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The Increment of Genoprotective Effect of Melatonin due to "Autooptic" Effect versus the Genotoxicity of Mitoxantron

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ABSTRACT

Background: Genotoxicity due to the effect of anti-cancer drugs on the healthy cells in cancerous patients is one of the problems of chemotherapy. Mitoxantrone is a chemotherapy anti-cancer drug, which can have side effects on the healthy cells like secondary cancers. On the other side, Melatonin is a hormone that is responsible for the daily rhythm adjustment and has several properties to be anti-cancer and anti-inflammation, and therefore it has a genoprotective effect against the genotoxicity. Recently, it has been shown that all living cells produce ultraweak photon emission (UPE) spontaneously and continuously. The intensity of UPE is in the order of a few, up to 104 photon/(cm^2 sec) (or $10-19$ to $10-14$ W/cm^2) measurable by photodetectors. UPEs are produced from diverse natural oxidative and biochemical reactions, especially free radical reactions and the simple cessation of excited molecules. Also, it has been evidenced that UPE has a signaling role at a distance among different cell cultures.

Objective: Here, we investigate the effect of UPE among similar cells (i.e. "Autooptic effect") by using mirrors around the cell plate(s). Here, we have used HepG2 as sample cancer cells.

Methods: In this study, the HepG2 cells were co-treated by melatonin as a genoprotective and silver nanoparticles as a carrier against mitoxantrone's genotoxicity. Our results are analyzed based on the Comet assay method, and the genoprotective effect of melatonin is investigated in presence of (and without) mirrors against the genotoxicity of mitoxantrone. Additionally, the autooptic effect is investigated in presence of Ag nanoparticles (NPs).

Results: The obtained results indicate that Ag NPs with lower concentrations of melatonin made more protection as genoprotective agent, and the same results obtained by increasing access' cells to drug.

Conclusion: The autooptic effect could increase the genoprotective effect of melatonin.

Keywords
Genoprotective effect, Melatonin, Genotoxicity, Mitoxantrone, Biophoton, Autooptic effect

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Introduction

Mitoxantrone (or Novantrone) is an injective anti-cancer drug [1-2] and though its connection to DNA causes cytotoxic and treating behavior in cancer cells, nevertheless this mechanism also has undesired consequences in the healthy cells like secondary cancers. For example, the treatment of MS and cancerous patients via mitoxantrone increased the risk of the secondary acute 'myeloid'

leukemia. Genotoxicity due to the effect of anti-cancer drugs on the healthy cells of cancerous patients, which increases the danger of the emergence of secondary cancers, is one of the problems of chemotherapy [3]. The DNA damage due to its contact to mitoxantrone via free radicals or direct connections has been proven in different studies. The free radical production by mitoxantrone metabolites had higher concentrations relative to by mitoxantrone [4-5]. Melatonin is a hormone that is responsible for the daily rhythm adjustment and has several properties to be anti-cancer and anti-inflammation [6-7]. Melatonin also has useful effects versus intense stress and it can keep the strength of the immune system against the weakening effects of the drugs. It is used besides the interleukin 2 for immunotherapy of cancer [8]. Moreover, Melatonin is an antioxidant that decreases the effect of free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) [9-12]. It has the ability to cross through the all membranes of living cells and penetrate the nucleus to be populated and protect DNA against harmful factors and even treat the harms [13].

Ultraweak Photon Emission in Living Cells

It has been clearly demonstrated that all living cells (without external excitation) spontaneously and continuously produce ultraweak photon emission (UPE) and the intensity of UPE is of the order of a few, up to 104photon/ (cm^2 s) (or equivalently 10-19 to 10-14W/cm^2) [21]. In fact, in different literature sources the UPE is referred to by different names such as ultraweak emission, biophotons, ultraweak bioluminescence, self-bioluminescent emission, photoluminescence, delayed luminescence, ultraweak luminescence, spontaneous chemiluminescence, ultraweak glow, biochemiluminescence, metabolic chemilumi-nescence, dark photobiochemistry, and bioluminescence [15]. It seems that biophotons are byproducts of metabolism inside the cells and

therefore they may be appeared as trivial signals. Nevertheless, increasing evidences are beginning to emerge that UPE may play important roles in cellular functions. The question about a functional role for weak intrinsic UPE has a rather long tradition [22], and particularly the question about a pure biological significance beyond the chemical role in electronic–photonic interactions is still very speculative and a matter of debate. Measuring this radiation requires single photon detectors, e.g., photomultipliers (PMTs). The measurement process itself has to take place in a dark room because the measurement of UPE, e.g., from the human hand surface, results in only a few photons per 100ms. UPEs are produced from diverse naturally occurring oxidative and biochemical reactions, especially free radical reactions and the simple quenching of excited molecules. Examples include non-enzymatic and enzymatic lipid peroxidation, the reactions in the mitochondrial respiration chain and peroxisomal reactions, oxidation of tyrosine and tryptophan residues in proteins, etc. [23, 24]. The main source of UPE derives from oxidative metabolism of mitochondria and lipid peroxidation that generate photon-emitting molecules such as excited triplet carbonyls R=O* and singlet oxygen1O2 [23, 24]. The spectrum of UPE from electronically excited species is mostly in the UV and visible region, i.e., 200–800nm. For example, Triplet excited carbonyls produce UPE in the range 350–550nm, singlet excited pigments in 360–560nm, dimolar singlet oxygen in 634nm and 703nm [17], and hydrogen peroxide in the range 520–650nm [25].

## Non-Chemical Distant Cellular Interaction (NCDCI)

Basically, the cell-to-cell communication involves chemical or electrical signaling. In contrast, our understanding of non-chemical, non-electrical and non-mechanical forms of communication is still under debate [14]. There is growing experimental evidence that

cells and tissues may interact over distances even when chemically and mechanically isolated, probably via electromagnetic (EM) fields [15]. Stemming from the pioneering experiments of Gurwitsch in 1923 and 1924 [16], some researchers confirmed that cellular interactions may be mediated by EM fields [14]. There is no doubt that different EM fields can affect living cells and no question that living cells can generate EM fields, but the question is whether cells can affect each other via their EM fields? One of the possible candidates for non-chemical distant cellular interaction is UPE [17]. In fact, the intensity of UPE is so weak and looks unlikely to affect neighboring cells since under light condition the "competition between UPE and room-light" is not in favour of UPE, depending on room light intensity there are billions of photons per biophoton. Taking into account the modulation through absorption, the negative feedback loop and the relative amount of photons, it is understandable that not a high amount of photons will have a high amount of information, but a low amount of photons is more effective and the ratios and therefore the shape of wavelength spectra of the emission patterns are the key factors, and not the high intensity of light.

Auto-optic Effect

One way to investigate the treatment based on UPE is to "optically" enclose the sample with mirrors (Figure 1) [18]. In this case, the sample cells receive their UPE and not from the distant isolated neighbor cells. It can be called auto-optic effect or self-NCDCI. Initially, this method was used to treat only psychosomatic problems. [18]

However, its modification proved to have a broader range of therapeutic applications [18]. An important point of the first clinical observations [18, 19] was the spectrometric detection of the response in patient blood. Namely, after treatment, there were significant increases in the enzymatic and antioxidant activities of the blood (absorption changes in the 230-
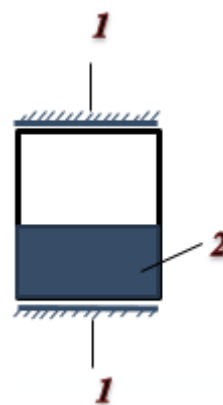
Figure 1: The scheme of placing (1) mirrors relative to (2) the sample (i.e. HepG2 cells).

240 and 400-415-nm regions of wavelengths, respectively). We attempted to explain cellular interactions via light. Roughly speaking, our approach can be described as follows: first, the UPE is produced by excited species in the emitter cells. The signal is then transformed through absorption or/and spectrally modified by auto-fluorescence, by the emitter cell's contents, to then escape the emitter cells. Finally, they hit the mirrors around the plate to increase the absorption of the light information to the nucleus or other biochemical pathways.

Material and Methods

Chemicals

Tris, Triton X-100, H_2O_2, NaCl, EDTA, NaOH and NaH_2PO_4 were obtained from Merck Co. (Germany), Melatonin and mitoxantrone were respectively purchced from Sigma (USA) and Kocak Farma (Istanbul). LMA and NMA were respectively gained from Sigma (USA) and CinnaGen (Iran). Melatonin kept at -20ºC, Mitoxantrone was selected for present study due to their extensive clinical use in Iran. Preparation of drug solution: The concentrations of 0.05 µM for Mitoxantrone and 50 µM for Melatonin in 0.01 % ethanol were prepared.

Cells

HepG2 cells (1×10^6cells/ml) were incubated at 37º C and cultured in dishes in modified medium supplemented with 10% fetal bovine serum (FBS) and antibiotics.

Groups of treatment

The present study includes the following experimental group (Figure 2):

1) The Positive control (Mitoxantrone: 0.05 µM)

2) Mirror free (Mitoxantrone: 0.05 µM + Melatonin: 5o µM)

3) With mirror (Mitoxantrone: 0.05 µM + Melatonin: 5o µM + mirror)

Cells were treated with MLT (melatonin) (5o µM), for one hour using 2 mirrors up and down of plate (group 3) and without mirror (group 2), then Mitoxantrone (0.05 µM) as a mutagenic factor was added for next one hour incubation.

After treatment, cells were washed with PBS (Phosphate buffered saline, pH= 7.4), then centrifuged, the supernatant discarded and cells were resuspended in low melting point agarose (LMA) and plated on slides which were covered with normal melting point agarose (NMA). The slides were placed in cool (-4 ºC) and dark condition for 10 minutes. Slides were immerged in lysing solution (pH=10.5) for 40 minutes and washed out with distillated water.
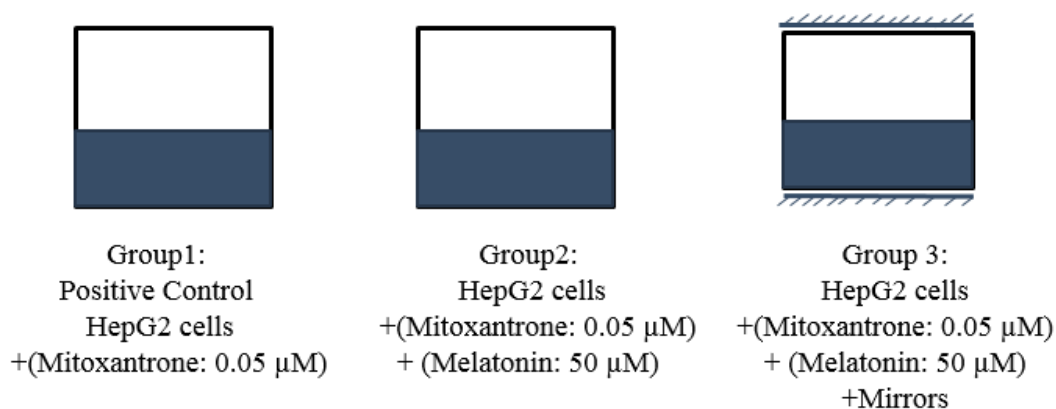
Then slides were purchased in electrophoresis buffer (PH=13-13.5) for 40 min. Electrophoresis process was carried out for 40 min (25 V and 280 mA).

At the next stage, slides were placed in tries buffer (PH=7.4) for 10 min. The slides were colored with ethidium bromide for 5 min and washed with PBS and water. A fluorescence microscope (CETI) was used in preparation of images. The comets were analyzed with the Comet Score program and three factors of tail length, Percentage of DNA in tail and Tail moment were used in statistical analysis.

Comet assay

We used the Comet method [20] for evaluation of chemotherapy drugs (e.g. mitoxantrone) genotoxicity, protection and recovery. The Comet assay (or SCGE) is a cheap, sensitive and standard experimental method for evaluation of DNA damage and has many applications in human biomonitoring, ecologic monitoring and DNA damage evaluation before (and during) the treatment. The comet assay procedure has been described in our previous studies [26-28]. Briefly, incubated cell suspensions (1×10^6 cells/ml) were mixed with LMA and were placed on the precoated slides (1 % NMA (Sigma)). The slides were respectively incubated with lysis solution (pH = 10.0) and electrophoresis buffer (pH > 13.0) for 40 minutes. Electrophoresis was done for 40 minutes (25 V, 280 mA). After this stage,

Group1:
Positive Control
HepG2 cells
+(Mitoxantrone: 0.05 µM)

Group2:
HepG2 cells
+(Mitoxantrone: 0.05 µM)
+ (Melatonin: 50 µM)

Group 3:
HepG2 cells
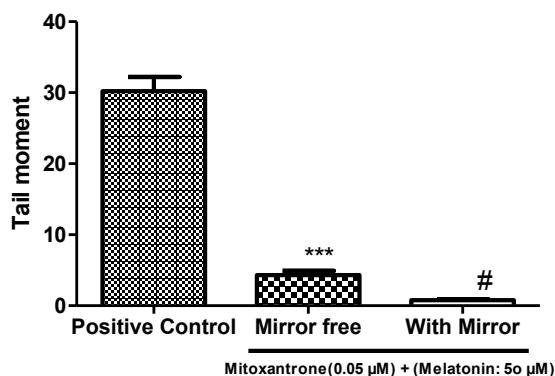+(Mitoxantrone: 0.05 µM)
+ (Melatonin: 50 µM)
+Mirrors

Figure 2: The scheme of experimental groups

the slides were placed in neutralization solution (pH = 7.5) for 10 minutes, covering by sufficient dye solution (20 µg/ml ethidium bromide) for 10 minutes and washed with PBS (two times 10 minute each) and distillated water. Finally comets were visualized under × 400 magnification using fluorescence microscopy with an excitation filter of 510-560 nm and barrier filter of 590 nm. All stages of comet assay were performed at cold place in dark conditions and all solutions were prepared freshly and used cool.
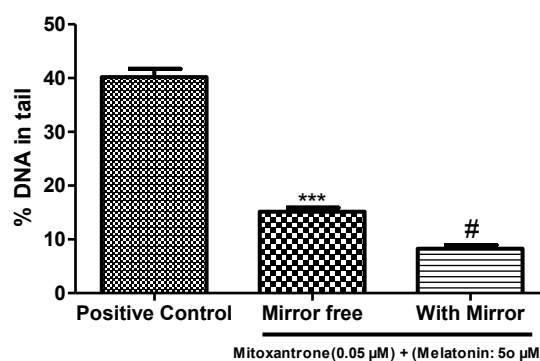
Statistical analysis

Results of Tail length, Percentage of DNA in tail and Tail moment were reported as mean ±SEM and were analyzed using Graphpad softwar Inc, USA.

The results of One way analysis of variance (ANOVA) for the Tail length and Percentage of DNA in tail showed significancy of $p<0.001$. Moreover, the results of Tukey's post hoc test determined significant differences (*** $p<0.001$) and (### $p<0.001$) respectively incompaired with the positive control group and Mirror free group for both of parameters (Figures 3 and 4).

Figure 4: The comparison of Percentage of DNA in tail for the studied groups (Positive Control, Mirror free and With mirror groups). Each group has represented as Mean ± SEM. The signes of *** and ### represents significancy of $p<0.001$ respectively in compare with the Positive control and the Mirror free group.

The result of One way analysis of variance (ANOVA) for the Tail moment showed significancy of $p<0.001$. Moreover, the results of Tukey's post hoc test determined significant differences (*** $p<0.001$) and (# $p<0.05$) respectively incompaired with the positive control group and Mirror free group (Figure 5).
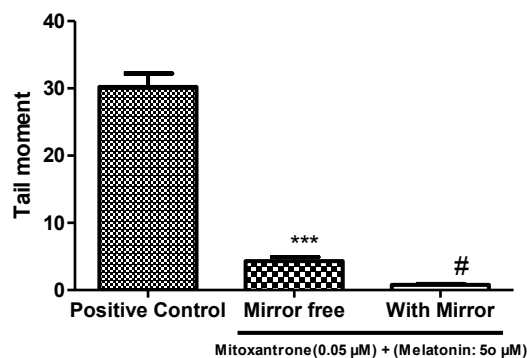
Figure 3: The comparison of tail length for the studied groups (Positive Control, Mirror free and with mirror groups). Each group has represented as Mean ± SEM. The signs of *** and ### represents significancy of $p<0.001$ respectively in compare with the Positive control and the Mirror free group.

Figure 5: The comparison of Tail moment for the studied groups (Positive Control, Mirror free and with mirror groups). Each group has represented as Mean ± SEM. The signs of *** and # represents significancy of $p<0.001$ and $p<0.05$ respectively comparing with the Positive control and Mirror free group.
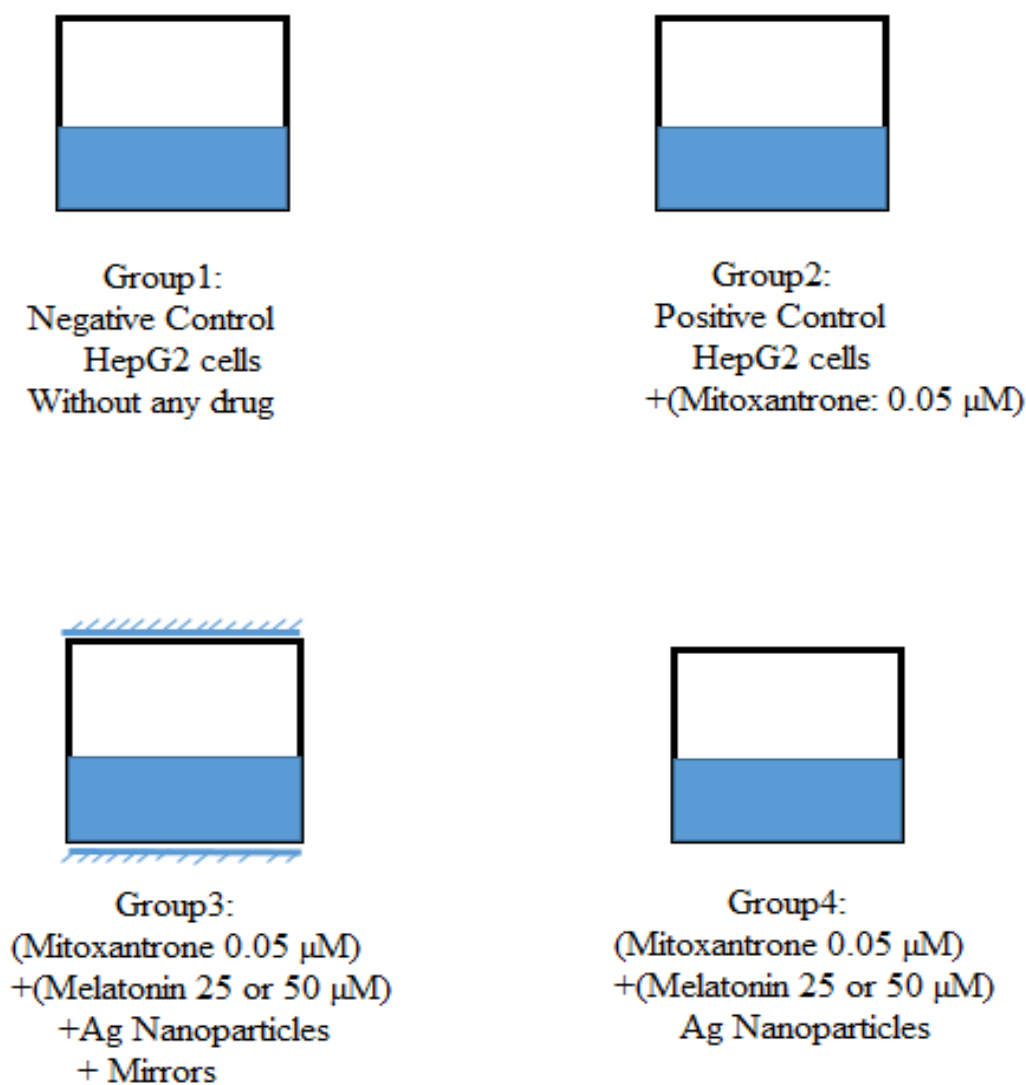
The Effect of Nanoparticles

Numerous studies have been published reporting DNA damage by silver nanoparticles (Ag NPs) [30, 31]. Despite having different surface chemistry type of Ag NPs induce different DNA damage response, Ag NPs' size is involved [32]. In this study, the HepG2 cells were co-treated by melatonin as a genoprotective and silver nanoparticles as a carrier against mitoxantrone's genotoxicity.

Materials and methods

The materials and methods regarding the chemicals and cells are the same as the section 4.

Groups of treatment

The following experimental groups were obtained: negative control group (HepG2 cells without drug or Mitoxantrone); positive control group (HepG2 cells + Mitoxantrone (0.05 μM)), different concentration of Ag NPs (0.00 5 or 0. 01 μl) + melatonin (25 or 50 μM) an one-hour period incubation (co-treatment) + Mitoxantrone (0.05 μM); different concentration of Ag NPs (0.00 5 or 0. 01 μl) + melatonin (25 or 50 μM) an one-hour period incubation (co-treatment) + Mitoxantrone (0.05 μM) + Mirror group (Figure 6).

Group1:
Negative Control
HepG2 cells
Without any drug

Group2:
Positive Control
HepG2 cells
+(Mitoxantrone: 0.05 μM)

Group3:
(Mitoxantrone 0.05 μM)
+(Melatonin 25 or 50 μM)
+Ag Nanoparticles
+ Mirrors

Group4:
(Mitoxantrone 0.05 μM)
+(Melatonin 25 or 50 μM)
Ag Nanoparticles

Figure 6: The scheme of treatment's groups

Statistical analysis

In order to comparison between the drug treated and untreated samples we used the analysis of variance (ANOVA), results of tail length, percentage of DNA in tail and tail moment were reported as mean ± SE. Furthermore, post hoc analysis using Graphpad softwar Inc, USA were performed [20]. One-way analysis of variance [29] was used to compare the results of comet assay, followed by Tukey's multiple comparison post hoc test. The significance level of 0.05 was considered as statistically significant.

Results

To determine the effect of Ag NPs on genoprotective concentration of melatonin, HepG2 cells were separately incubated with different concentrations of Ag NPs for 2 hours fallowed by the comet assay. Three factors including tail length, percentage of DNA in tail and tail moment (% DNA in tail × tail length) were measured and compared with the control group. Concentrations of melatonin (25 and 50μM) (Figure 7), Mitoxantrone (0.05μM) and Ag NPs (0.005 and 0.01μl) were selected as a suitable dose and comet properties were studied (Figure 8).

Based on our analysis it looks that the high protection could take more melatonin up by penetration of membrane which created by AG NPs [33]. The three comet assay parameters of DNA damage (tail length, %DNA in tail and tail moment) are utilized, and our data revealed a significant decrease in all three parameters in presence of silver nanoparticles comparing with the control group. When we examined the relationship between dosages of nanoparticles, significant correlations were found between all comet parameters and dosage of silver nanoparticles. Two concentrations of Ag NPs were compared, which the higher dosage was significantly more protecting contrast to half-concentration of Ag NPs subjects.
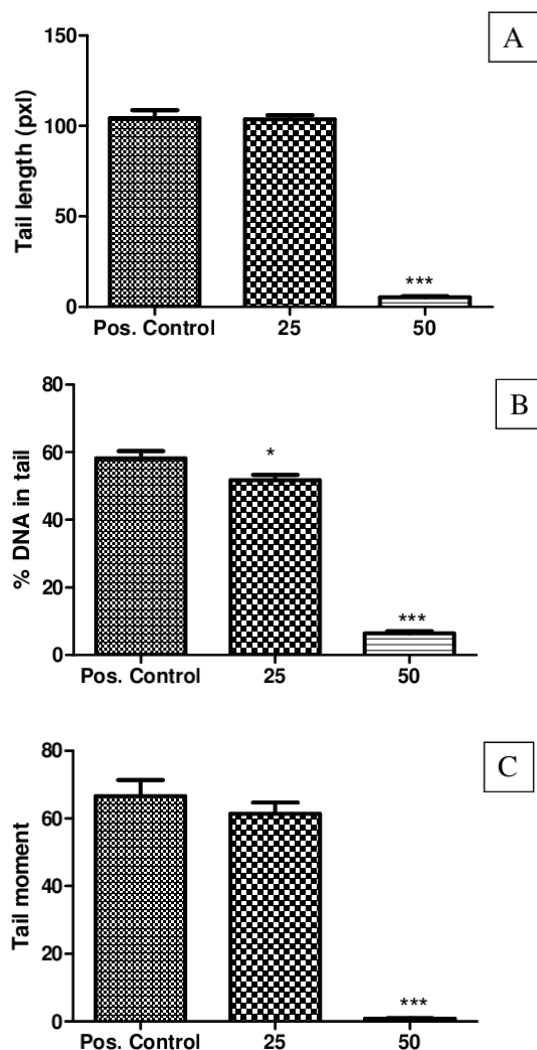

Figure 7: Comparison of (A): Tail length, (B): % DNA in tail and (C): Tail moment of concentrations of melatonin (25 and 50 μM) and mitoxantrone (0.05 μM) as positive control. Each graph has been represented as Mean ± SEM. Signs (***) and (*) show respectively significancy of (P<0.001) and (P<0.05) in comparison with the positive control group.

Conclusion

Our results indicated that the genoprotective effect of melatonine in presence of mirrors had significant difference with one without mirrors (p<0.05) against the genotoxicity of mitoxantrone (Figure 9). Therefore, it appears that
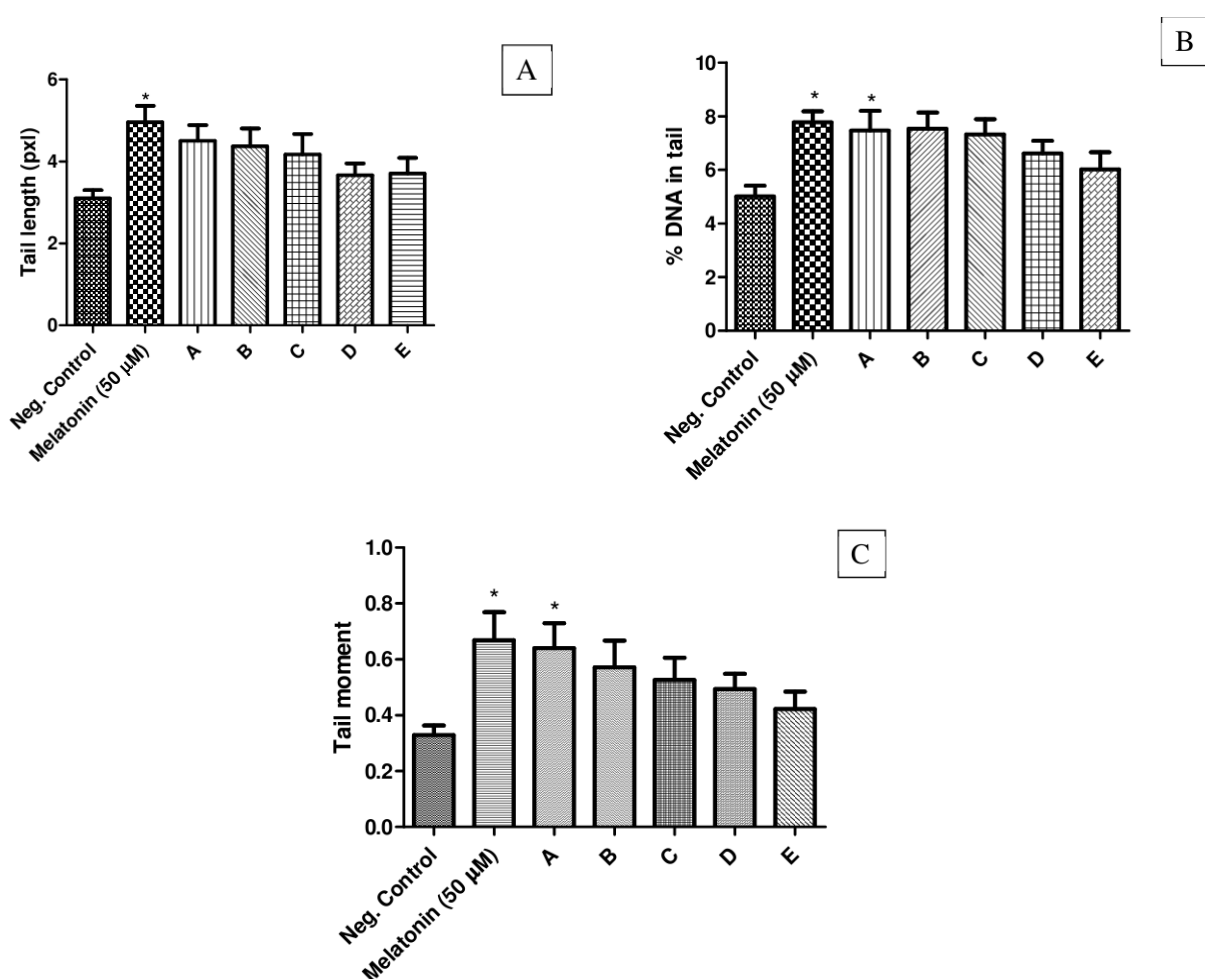
Figure 8: Comparison of (A): Tail length, (B): % DNA in tail and (C): Tail moment of Melatonin (50 μM), A: Melatonin (25 μM) in presence of mirror, B: Melatonin (25 μM) + Ag nanoparticles (0.005 μl), C: : Melatonin (25 μM) + Ag nanoparticles (0.01 μl), D: : Melatonin (25 μM) + Ag nanoparticles (0.005 μl) in presence of mirror, E: Melatonin (25 μM) + Ag nanoparticles (0.01 μl) in presence of mirror. Each graph has been represented as Mean ± SEM. Sign (*) shows significancy of (P<0.05) in compare with the negative control group.
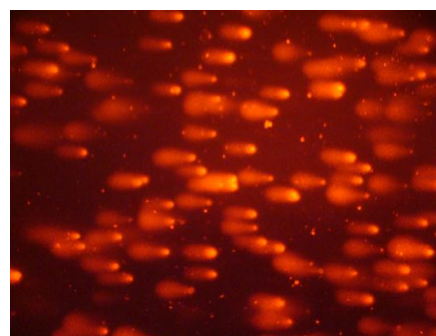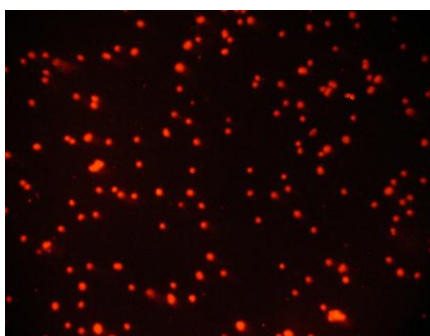
Figure 9: Left) The image of HepG2 cells taken by fluorescent microscope (400x), Right) The image of HepG2 cells imposed to Mitoxantrone.

the autooptic effect could increase the geno-protective effect of melatonin. Regarding the presence of Ag NPs, the genoprotective effect of Ag NPs is dose dependent. Indeed, Ag NPs with lower concentrations of melatonin made more protection as genoprotective agent, and the same results obtained by increasing access' cells to drug.

Acknowledgment

Conflict of Interest

None

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