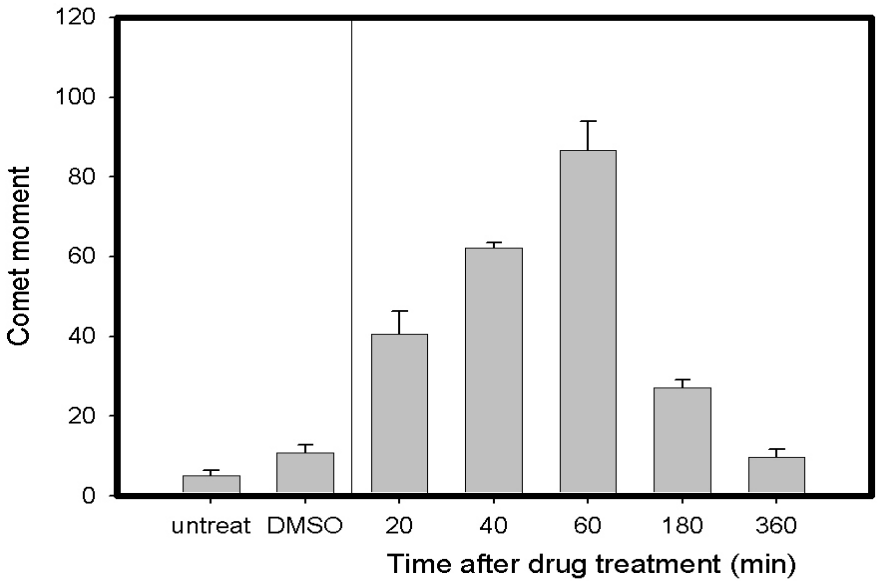


Figure 3 (amox)

Fig. 3.

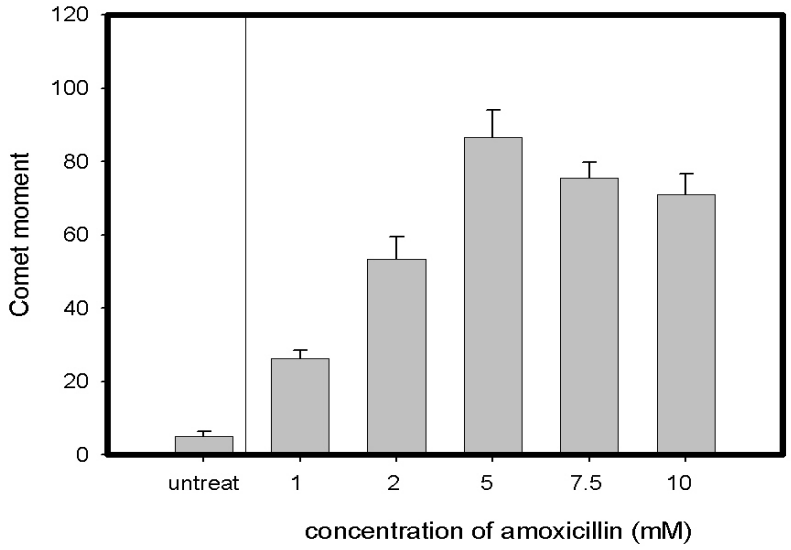


Figure 4 (amox)

Fig. 4.

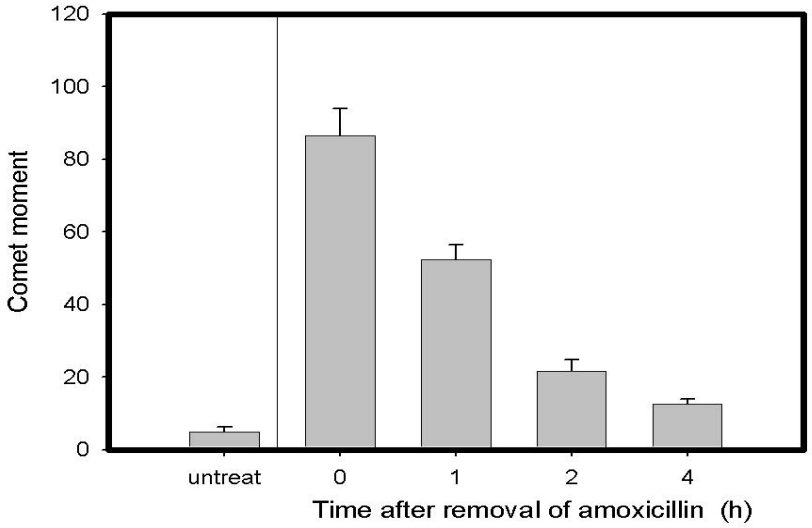
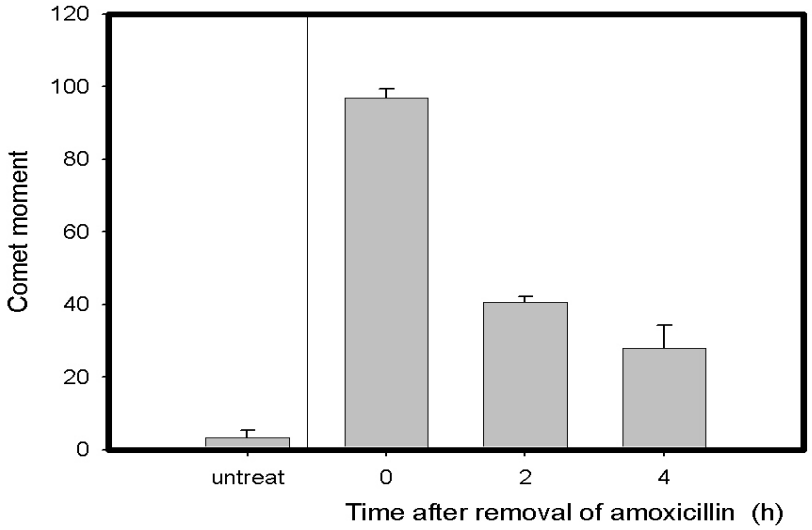


Figure 5 (amox)

Fig. 5.

(A)



(B)

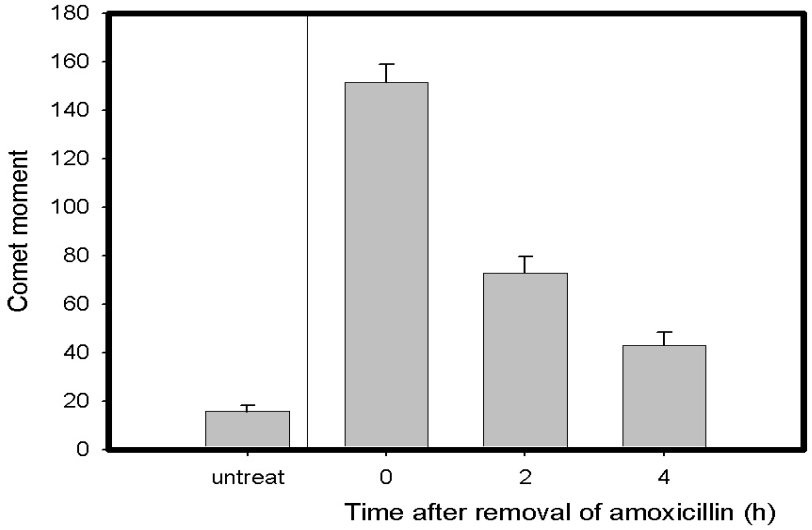


Figure 6 (amox)

Fig. 6.

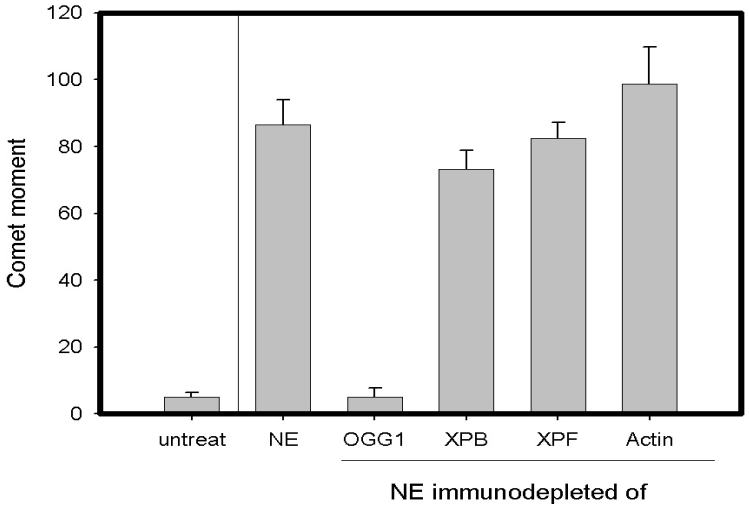


Figure 7 (amox)

Fig. 7.

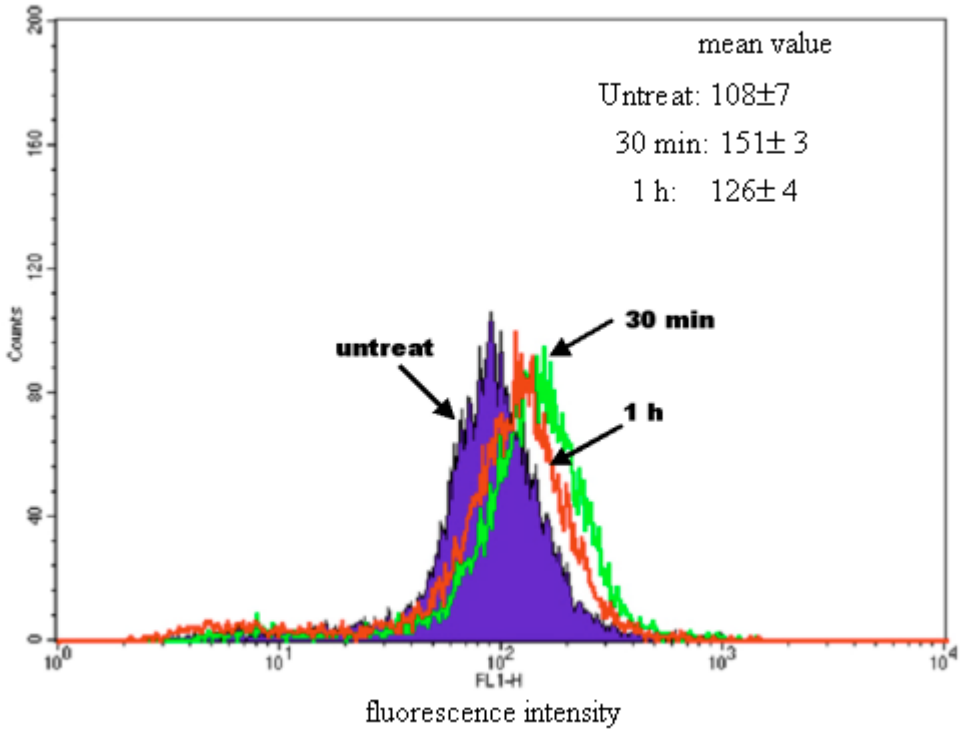


Figure 8 (amox)

Fig. 8.