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IN-VITRO CYTOTOXIC, MUTAGENIC AND GENOTOXIC EVALUATION OF TILMICOSIN

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ABSTRACT

The efficacy of antibiotics is endangering worldwide due to rapid development of resistant bacterial strains. It is of worth to re-evaluate previously developed antibiotics to alleviate the pressure of microbial resistance on recently developed antibiotics. In this study *in vitro* toxicological evaluation (cytotoxicity, mutagenicity and genotoxicity) of tilmicosin was carried out. Different concentrations (3.12, 6.25, 12.5, 25, 50, 100, 200, 400 and 800µg/mL) of tilmicosin were used. *In vitro* cytotoxicity was evaluated using MTT Assay. BHK-21 cells and cardiac cells from chicken embryo were used. The mutagenic potential was evaluated using Ames test. Auxotroph strains of *Salmonella typhemurium* (TA-98 and TA-100) were used. The genotoxicity was assessed using comet assay. Cell survival percentage of BHK-21 cells at 50µg/mL was 50.64% and IC_{50} was 49.64µg/mL indicating that below 49.64µg/mL all concentrations were not cytotoxic. While the cell survival percentage of chicken cardiac cells at 25µg/mL was more than 50% and at 50µg/mL was less than 50%. The IC_{50} calculated was 52.75µg/mL indicating that below 52.75µg/mL all concentrations are not cytotoxic for cardiac cells. The mutagenic indices of all concentrations of tilmicosin with and without S9 fraction were ≤ 2 both for TA98 and TA100. So all the concentrations are non-mutagenic for both TA 98 and TA 100. For genotoxicity, comets were analyzed for head diameter and tail length. The damage indices were calculated. The damage was concentration dependent. The damage indices at concentrations 3.12, 6.25, 12.5 25 and 50µg/ml were not significant ($P<0.01$) while significant at 100, 200, 400 and 800µg/ml when compared with control ($P<0.01$). It may be concluded from this study that tilmicosin has not produced cytotoxicity, mutagenicity and genotoxicity at low concentrations under *in vitro* conditions.

Key words: Tilmicosin, MTT Assay, Chicken cardiac cells, Ames test, Comet Assay

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INTRODUCTION

Antibiotics have performed an overwhelmingly a significant role in staving off bacterial infections since by Alexander Fleming who first time discovered penicillin in 1928. Macrolides belongs to the group of antibiotics which were isolated from bacteria present in the soil i.e., genus *Streptomyces* (Riviere and Papich, 2017). They are efficacious against *Mycoplasma* spp. and many gram-positive organisms, including *Staphylococcus* and *Streptococcus* spps. Tilmicosin a macrolide antibiotic prepared by chemical alteration of desmycosin and it interferes with bacterial protein synthesis (Yazar *et al.,* 2001). It is a broad-spectrum antibiotic (Ziv *et al.,* 1995). It is available in injectable and feed premix mixture. Tilmicosin is usually recommended for veterinary use in the prevention and treatment of pneumonia in sheep, pigs and cattle, associated with *Actinobacillus pleuropneumoniae, Pasteurella multocida, Pasteurella haemolytica.* Many species of Mycoplasma and many other microorganisms which are susceptible to this compound. The dosage of tilmicosin as injectable in sheep and cattle is single subcutaneous (SC) administration at the dose of 10mg per kg body weight. In poultry the recommended dosage of tilmicosin is 10-20mg per kg bodyweight orally (Kempf *et al.,* 1997). It has some diverse side effect as from other antibiotics of macrolide's group. It induces cardio toxicity (Yapar *et al.,* 2006). Cardioxicity produced by tilmicosin is mostly obvious with injectable dosage forms (Prescott, 2009).

Due to rapid emergence of microbial resistance against newly developed antibiotics doctors have started to prescribe old antibiotics as they may still be effective against many of MDR or XDR pathogens. These early, now resurgent, antibiotics were developed during 1950s–70s and were never developed fulfilling the due process of drug development and after necessary regulatory approval. Because the existing requirements and standards for clinical testing have evolved over the time (Theuretzbacher *et al.,* 2015).

Candidate drugs required *in vitro* testing for their toxicity evaluation during pre-clinical phase and there is a need of more reliable models for testing drug toxicity, as too many drug approval failures occur with the current models (Madorran *et al.,* 2020; Legrt, *et al.,* 2016). Cytotoxicity evaluation procedures are used *in*

vitro to evaluate the efficacy and safety of different drugs on the growth, reproduction and morphology of the cells. These procedures are preferred as a pilot project testing and as significant indicators for toxicity evaluation of drugs as they are simple, fast, having high sensitivity and may save animals from toxicity (Li *et al.,* 2015).

As bacteria are becoming resistant against antibiotics world-widely. It is the need of hour to develop new antibacterial agents and to re-evaluate the previously developed antibiotics to alleviate the pressure of microbial resistance on recently developed antibiotics (Theuretzbacher *et al.,* 2015). A very limited data is available regarding the toxicity of tilmicosin under *in vitro* conditions. In this study *in vitro* cytotoxicity, mutagenicity and genotoxicity evaluation of tilmicosin was carried out. Cytotoxicity was evaluated on BHK-21 cells and cardiac cells from chicken embryo using MTT Assay. Auxotroph strains of *Salmonella typhemurium* (TA 98 and TA 100) were used to evaluate the mutagenic potential of tilmicosin using Ames test. The genotoxicity was assessed using comet assay.

MATERIALS AND METHODS

The research work was conducted in Pharmacology Department in collaboration with Quality Operational Laboratory, UVAS, Lahore.

Experimental protocol: The concentrations of tilmicosin selected for this study were based on toxicokinetics data which provides prescribed data of drug's action at peak plasma concentrations and enhancement in dose to assess acute, sub-acute, sub-chronic and chronic drug toxicity. Lastly, the relation to effective and lethal doses of tilmicosin (100-800 µg/mL) was also taken under consideration (Moore *et al*., 1996). Hence, selective range of concentrations of tilmicosin (3.12, 6.25, 12.5, 25, 50, 100, 200, 400 and 800µg/mL) were evaluated for cytotoxicity, mutagenicity and genotoxicity.

Cytotoxicity determination through MTT Assay: Cytotoxicity was evaluated using MTT Assay. BHK-21 cells and cardiac cells from chicken embryo were used. BHK-21 cells line was taken from Quality Operational Laboratory of university of Veterinary and Animal Sciences, Lahore Pakistan. The cell line was revived and percentage viability was determined. For cardiac cells the heart was isolated from 19 days old live chicken embryos and cut into small pieces. It was then mixed with 0.25% trypsin and shifted to flask for continuous stirring. The cell suspension was then centrifuged. The pellet was taken and suspended in DMEM having 10% fetal bovine serum. Cell culture flasks were then incubated for 48 hours and quantified on hemocytometer. Percentage cell viability of cardiac cells was determined.

% viable cells = Number of viable cells/mL x 100 Total number of cells /mL

The assay was carried out by seeding confluent monolayer of chicken cardiac cells in the flat bottom 96 well cell culture plates and after that treated these cells with 3.12, 6.25, 12.5, 25, 50, 100, 200, 400 and 800µg/mL concentrations of tilmicosin. DMSO 20% was negative control and cell culture media was positive control. The strength of viable cells was calculated with the help of ELISA reader and optical density at 570nm of every well was taken (Seidl and Zinkernagel, 2013). The cell survival percentage (CSP) values were calculated and used for determination of inhibitory concentration (IC_{50}) using Graph Pad Prism.

$$CSP: \frac{\text{Mean OD of test Chemical – Mean OD of negative Control}}{\text{Mean OD of Positive Control}} \times 100$$

Mutagenicity determination through Ames test: Mutated strains of *Salmonella typhimurium* TA-98 and TA-100 were used. The bacterial culture was kept in the incubator at least for 24 hours. Single colony was selected and splashed on agar plates. Purification of tester strain and genetic analysis was done. Mutagenicity testing of all the concentrations (3.12, 6.25, 12.5, 25, 50, 100, 200, 400 and 800µg/mL) of tilmicosin was performed using plate incorporation assay with and without S9 mixture. Both strains were inoculated in a freshly prepared and sterilized nutrient broth for 15-18 hours prior to experiment. The numbers of bacteria were adjusted at 0.5 mcfarland (mcf) by amending the optical density with the help of spectrophotometer. Each drug concentration was treated with 0.1 mL suspension of bacteria (2 x 10^8 bacteria), incubated for 20 minutes and then spread over glucose minimal ager plates. Similarly, the procedure was performed again with S9 mixture by incorporating 0.5mL of S9 mixture in every drug concentration. To end with, histidine independent revertant colonies were counted manually and mutagenic index (M.I) was calculated with following formula (Mortelmans, Zeiger, 2000).

$$(M.I) = \frac{\text{Number of Revertant colonies per plate with test chemical dose}}{\text{Number of natural Revertant colonies of negative control plate}}$$

Results were interpreted as;

- Mutagenic index if ≥ 2 indicates that test concentration is not mutagenic.
- Mutagenic index if ≥ 3 indicates that test concentration is significantly mutagenic.

- Mutagenic index if ≥ 4 indicates that test concentration is very strongly mutagenic.

Genotoxicity determination through Comet Assay: Peripheral blood of healthy sheep was collected and was layered over 5 mL of lymphocyte separating media. Centrifugation was done at 8000g for 45 minutes at 25 °C. The buffy coat was pipetted out into 5 mL of RPMI 1640. Centrifugation was again done and pellet was re-suspended in RPMI 1640. Counting of lymphocytes was done using hemocytometer and final count was adjusted to $2x10^4$ cells per 100µL. The 1 mL of all the concentrations (3.12, 6.25, 12.5, 25, 50, 100, 200, 400 and 800µg/mL) of tilmicosin and 100µL of cell suspension were taken in eppendorf tube and incubated for 3 hours. Positive and negative controls were 20% and 1% DMSO respectively. Centrifugation was done for 5 minutes at 3000 rpm and pellets of lymphocyte were formed at bottom. The drug was extracted out and pellets were re-suspended in RPMI 1640. LMPA Layering was done, and then slides were dipped in lysis solution and then electrophoresis was done. Slides were neutralized and stained with ethidium bromide and observed under fluorescent microscope. Every slide was visualized under the microscope and 25 images of comet per slides were evaluated. Damage indices (D.I) were calculated.

D.I = number of cells in class1 + (2x number of cells in class 2) + (3 x number of cells in class 3)

The comets were classified into 4 classes depending on length of tail.
Class 0: Unharmed cells
Class 1: Length of tail was smaller or equivalent to diameter of head
Class 2: Length of tail was larger than diameter of head, but less than double diameter of head
Class 3: Length of tail was bigger than double of diameter of head

Data analysis: The results of MTT assay were analyzed by Graph Pad Prim 5.00 for windows. The results were explained in terms of Mean±SD. The results of Ames test and COMET assay were considered significant if the ($p<0.05$), ($p<0.01$) and ($p<0.001$). Analysis of variance (One-way ANOVA) was applied to the data to find the variance between tail lengths and DNA diameter followed by Tukey's multiple comparison *post hoc* tests, using Graphpad Prism V 5.0 (Graphpad Software, San Diego, CA) software. $P \leq 0.01$ were considered as statistically significant. (Etebari *et al.,* 2014).

RESULTS AND DISCUSSION

Cytotoxicity determination through MTT Assay: Cytotoxic effects of tilmicosin were evaluated on BHK-21 cell line and cardiac cell of chicken embryo. Percentage viability of BHK-21 cell line and cardiac cell of chicken embryo was 92.59% and 86.48% respectively. The cell survival percentages of BHK-21 cells treated with different concentration of tilmicosin were calculated (Figure 1). The cell survival percentage of tilmicosin at concentration of 50µg/mL was 50.64% and IC-50 calculated was 49.64µg/mL. This indicated that below 49.64µg/mL all concentrations of tilmicosin are not cytotoxic for BHK-21 cells. The cell survival percentages of chicken cardiac cells treated with different concentration of tilmicosin were calculated (Figure 2). The cell survival percentage of chicken cardiac cells at 25µg/mL was more than 50% and at 50µg/mL was less than 50%. The IC-50 calculated was 52.75µg/mL. This indicated that below 52.75µg/mL all concentrations of tilmicosin are not cytotoxic for cardiac cells. At concentrations 3.12, 6.25, 12.5, 25 and 50µg/mL, the cell survival percentages of BHK-21 cells were 81.29%, 73.24%, 69.35%, 62.69% and 50.64% and of chicken cardiac cells were 93.24%, 85.95%, 69.79%, 65.78% and 49.84% respectively. At 50µg/mL concentration had BHK-21 cell survival percentage 50.64%, while chicken cardiac cells have cell survival percentage 49.84%. The difference in the percentages of cell survival of both the cell lines is comparable with Fülöpová (2012) who evaluated the cytotoxicity of various concentrations of macrolides on different cell cultures *in vitro*. BHK cells proved to be most sensitive to tilmicosin. Mencucci *et al* (2013) compared the cytotoxic effects of azithromycin (preservative free) on epithelial cells of cornea *in vivo* with those of preservative free netilmicin and levofloxacin, and the benzalkonium chloride (BAK) having the preservative.

In this study the cell survival percentages in BHK-21 cells were 47.59%, 34.39%, 19.81% and 16.20% while chicken cardiac cells have cell survival percentages 43.82%, 31.57%, 21.75% and 11.93% when exposed to different higher concentrations (100, 200, 400 and 800µg/mL) respectively. The results were comparable with (Fülöpová 2012) where considerable reduction in vero cells was noted at concentration of 300 micro grams per ml. There a considerable decrease was seen in the count of vital BHK cells at the concentration of 150 micro grams per ml of spiramycin (Fülöpová 2012). While spiramycin at 300 micro grams per ml, no vital cells and only 17.53% of subvital cells were seen. The 500 micro grams per ml concentration reported 10.34% of vital FE cells. At 500 and 1000 micro grams per ml 22.48% of vital and 71.16% and 3.13% for vital and 70.52% of sub-vital vero cells were observed (Fülöpová 2012). According to our results the 100, 200, 400 and 800µg/mL concentrations of tilmicosin were toxic for both BHK-21 and chicken cardiac cells. The results are also in accordance with Fülöpová (2012). The findings are in comparison with Otoguro *et al* (1991) that the cytotoxicity of tilmicosin is dose dependent.

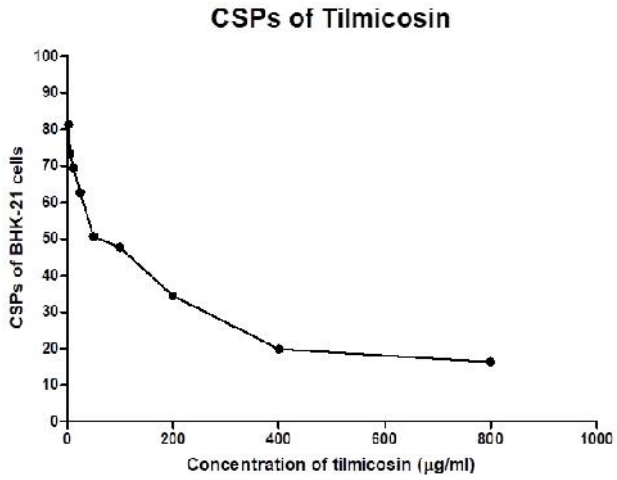

Figure 1: The Cell survival percentages of BHK-21 cells using 3.12, 6.25, 12.5, 25, 50, 100, 200, 400 and 800µg/mL concentrations of tilmicosin

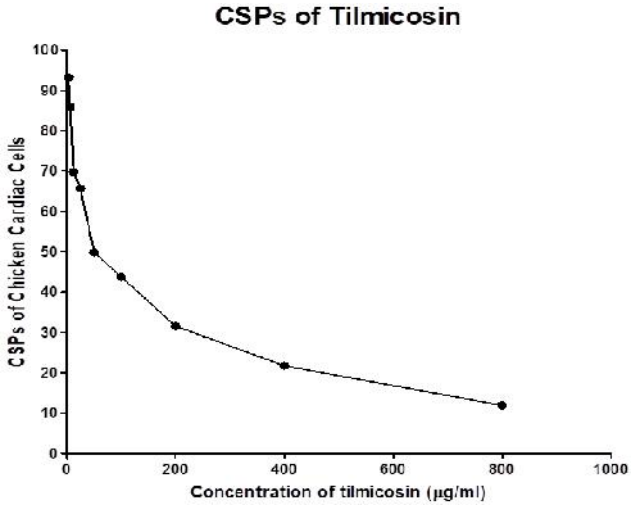

Figure 2: The Cell survival percentages of chicken cardiac cells using 3.12, 6.25, 12.5, 25, 50, 100, 200, 400 and 800µg/mL concentrations of tilmicosin

Mutagenicity determination through Ames test: The mutagenic indices of tilmicosin (Table 1) concentrations 3.12, 6.25, 12.5 and 25µg/ml were ≤ 2 both for TA 98 and TA 100. Isidori *et al.* (2005) investigated six antibiotics on aquatic organisms including Erythromycin, Oxytetracyclin, Sulfamethoxazole, Ofloxacin, Lincomycin and Clarithromycin. Erythromycin and clarithromycin showed no mutagenic effects. In this present study 25µg/ml treated tilmicosin group, the mutagenic indices of TA 98 were 1.27 and 1.14 with and without metabolic activation (S9) respectively and for TA 100 the indices were 1.09 and 1.12 with and without metabolic activation (S9 mix) respectively. Our results are in accordance with (Isidori *et al.* 2005) that different concentrations including 25µg/ml and below (3.12, 6.25, 12.5 µg/ml) were non-mutagenic on TA 98 and TA 100 with and without metabolic activation (S9 mix). The mutagenic indices of all the tilmicosin treated plates showed no increase when metabolic activation (S9) mixture was used. The S9 fraction has been used in conjunction with the Ames test (Mortelmans and Zeiger 2001) to observe the mutagenic abilities of chemical compounds (*Sakura et al. 2004)*. The S9 mixture has been added to evaluate the metabolic firmness of the contestant agents (Wu and McKown 2004). Our research findings are in comparison with (Hirano and Takeda 1981) who tested a new macrolide antibiotic, Miokamycin (MOM), for mutagenicity through Ames *Salmonella* test and the dominant lethal assay. The drug has shown no mutagenic activities. David *et al* (1993) also studies the new macrolide, clarithromycin for mutagenic potential. No evidences of mutagenic potential were revealed from various *in vitro* and *in vivo* study assays. Tilmicosin is non-mutagenic. This is in accordance with the findings of David *et al* (1993). The findings of present study indicated that 50 µg/ml concentration of tilmicosin showed the mutagenic index ≤ 2 both for TA 98 and TA 100. The mutagenic indices were 1.17 and 1.14 for TA 98 with and without S9 mixture and 1.03 and 1.06 for TA 100 with and without S9 mixture respectively. Our results are in accordance with (Isidori *et al.* 2005). Tilmicosin at low concentrations is not mutagenic which is in accordance with David *et al* (1993), while at higher concentrations 100, 200, 400 and 800µg/ml, a drastic reduction in reversion of colonies was recorded (Table 1). This might be due to lethal concentrations of drugs that might cause death of bacteria.

Genotoxicity determination through Comet Assay: Comet assay was performed on 3.12, 6.25, 12.5, 25, 50, 100, 200, 400 and 800µg/mL concentrations of tilmicosin. Scoring of damage induced was determined by using image J software. The mean diameter of DNA head and tail lengths were determined (Figure 3). The comets were analyzed by evaluating tail lengths which came out following the exposure to different concentrations of tilmicosin. One way analysis of variance was applied for the comparison of means. The result exhibited considerable difference when compared ($p < 0.05$) with control group as described in (Figure 3). The damage of DNA calculated was concentration dependent. The damage indices of tilmicosin concentrations at 3.12, 6.25, 12.5 and 25, 50 µg/ml were not statically significant but 100µg/ml exhibited significant ($P<0.01$) damage to DNA when compared with control. Tilmicosin showed significant damage indices ($P<0.01$) at 200, 400 and 800µg/ml concentrations (Table 2). No previous studies have shown the genotoxic potential of any concentration of tilmicosin. Our results suggested that these

concentrations of tilmicosin have no genotoxic effects, which is evident from comet tail length and head diameter of lymphocyte DNA. The research findings are also similar with Kayrald *et al* (2015) where genotoxicity of a macrolide antibiotic i.e. dirithromycin was evaluated in cultured human lymphocytes. No significant genotoxicity was observed. Our results suggested that tilmicosin at concentration of 100µg/ml caused significant damage to DNA. The damage index was 43, which is obvious from tail formation of comet and alterations in diameter of head of lymphocyte DNA. Rocco *et al* (2011) assessed genotoxicity of erythromycin and lincomycin by performing micronucleus test on red blood cells and done a comet assay on red blood cells and hepatocytes in fish. Both of drugs caused a significant enhancement in DNA tail moment and a significant increase in the frequency of micronucleus. Tilmicosin showed significant genotoxic damage indices at 200, 400 and 800µg/ml concentrations which were 52, 59 and 61 respectively. The results are in contrast with (Aziza *et al.* 2005) who investigated the effect of clarithromycin, a macrolide drug on the induction of chromosome aberrations in bone marrow of mice, spermatocyte and splenocyte cells. Its high doses produced a momentous enhancement in sister chromatid exchanges frequency in mice bone-marrow.

Conclusion: Tilmicosin was not cytotoxic at lower concentrations (3.12, 6.25, 12.5, 25 and 50µg/mL) while it is cytotoxic at higher concentrations *in-vitro*. It is non-mutagenic on all concentrations but addition of S9 mixture for both strains increased the mutagenic indices. For genotoxicity the concentrations including 3.12, 6.25, 12.5 and 25, 50 µg/ml are non-genotoxic while higher concentrations exhibited the genotoxicity.

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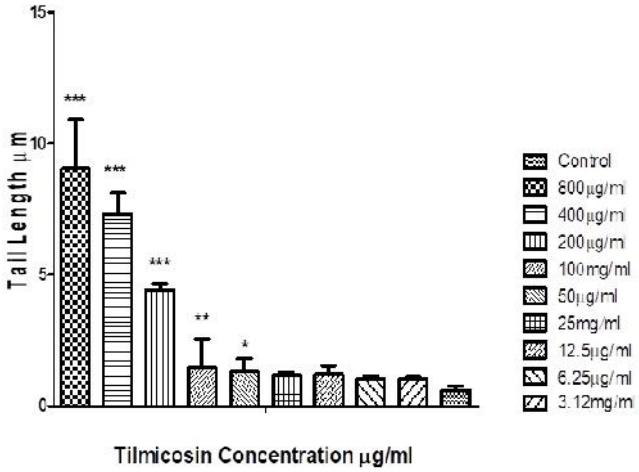

Figure 3: Tail lengths of damage induced in lymphocytes at 3.12, 6.25, 12.5, 25, 50, 100, 200, 400 and 800µg/mL concentrations of tilmicosin.

Table 1: Revertant colonies and mutagenic indices obtained after exposure with different concentration of tilmicosin.

Sr. #	Drug Conc. µg/ml	Revertant Colonies per Plate							
		TA98				TA100			
		-S 9	M.I.	+S 9	M.I.	-S 9	M.I.	+S 9	M.I.
1	3.12	53	1.35	81	1.40	140	1.42	176	1.49
2	6.25	52	1.30	77	1.35	143	1.45	143	1.21
3	12.5	49	1.22	70	1.22	124	1.26	166	1.25
4	25	51	1.27	65	1.14	112	1.09	121	1.12
5	50	47	1.17	65	1.14	101	1.03	126	1.06
6	100	38	0.95	60	1.05	92	0.93	112	0.94
7	200	33	0.82	55	0.96	84	0.85	115	0.97
8	400	34	0.85	54	0.94	81	0.82	98	0.72
9	800	30	0.75	48	0.84	80	0.81	100	0.79
10	Control +ve	540	13.50	779	13.66	756	7.71	1540	13.05
11	Control -ve	40		57		98		118	

$^{*}p<0.05$ $^{**}p<0.01$ $^{**}p<0.001$

Table 2: Damage indices of different concentrations of Tilmicosin.

Sr. No.	Conc. µg/ml	Class 0	Class 1	Class 2	Class 3	Damage Indices
1	3.12	24	1	0	0	1
2	6.25	24	1	0	0	1
3	12.5	23	2	0	0	2
4	25	19	5	1	0	7
5	50	15	4	5	1	17
6	100	7	4	3	11	43*
7	200	3	4	7	11	52*
8	400	2	2	6	15	59*
9	800	2	2	4	17	61*
+ve control	DMSO 20%	1	1	2	21	68
-ve control	RPMI-medium	25	0	0	0	0

N = 25 nuclei in two experiments. Nuclei with damage DNA from 0 (undamaged nuclei) to 3 (Damaged nuclei) * = significant difference ($p<0.01$) as compared to control analyzed by Graph Pad Prism.

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