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DNA Binding and its Degradation by the Neurotransmitter Serotonin and its Structural Analogues Melatonin and Tryptophan: Putative Neurotoxic Mechanism

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Abstract

Over the years, DNA damage has been suggested to be directly linked to mutagenesis, carcinogenesis and ageing. There is ample evidence suggesting that considerable DNA damage may be induced by metabolites produced during normal or aberrant metabolic processes. Herein, we examine the chemical basis of cytotoxicity of some endogenous metabolites. Towards this goal, we study the DNA reactive activities of the neurotransmitter serotonin and its structural analogues tryptophan and melatonin in the presence of copper. Fluorescence spectroscopy reveals a simple mode of interaction of these metabolites with calf thymus DNA and copper ions. The results of agarose gel electrophoresis demonstrate copper mediated strand scission in plasmid pBR322 DNA by these metabolites. Further, the relative DNA binding affinity order was affirmed using molecular docking studies involving the interaction of these metabolites with calf thymus DNA. We employ single cell alkaline gel electrophoresis and determine the relative efficiency of cellular DNA breakage as Serotonin>Melatonin>Tryptophan. We hypothesize that such cellular DNA breakage is mediated by mobilization of copper ions bound to chromatin. Consistent with previous observations that many known antioxidants of plant and animal origin also exhibit pro-oxidant properties, these metabolites might contribute to oxidative DNA breakage in the presence of redox active metals such as copper. Our findings reveal a putative mechanism by which some endogenous metabolites may cause DNA damage leading to mutations and genetic diseases.

Keywords: Endogenous metabolites; Copper; DNA damage; Neurotransmitters

Introduction

Although the major impetus of research into the role of DNA damaging agents has been primarily on exogenous toxic chemicals, products of endogenous origin have recently garnered significant research appeal [1]. It has been reported that the frequency of occurrence of endogenous DNA damage is higher than exogenous damage and the variety of damage induced owing to endogenous cellular processes is similar in nature to those produced by exogenous agents [2]. Several technological developments in this realm made it possible to identify adducts of endogenous origin in cellular DNA highlighting the role of endogenous genotoxins [3-7]. Interestingly, research studies examining exogenous carcinogens in animal models also identified analogous DNA adducts in control groups [8]. Such reports stimulated the search for the physiological products and associated pathways that generate these DNA adducts causing endogenous genotoxicity.

Serotonin (5-hydroxytryptamine), a neurotransmitter synthesized from tryptophan, affects several behaviors such as mood, hunger, aggressiveness, sleep, and consciousness [9]. Serotonin has been found to exert pro-oxidant effects as assessed by the deoxyribose and bleomycin assays [10]. Further, there has been growing concern related to Serotonin Toxicity (ST), also referred to as Serotonin Syndrome (SS), a drug induced manifestation of elevated intrasynaptic serotonin [11]. Melatonin (N-acetyl-5-methoxy-tryptamine), an indoleamine

derived from tryptophan, is produced in the pineal gland, mostly during the night [12]. This hormone is involved in the synchronization of circadian and seasonal alterations in physiology and neuroendocrine function [12,13]. Moreover, this endogenous secretory product is well-established as a strong reducing agent of oxidant species produced in the cell medium. Since 1993, when melatonin was initially classified as a scavenger of free radicals [14], numerous studies have substantiated that melatonin possesses the ability of protecting DNA from free radical damage. Therefore, melatonin is known to possess anti-carcinogenic, anti-ageing and anti-tumor properties. Nevertheless, although the majority of studies corroborate the antioxidant action of melatonin, it has been documented to exhibit pro-oxidant properties, in certain cases [15].

The accumulation of spontaneous mutations or mutations evoked by endogenous mutagens can cause genome instability and, hence, increases the sensitivity to exogenous carcinogens. As per the free radical theory of aging, free radicals produced as a result of oxidative reactions cause significant damage to macromolecules, eventually leading to aging [15]. It is noteworthy that the major endogenous source of oxidizing agents within cells is provided by transition metal-driven Fenton reactions which generate Reactive Oxygen Species (ROS) [16].

Copper is present in chromatin in close association with DNA bases, particularly guanine [17]. Although the concentration of iron is the highest in cells, copper and zinc are the major metals available in the nucleus [17,18]. Further, copper is known to possess the highest redox activity among transition metal ions [19], aiding rapid recycling, in the presence of molecular oxygen and compounds such as plant

polyphenols, resulting in the formation of ROS. Additionally, copper chelators such as 1,10-phenanthroline can mobilize chromatin bound copper leading to internucleosomal DNA fragmentation [20]. Evidence suggests that the copper levels in serum and tissue are significantly raised in cancer patients [21-25]. Copper is also implicated in the pathogenesis of several neurological diseases, including Alzheimer's disease, Amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, Prion disease, and Wilson's disease [26,27].

Previously, it has been shown that various antioxidants of both animal and plant origin are themselves capable of pro-oxidant action, and lead to the production of ROS, particularly in the presence of metal ions, such as copper [28-30]. Similarly, we have also shown that such properties are also exhibited by endogenous molecules of animal origin such as uric acid [31], L-DOPA [32] and bilirubin [28]. Copper ions have been demonstrated to bind with melatonin as well as serotonin [33]. It was therefore of interest to examine and compare the DNA binding and degradation by the neurotransmitter serotonin and its structural analogues tryptophan and melatonin mediated by copper. Our results imply that serotonin and to a lesser extent melatonin is capable of causing oxidative DNA breakage in the presence of redox active copper. Our results suggest an important mechanism underlying endogenous DNA damage. The structures of the compounds studied have been shown in Figure 1.
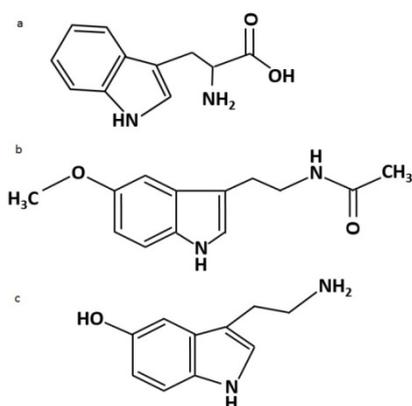

Figure 1: Chemical structures of the metabolites a) tryptophan, b) melatonin and c) serotonin.

Materials and Methods

Chemicals

Serotonin, melatonin, tryptophan, calf thymus DNA, agarose, Low Melting Point Agarose (LMPA), RPMI 1640, Histopaque 1077, phosphate buffered saline (PBS) Ca^{2+} and Mg^{2+} free, Triton X-100 and Trypan blue were obtained from Sigma (St. Louis, USA). Supercoiled plasmid pBR322 DNA was prepared according to the standard methods [34]. Serotonin, melatonin, tryptophan were dissolved in sterilized double distilled water as 1-2 mM stock solutions before experimentation. Upon addition to reaction mixtures, in the presence of buffers and at the concentrations used, the compounds remained in solution. Further, the volumes of stock solution added did not lead to any observable change in the pH of reaction mixtures.

Fluorescence spectroscopy

Fluorescence studies were carried using a Shimadzu spectrofluorometer RF-5310 PC (Kyoto, Japan) equipped with a plotter and a calculator. An excitation wavelength of 280 nm (approximate absorption maximum) was used for the compounds- tryptophan, melatonin and serotonin. Emission spectra were recorded in the wavelength range indicated in legends.

Molecular docking

Docking studies between double strand DNA with a sequence of d(CGCGAATTCGCG)2 dodecamer (PDBID: 1BNA) and compounds (tryptophan, melatonin and serotonin) were performed with the standard AutoDock (v4.2) suit employing Lamarckian Genetic Algorithm [35,36]. Prior to starting the docking protocol, the target B-DNA molecule and individual ligands were prepared using standard docking protocol and saved into 'PDBQT' format. The docking calculations involved ranking the target-ligand poses using energy based scoring function. In order to determine the potential binding sites of individual ligands in DNA as target, blind docking was performed. The input 'grid parameter' files were modified and the grid size was adjusted to X=56, Y=66 and Z=110 with 0.375 Å grid spacing. Rest all the docking parameters were set to default values. After docking, PyMOL software (Molecular Graphics System, version 1.5.0.1, Schrodinger LLC) was used to visualize the top pose conformation of each docked ligand for determining the possible interactions between ligand and DNA [37].

Agarose gel electrophoresis

To determine the reaction with plasmid pBR322 DNA, the reaction mixtures (30 μl) contained 10 mM Tris- HCl, pH 7.5, 0.5 μg plasmid DNA, and other components as mentioned in the figure legend. Incubation at room temperature was carried out for 1 hr at 37°C. Following incubation, 10 μl of a solution comprising of 40 mM EDTA, 0.05% bromophenol blue tracking dye, and 50% (v/v) glycerol was added, and electrophoresis was performed using 1% agarose gel. The gel was stained with ethidium bromide (0.5 mg/l), visualized and photographed using a UV transilluminator.

Isolation of lymphocytes

Fresh heparinized blood samples (2 ml) from a nonsmoking healthy donor were obtained by venepuncture and diluted appropriately in Ca^{2+} and Mg^{2+} free PBS. Histopaque 1077 (Sigma, St. Louis, MO, USA) was used to isolate lymphocytes from blood and once collected, isolated cells were suspended in RPMI 1640. All experiments were conducted using blood from the same donor (Nida Rehmani: author itself), in order to minimize variation between individual assays.

Viability Assessment of lymphocytes

The Trypan Blue Exclusion test by Pool-Zobel et al. was employed to assess the viability of the lymphocytes at the beginning and end of each reaction [38]. The viability of the cells was found to be greater than 93%.

Lymphocytes treatment and estimation of DNA breakage by comet assay

The isolated lymphocytes from 2.0 ml blood sample were diluted to the count of 2×10^5 cells/2 ml using RPMI 1640. About, 10,000 cells were then mixed with 75 μl of (pre-warmed) 1% low melting point agarose in PBS. Subsequently, this mixture was applied to a frosted microscopic slide layered with 75 μl of 1% normal agarose in PBS. The slides were then covered with coverslips and allowed to gel at 4°C for 10 mins for solidifying the agarose. These lymphocytes were treated with varying concentrations of compounds in a total reaction volume of 1 ml (400 μl RPMI, Ca^{2+} and Mg^{2+} free-PBS, and indicated concentrations of compounds) and processed for comet assay.

Comet assay was performed under alkaline conditions [39] with slight modifications as previously described [40]. Slides were scored employing an image analysis system (Komet 5.5, Kinetic Imaging, Liverpool, UK) equipped with a Olympus (CX41) fluorescent microscope and a COHU 4910 (equipped with a 510-560 nm excitation and 590 nm barrier filters) integrated CC camera. Comets were scored at a magnification of 100X. Images from 50 cells (25 from each replicate slide) were analyzed. Comet tail length (μm) was used as a parameter to evaluate lymphocytes DNA damage and was generated by the Komet 5.5 image analysis system.

Statistics

The statistical analysis was conducted according to the method described by Tice et al. [41] and is indicated as mean ±SEM/SD of three independent experiments.

Results

Interaction of calf thymus DNA with tryptophan/melatonin/serotonin

Figure 2 demonstrates the fluorescence emission spectra:
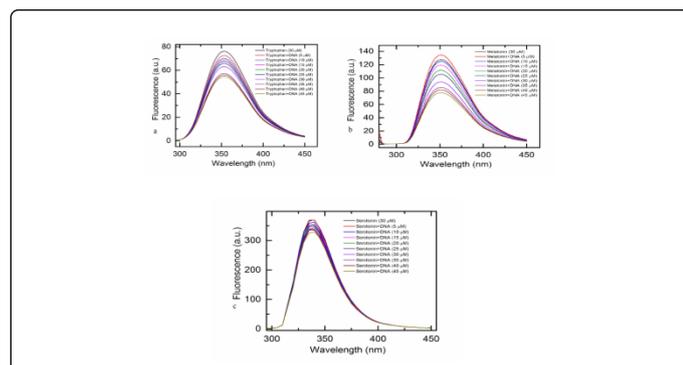

Figure 2: Effect of increasing concentrations of native calf thymus DNA on the fluorescence emission spectra of a) tryptophan, b) melatonin and c) serotonin. The compounds (in 10 mM Tris–HCl, pH 7.5) were excited at 280 nm in the presence of increasing native DNA base pair molar ratios. The emission spectra was recorded between 300-450 nm.

A) Tryptophan B) Melatonin and C) Serotonin in the presence of increasing molar base pair ratios of calf thymus DNA.

The compounds were excited at the wavelength indicated in the legends. A dose dependent quenching in the emission spectra of all the three metabolites is observed in the presence of DNA. However, no observable shift in the λ_{max} emission suggests a simple mode of DNA binding with these metabolites.

Binding of copper ions to tryptophan, melatonin and serotonin

The interaction of copper ions with tryptophan, melatonin and serotonin was investigated by studying the effect of increasing concentrations of Cu(II) on the fluorescence emission spectra of the compounds.
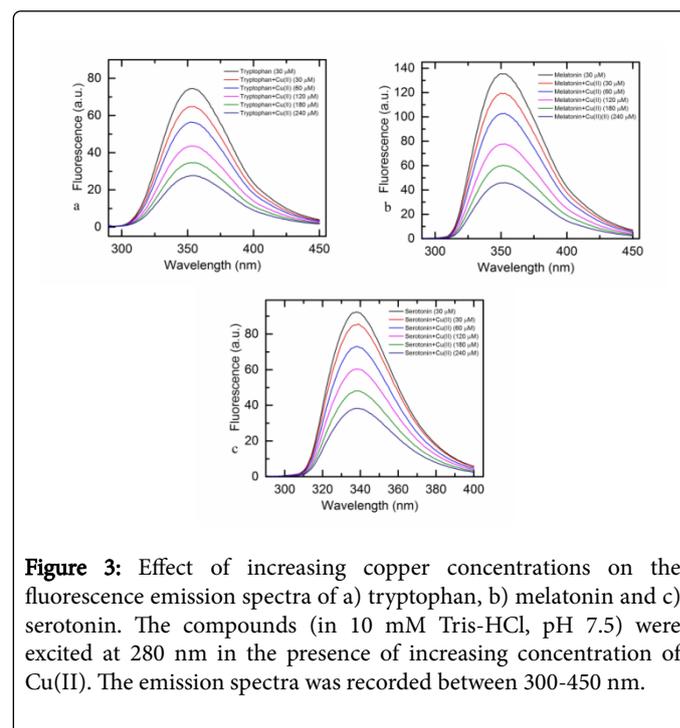

Figure 3: Effect of increasing copper concentrations on the fluorescence emission spectra of a) tryptophan, b) melatonin and c) serotonin. The compounds (in 10 mM Tris-HCl, pH 7.5) were excited at 280 nm in the presence of increasing concentration of Cu(II). The emission spectra was recorded between 300-450 nm.
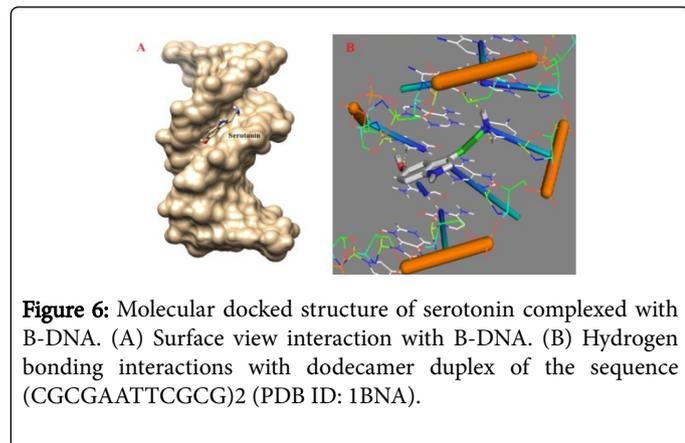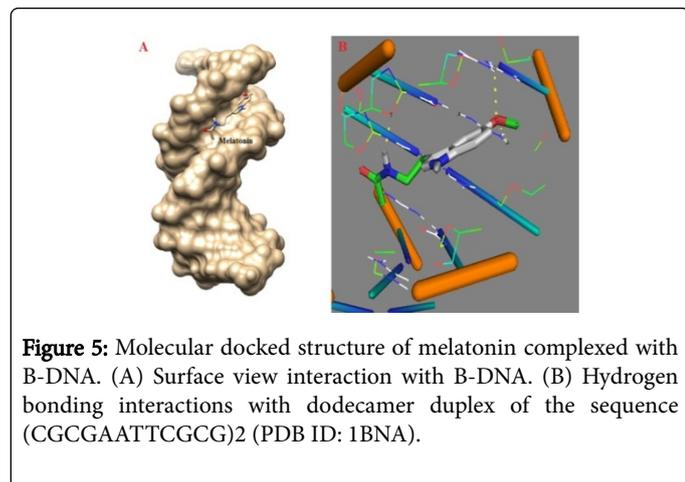
The results indicate that upon addition of Cu(II) to tryptophan (Figure 3a), melatonin (Figure 3b) and serotonin (Figure 3c), a quenching in the fluorescence spectra of these compounds is observed. Further, a decrease in the degree of fluorescence is observed with increasing concentrations of Cu(II). These results suggest that these metabolites are able to bind to copper ions.

Molecular docking studies

As evident from results (Figures 4-6 and Table 1), serotonin forms 4 hydrogen bonds with B-DNA leading to more negative AutoDock binding energy (−5.77 kcal/mol) and lesser AutoDock inhibition constant (59.4 μM). As compared to serotonin, melatonin forms only 3 hydrogen bonds with nitrogenous bases of B-DNA. This decreased hydrogen bonding caused less negative binding energy (−4.97 kcal/mol) and higher inhibition constant (227.88 μM).

On the other hand, tryptophan (Figure 4) holds a tendency to form only 2 hydrogen bonds with nitrogenous bases of B-DNA as compared to melatonin (Figure 5) and serotonin (Figure 6). Hence, formation of only 2 hydrogen bonds with nitrogenous bases of B-DNA renders the least stability to tryptophan with B-DNA amongst all three

metabolites. These results commensurate the relative cellular DNA breakage inducing capability of the three compounds employed in this study.
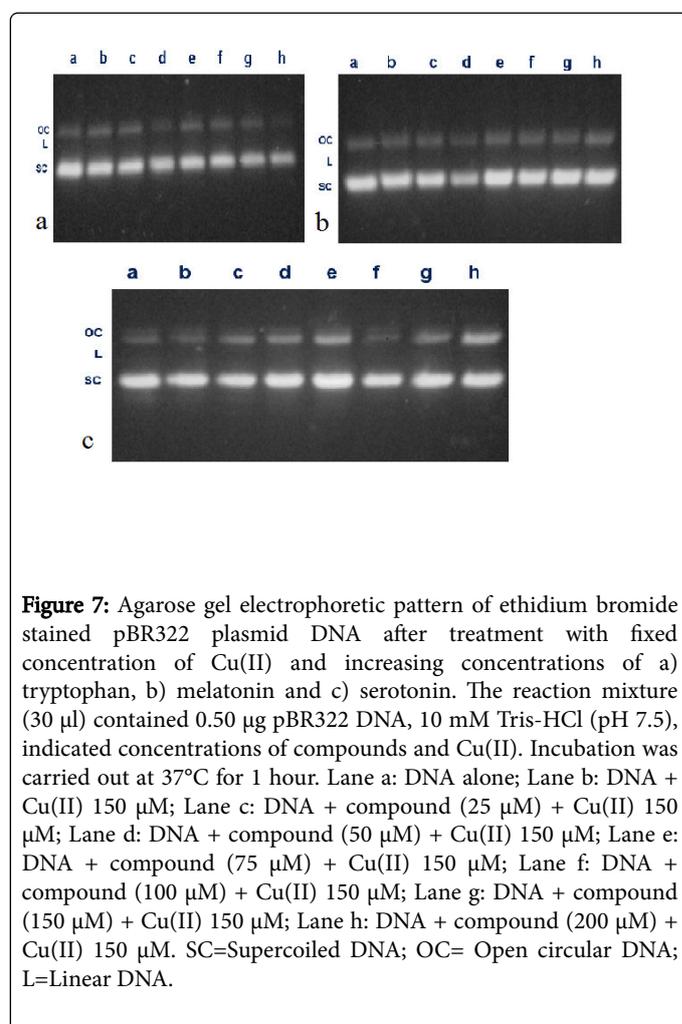

Figure 4: Molecular docked structure of tryptophan complexed with B-DNA. (A) Surface view interaction with B-DNA. (B) Hydrogen bonding interactions with dodecamer duplex of the sequence (CGCGAATTCGCG)2 (PDB ID: 1BNA).

Figure 5: Molecular docked structure of melatonin complexed with B-DNA. (A) Surface view interaction with B-DNA. (B) Hydrogen bonding interactions with dodecamer duplex of the sequence (CGCGAATTCGCG)2 (PDB ID: 1BNA).

Figure 6: Molecular docked structure of serotonin complexed with B-DNA. (A) Surface view interaction with B-DNA. (B) Hydrogen bonding interactions with dodecamer duplex of the sequence (CGCGAATTCGCG)2 (PDB ID: 1BNA).

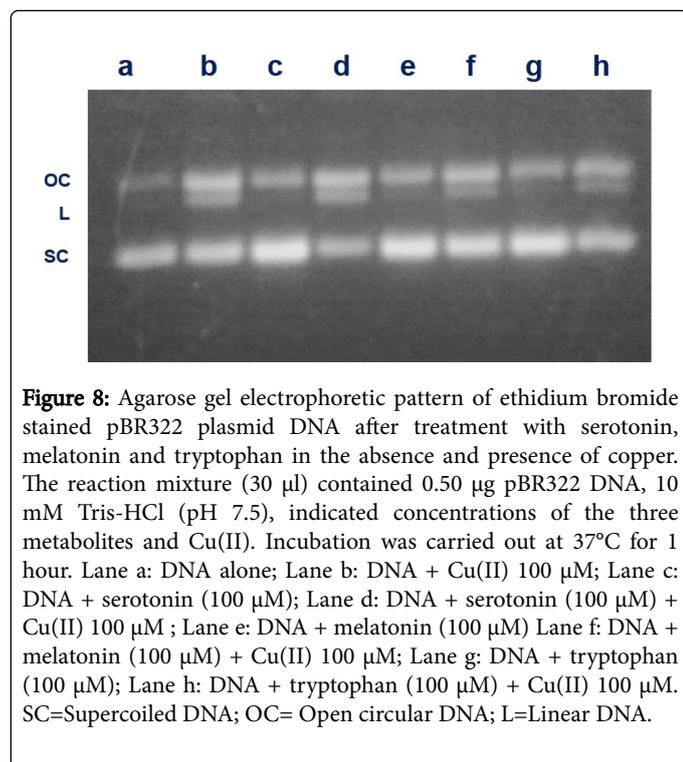| Ligand	AutoDock Binding Energy (Kcal/mol)	AutoDock Inhibition Energy (µM)	No. of Hydrogen bonds
Serotonin	−5.77	59.4	4
Melatonin	−4.97	227.88	3
Tryptophan	−4.31	692.18	2

Table 1: Hydrogen bonds, inhibition constant and binding energy as obtained by AutoDock results of three docked ligands.

Agarose gel electrophoresis of pBR322 plasmid DNA treated with compounds and copper

We examined the efficacy of tryptophan/melatonin/serotonin-Cu(II) system in cleaving plasmid DNA. For this, pBR322 plasmid DNA was treated with the compounds in presence of fixed concentrations of copper. As demonstrated in (Figure 7A) and (Figure 7B), tryptophan and melatonin do not induce significant DNA strand scission in plasmid DNA which correlates with their relative DNA binding affinity order. However, in case of (Figure 7C), the generation of open circular topological form of plasmid DNA suggests that serotonin is more potent in inducing DNA strand scission than its structural analogues.

Figure 7: Agarose gel electrophoretic pattern of ethidium bromide stained pBR322 plasmid DNA after treatment with fixed concentration of Cu(II) and increasing concentrations of a) tryptophan, b) melatonin and c) serotonin. The reaction mixture (30 µl) contained 0.50 µg pBR322 DNA, 10 mM Tris-HCl (pH 7.5), indicated concentrations of compounds and Cu(II). Incubation was carried out at 37°C for 1 hour. Lane a: DNA alone; Lane b: DNA + Cu(II) 150 µM; Lane c: DNA + compound (25 µM) + Cu(II) 150 µM; Lane d: DNA + compound (50 µM) + Cu(II) 150 µM; Lane e: DNA + compound (75 µM) + Cu(II) 150 µM; Lane f: DNA + compound (100 µM) + Cu(II) 150 µM; Lane g: DNA + compound (150 µM) + Cu(II) 150 µM; Lane h: DNA + compound (200 µM) + Cu(II) 150 µM. SC=Supercoiled DNA; OC= Open circular DNA; L=Linear DNA.
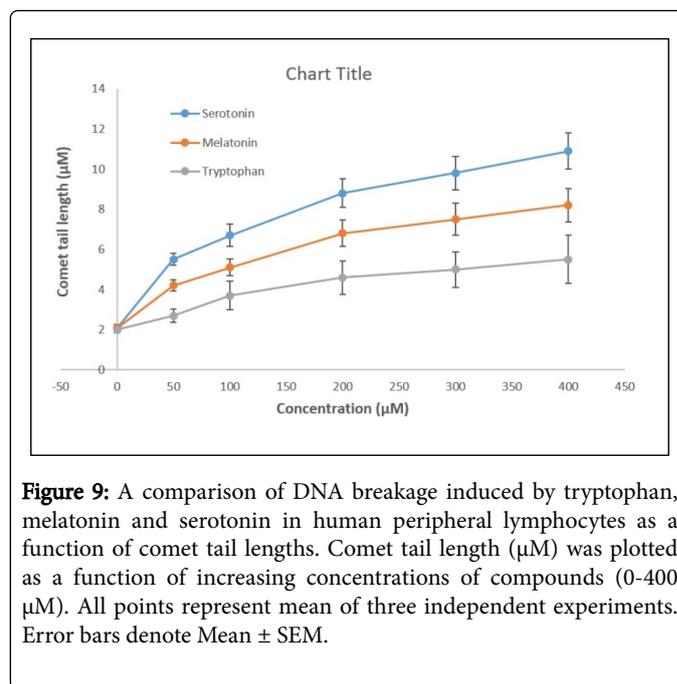
Further, for comparing DNA strand scission caused by serotonin and related metabolites in the presence of copper ions, we performed plasmid nicking assay with the three metabolites (serotonin, melatonin, and tryptophan) under identical conditions as depicted in (Figure 8).

Figure 8: Agarose gel electrophoretic pattern of ethidium bromide stained pBR322 plasmid DNA after treatment with serotonin, melatonin and tryptophan in the absence and presence of copper. The reaction mixture (30 µl) contained 0.50 µg pBR322 DNA, 10 mM Tris-HCl (pH 7.5), indicated concentrations of the three metabolites and Cu(II). Incubation was carried out at 37°C for 1 hour. Lane a: DNA alone; Lane b: DNA + Cu(II) 100 µM; Lane c: DNA + serotonin (100 µM); Lane d: DNA + serotonin (100 µM) + Cu(II) 100 µM ; Lane e: DNA + melatonin (100 µM) Lane f: DNA + melatonin (100 µM) + Cu(II) 100 µM; Lane g: DNA + tryptophan (100 µM); Lane h: DNA + tryptophan (100 µM) + Cu(II) 100 µM. SC=Supercoiled DNA; OC= Open circular DNA; L=Linear DNA.

As evident from (Figure 8), serotonin and its structural analogues (at higher concentration) are capable of inducing strand scission in plasmid DNA manifested by the formation of linear and open circular forms of plasmid DNA. Further, serotonin is relatively more efficient in inducing strand scission in plasmid DNA than its structurally related metabolites, melatonin and tryptophan.

Cellular DNA breakage by tryptophan/melatonin/serotonin in human lymphocytes as measured by comet assay

It has been previously shown that several types of polyphenols cause cellular DNA damage and that such degradation can be measured by comet assay [42,43]. Similarly, several other studies have employed comet assay to evaluate DNA breakage induced by estrogen-like compounds such as diethylstilbestrol, diadzein and genistein [44]. In the present study, increasing concentrations of the metabolites-serotonin, melatonin and tryptophan (0-400 µM) were tested for DNA breakage in isolated human peripheral lymphocytes using alkaline single cell gel electrophoresis. The corresponding tail length was plotted as a function of the concentrations of compound. As evident from (Figure 9), there is a dose dependent increase in the DNA breakage induced by tryptophan, melatonin and serotonin as shown by increasing comet tail lengths indicative of significant breakage of cellular DNA. However, these compounds differ in the ability to induce breakage of cellular DNA. It is observed that serotonin causes a significantly greater degree of DNA breakage in whole lymphocytes. Therefore, it can be deduced that the highest level of DNA degradation is caused by serotonin followed by melatonin and tryptophan. We propose that such cellular DNA breakage is biologically significant and is physiologically feasible.

Figure 9: A comparison of DNA breakage induced by tryptophan, melatonin and serotonin in human peripheral lymphocytes as a function of comet tail lengths. Comet tail length (µM) was plotted as a function of increasing concentrations of compounds (0-400 µM). All points represent mean of three independent experiments. Error bars denote Mean ± SEM.

Discussion

Progressive accumulation of genetic mutations is thought to be central to the process of tumorigenesis and aging [45]. Sufficient evidence exists to indicate the induction of DNA damage by adducts produced from endogenous substrates arising as a result of metabolic processes which may contribute to the etiology of genetic diseases and ultimately cancer [46]. Recent evidence indicates that prominent mechanisms of aging and cancer are related to endogenously induced DNA damage caused by ROS [47-51]. It is noteworthy that some "antioxidants" are known to exert pro-oxidant actions *in vitro*, by interacting with transition metal ions [52,53].

Herein, we have investigated the chemical basis for DNA breakage activity of serotonin-Cu(II) complex by comparing its relative efficiency with the structural analogues tryptophan and melatonin in the presence of copper. We demonstrate that the neurotransmitter serotonin and its structural analogue melatonin may also have pro-oxidant properties, and thus may contribute to endogenous DNA damage. As already mentioned, antioxidant metabolites such as uric acid also exhibit pro-oxidant properties under appropriate conditions [28-30]. This also appears to be the case with the endogenous metabolites serotonin and melatonin.

It has been reported that copper can be neurotoxic as evidenced by the brain pathology produced in patients with copper overload in Wilson's disease [54]. Copper ions are therefore susceptible to binding with endogenous metabolites in the presence of molecular oxygen to generate ROS by Haber-Weiss or Fenton reactions, eventually leading to oxidative damage to biomolecules [55]. Therefore, serotonin/melatonin, which has the potential of DNA binding, may form a ternary complex with Cu(II) and DNA as demonstrated by several other ROS generating and DNA breakage systems [19,56]. Presumably, in this ternary complex, the compounds serotonin/melatonin would reduce Cu(II) to Cu(I), and lead to hydroxyl radicals formation by Fenton reaction mechanism. These radicals would eventually cause significant damage to cellular DNA. Hence, this study provides the

mechanism of copper dependent cellular DNA breakage by the neurotransmitter serotonin and its structural analogues. It further emphasizes the putative role of endogenously synthesized neurotransmitters and copper ions as a source of oxidative DNA damage and neurotoxicity *in vivo*.

Although the concentrations of serotonin used in the above experiments are higher that the *in vivo* levels (0.2-1.14 µM in blood), DNA breakage in presence of copper ions is shown by other metabolites also [32,57]. Similarly, we should emphasize that the concentration of melatonin used in the study does not approach the levels found in the physiological medium. However, this concentration, or higher, have been used in several biological and toxicological studies. For instance, such levels can be attained when melatonin is applied on the skin topically [58,59]. Therefore, in these cases, this property of melatonin should be taken into consideration. We believe that the study of long-term effects of melatonin at diverse doses in different animal models will be useful for making a conclusion on its safety. It is plausible that the combined effect of such endogenous metabolites could hold physiological significance and contribute to the etiology of genetic diseases. Taken together, these findings are suggestive of a major contribution of endogenous DNA damage in mutagenesis, carcinogenesis and ageing.

Acknowledgment

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

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