European Journal of Biotechnology and Bioscience
Online ISSN: 2321-9122
Received: 15-12-2015: Accepted: 18-01-2016
www.biosciencejournals.com
Volume 4; Issue 1; January 2016; Page No. 63-69

Comparative study of UV- Vis spectra and restriction digestion gel patterns of DNA and nitrated DNA

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Abstract
DNA is known to be cleaved by nitration process, other than endonucleases. Here in this study, it has been found that DNA undergoes both non-enzymatic by peroxynitrite and enzymatic degradation by restriction endonucleases (EcoRI, BamHI, HindIII and NdeI). This is due to the nitration process on guanine residues makes DNA susceptible for the hydrolytic cleavage. It has been found that DNA damage due to peroxynitrite treatment is mostly depend on the presence of guanine and hence DNA damage decreases in the order of BamHI (5'- GGATCC-3')> EcoRI (5'-GAATTC-3')> HindIII (5'- AAGCTT-3')> NdeI (5'-CATATG-3'). Further it has been found that the UV Visible characteristics of nitrated product do not changes. The nitration process increases as the concentration of peroxynitrite being increased. It means that, DNA undergoes hydrolytic cleavage and hydrolytic product will remain stable. So to neutralize these nitration products further studies need to be done.

Keywords: Peroxynitrite, Restriction endonucleases, Non-enzymatic digestion, enzymatic digestion, Guanine, Base modification.

1. Introduction

Peroxynitrite (PXN) is a potent oxidising and nitrating species [1]. Activated microphages, neutrophils and endothelial cells can release both nitric oxide ($^{\cdot}NO$) and superoxide (O_2^{-}) and rapid reaction of these two species yield the potent oxidant peroxynitrite anion ($ONOO^-$) [2, 3]. PXN has high diffusion constant and high permeability [4, 5]. $ONOO^-$ is most toxic of reactive nitrogen species (RNS) and can potentially oxidise and nitrate many biological molecules such as proteins of tyrosine residues, thiols, unsaturated fatty, acid-containing phospholipids and DNA [6, 7, 8, 9]. The modification of these biomolecules leads to the inhibition, inactivation and sometimes activation of enzymes and channels, as well as disturbances in cellular energetic and signalling processes [10]. Thus, $ONOO^-$ has been implicated as a key pathophysiological intermediate in various diseases, including inflammatory processes, neurodegenerative disorders and atheroma [2, 11]. It has been reported that deleterious effect of ROS/ RNS on human cells may end in oxidative injury leading to programmed cell death i.e. apoptosis. Apoptosis, or programmed cell death, plays convincing role in mediating neurodegenerative diseases [12]. Those neurons that fail to complete apoptotic phase become necrotic and inflamed nearby neurons which, in turn, leads to neurodegeneration [13]. Evidence suggest that, certain condition favor escape of some of the peroxide from degradation, consequently releasing it into other compartment of the cell and increasing oxidative stress leading to DNA damage [10].

Brain is particularly susceptible to oxidative damage [14]. As compared to other body tissues, neural cells are considered to be more susceptible to oxidative damage due to more number of mitochondria, high oxygen consumption, low activity of antioxidant enzymes, elevated level of polyunsaturated fatty acids in the cell membrane, unfavorable space/volume ratio and presence of microglia cells nearby, which are more likely to produce increased amount of superoxide radical [15, 16]. Excess of oxidation in neuronal microenvironment causes oxidation of lipid, proteins and DNA and generates many byproducts such as cholesterol oxides, aldehydes, peroxides, alcohols and ketones. Most of them are toxic to blood lymphocyte and macrophages, paralyzing *in vivo* defense system which ultimately leads to various diseases [17]. DNA single strand breaks are discontinuities in a single strand of the DNA double helix and are one of the most common occurring type of DNA lesion [11]. Approximately, 10^3 single strand breaks can arise in a single cell per day [18]. Although they can arise from a variety of sources, those arising from the disintegration of sugar phosphate backbone of DNA following oxidative attack by reactive oxygen species and reactive nitrogen species are some of the most frequent types of single strand break [19]. G is most susceptible for oxidation by peroxynitrite because ionization potential for guanine (G; 1.29V) is lowest followed by Adenine (1.42V) <Cytosine (1.6V) <Thymine (1.7V) [5, 20, 21, 22]. It has been found that the 5′ G of GG and GGG sequences are especially susceptible to one-electron oxidation [5, 19, 23]. When guanine react with peroxynitrite leads to the generation of various products such as 2,6-diamino-4-oxo-5-formamidopyrimidine, 2,5-diamino-4H-imidazolone, 5-guanidino-4-nitroimidazole, guanidinohydantoin, spiroiminodihydantoin, cyanuric acid, parabanica acid, oxaluric acid, urea, 8-oxoguanine, FapyG, and oxazolone, 2,5-diaminoimidazol-4-one, 2,2-diamino-4-[(2-deoxy-*â*-D-*erythro*-pentofuranosyl)amino]-5-(2*H*)-oxazolone, and 4,5-dihydro-5-hydroxy-4-(nitosooxy)dG. These products are formed either from ring opening or subsequent rearrangement of open ring structure [5, 24, 25]. In this consequence here an attempt is made to assess the capacity of peroxynitrite to damage supercoiled DNA and stability of nitrated product of DNA.

2. Materials and Method

2.1 Chemicals:

pBR 322, EcoRI, BamHI, HindIII and NdeI were purchased from New England Biolabs. Agarose, TEB running buffer, ethidium bromide, and loading dye were obtained from Invitrogen. Diethylene triamine penta acetic acid (DTPA), NaOH, Manganese dioxide, Salmon DNA and Guanine extrapure from HiMedia. Dichloro methane from SRL. Isoamyl nitrite from sigma. Hydrogen peroxide was obtained from Fischer Scientific. All the reagents were of AR grade and double distilled water was used throughout. Peroxynitrite was synthesized as per the reported literature [26, 27].

2.2 Apparatus

All the absorbance were measured using Ocean optics (USA) spectrofluorimeter with 1 cm quartz cuvettes. All pH measurements were carried out using a Control Dynamics digital pH meter (model APX 175). Gel was imaged using gel doc system, G: BOX, Chemi XX9, Syngene, Haryana, India.

2.3. Peroxynitrite mediated nitration of DNA

200µg of pBR 322 DNA solution was treated with 0.05mM concentration of peroxynitrite. Since the half-life of PXN is less than one second so, soon after the addition of peroxynitrite, immediately spin for 1-2 min and incubated at room temperature for 10 mins.

2.4. Enzymatic Digestion

To the peroxynitrite treated DNA sample, restriction enzymes (1U/ 1µg) was added along with double distilled water, 10X assay buffer to make total volume of 20µl. spin immediately for 1-2 sec and incubate at 37 ˚C for 1 hr in water bath. For control, same reaction was performed with peroxynitrite untreated DNA.

2.5. Agarose gel electrophoresis of pBR 322

After 1 hr of incubation, loading dye was added into each eppendrof tube to stop the reaction. Tapped to mix and then spin. Sample was loaded on 0.8% agarose gel which was prepared in 20ml of 1X TAE buffer and DNA was separated with an applied voltage of 80 V for 140 mins at room temperature. Gels were imaged using the Gel Doc System.

2.6. Spectral analysis of Nitrated DNA:

2.6.1. Preparation of DNA sample

Stock solution of salmon DNA (mg/ml) was prepared in 1X TAE buffer. For spectral analysis, 1980µl of 1X TAE buffer and 20µl of DNA from stock were added. DNA shows peak at 260nm (See Fig. 1).

2.6.2. Reaction with Peroxynitrite

Salmon DNA was nitrated with peroxynitrite. Spectral analysis of nitrated DNA, 1980µl of 1X TAE buffer and 20µl of DNA from stock were added in vials. This solution was treated with different concentration of peroxynitrite (50µM to 1mM).

2.6.3. Guanine and its reaction with Peroxynitrite

For Guanine (G), 4mM of guanine was dissolved in 0.5 M, pH 7 sodium phosphate buffer and peak was observed at 277nm. Nitration of guanine was carried out by treating 4mM of G with 10mM of peroxynitrite and spectral analysis was done.

2.6.4. Time Scale Study on Nitrated DNA

To study the effect of time (from 1 min to 10 min) on nitrated DNA product, 1980µl of 1X TAE buffer and 20µl of DNA from stock was taken in vials. This solution was treated with 50µM to 1mM concentration of peroxynitrite and absorbance at 362nm (other than DNA peak at 260nm) was noted after every 1 min till 10 mins for each concentration of peroxynitrite

2.6.5. Temperature Scale Study on Nitrated DNA

To study the effect of temperature on nitrated DNA product, 1980µl of 1X TAE buffer and 20µl of DNA from stock were added in vial. This solution was treated with 500µM concentration of peroxynitrite and absorbance at 362nm was recorded at every 10 ˚C rise in temperature starting from room temperature 25 ˚C.

2.6.6. Time and Temperature Scale Study on Nitrated Guanine

To study the effect of time on nitrated G product, 1980µl of sodium phosphate buffer and 4mM of G was taken in vial. This solution was treated with 10mM concentration of peroxynitrite and absorbance was noted after every 1min till 10 min. For temperature scale study same reaction was carried out and absorbance was measured at every 10 ˚C rise in temperature from room temperature (RT).

3. Result and Discussion

Peroxynitrite Preparation

Peroxynitrite (PXN) was prepared as per the previous protocol. Peroxynitrite is pale yellow color whose concentration was measured at 302nm. Concentration of prepared PXN was found to be 0.2M.

Restriction digestion analysis of peroxynitrite treated DNA

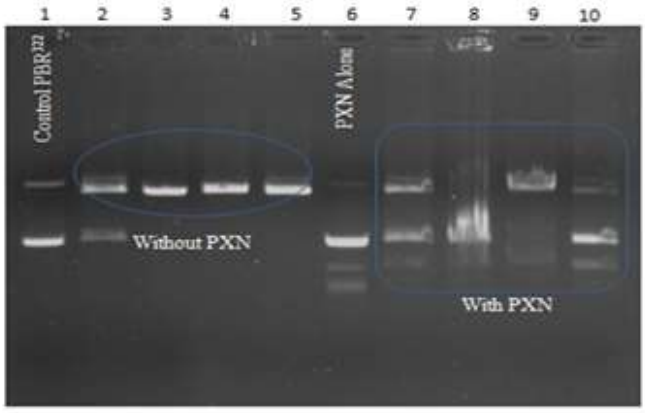


Fig1: Restriction Digestion of pBR322 with and without PXN. Lane 2, 3, 4, 5 are pBR322 treated with EcoRI, BamHI, HindIII, and NdeI respectively: -ve PXN, Lane 7, 8, 9, 10 are pBR322 treated with EcoRI, BamHI, HindIII and NdeI respectively: +ve PXN

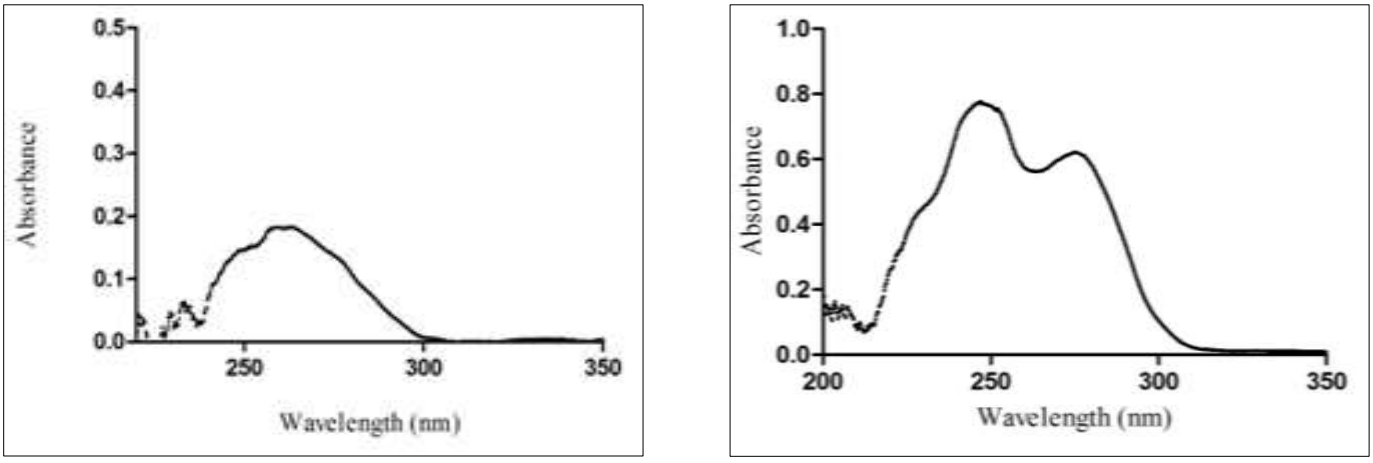
Figure 1 shows the effect of nitrated DNA (pBR 322) on the activity of restriction endonucleases by traditional agrose gel electrophoresis. pBR 322 and PXN treated pBR 322 was treated with four different enzymes i.e. EcoRI, HindIII, BamHI and Nde I. These enzymes are selected because of unique restriction site in pBR 322 and have guanine in their restriction site (Table 1).

It has been observed that there is difference in activity of restriction enzymes for PXN treated DNA (pBR 322) and without treated. As can be seen in lane 1 (Fig. 1) supercoiled plasmid runs as a single band. When lane 1 and lane 6 was compared, it was found that PXN may further coiled the supercoiled DNA (Fig. 1.). A lower yield of linearized form also formed due to break in double stranded supercoiled DNA. Guanine is the preferable site for peroxynitrite and this difference in cleavage pattern may be because in EcoRI single G is present but in BamHI double G (GG) is present in cleavage site at restriction site. So DNA damage is more in BamHI as compared to EcoRI. But in case of HindIII and NdeI, G is present in restriction site but away from cleavage site. In NdeI, G is present 3 nucleotides away from cleavage site. This may be the reason that NdeI has worked in similar manner for PXN treated pBR 322. So, it may be possible that DNA damage due to PXN treatment is mostly depend on the presence of G and hence DNA damage decrease in the order of Bam HI (5'-GGATCC-3')> EcoRI (5'-GAATTC-3')> HindIII (5'-AAGCTT-3')> Nde I (5'-CATATG-3'). This experiment reveals the susceptibility of different guanine sites toward peroxynitrite induced damage and co-relates with the previous finding by Madison *et al.,* which also shows a consistent trend for guanine to undergo one-electron oxidation: 5'-GGG-3' > 5'-GG-3' > 5'-G-3'. Thus, it appears that in mixed DNA sequences the guanine sites are most vulnerable to oxidize.

Table 1: Restriction enzymes with Guanine (G) in their recognition sequences and having unique restriction site in pBR 322.

Restriction Enzyme	Recognition Site	Cleavage Position in pBR 322
EcoRI	5'-GAATTC-3' 3'-CAATTC-5'	4359
BamHI	5'-GGATCC-3' 3'-CCATGG-5'	375
HindIII	5'- AAGCTT-3' 3'-TTGCAA-5'	29
NdeI	5'-CATATG-3' 3'-GTATAC-5'	2295

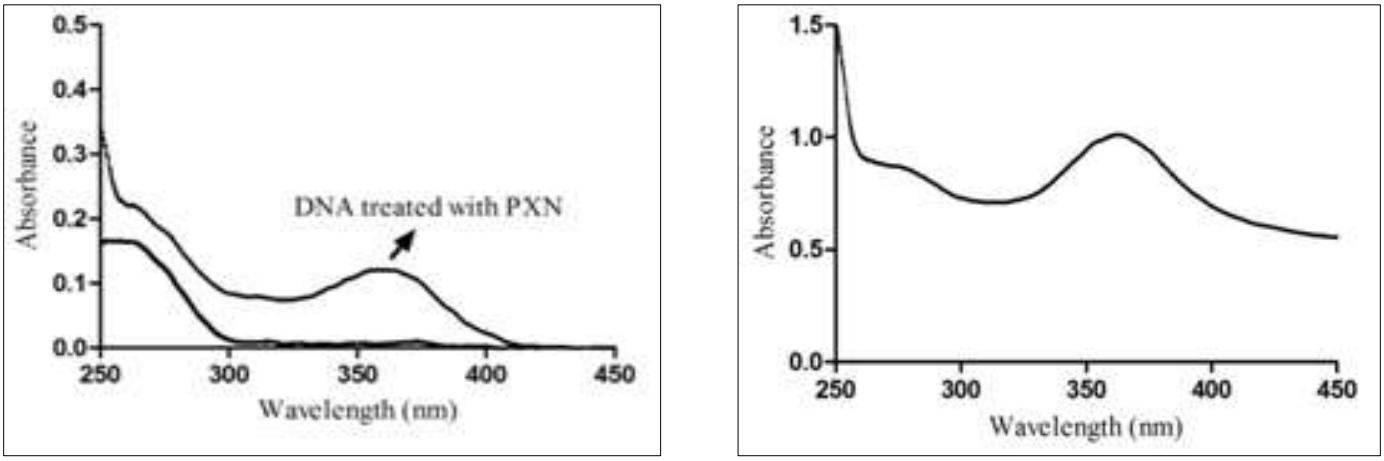
Spectral study of Nitrated DNA and Guanine



(A) (B)

Fig 2: UV-Vis absorbance spectra of DNA showing λ_{max} at 260nm and Guanine showing λ_{max} at 277nm.

Nitration of DNA with peroxynitrite was standardized. It has been observed that DNA treated with peroxynitrite shows peak at 362nm including peak for DNA at 260nm (Fig.2; A & 3; A). DNA was treated with 50µM to 1mM concentration of peroxynitrite (Fig. 4). As the concentration of PXN increase the peak at 362nm also increases and the highest peak for DNA nitration is at 1mM. So DNA nitration product increases dose dependently as the concentration of peroxynitrite increases. To compare nitrated product of DNA, guanine (G) was used as standard. Guanine was dissolved in sodium phosphate buffer which shows peak at 277nm (Fig.2; B) but peroxynitrite treated guanine shows peak at 362nm (Fig.3; B) which is similar to DNA nitration peak. So we can say that during DNA nitration, Guanine is the major site for nitration.



(A) (B)

Fig 3: UV Absorption Spectra of nitrated DNA showing λ_{max} at 362nm and nitrated Guanine also showing λ_{max} at 362nm.

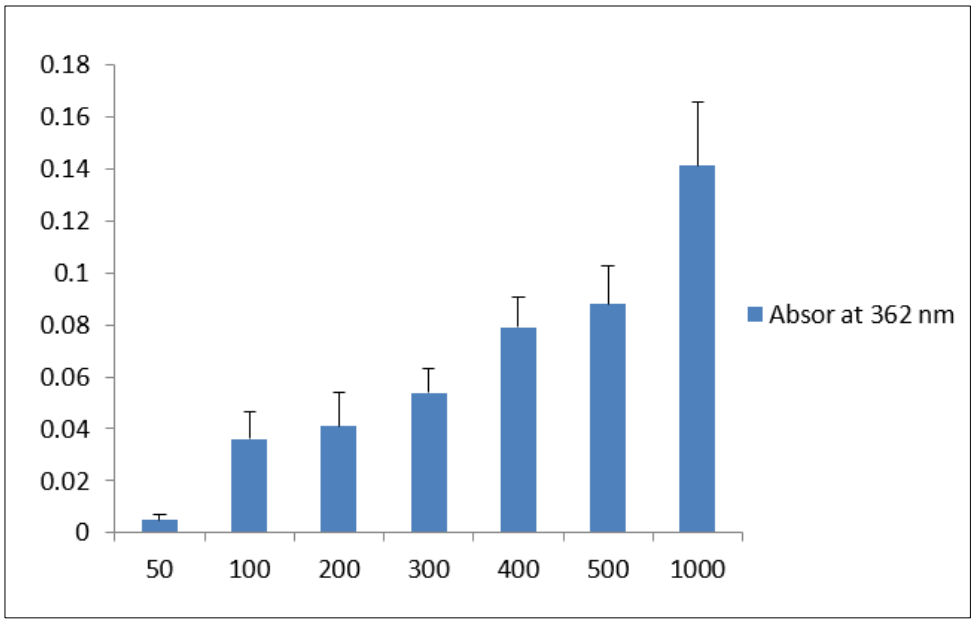
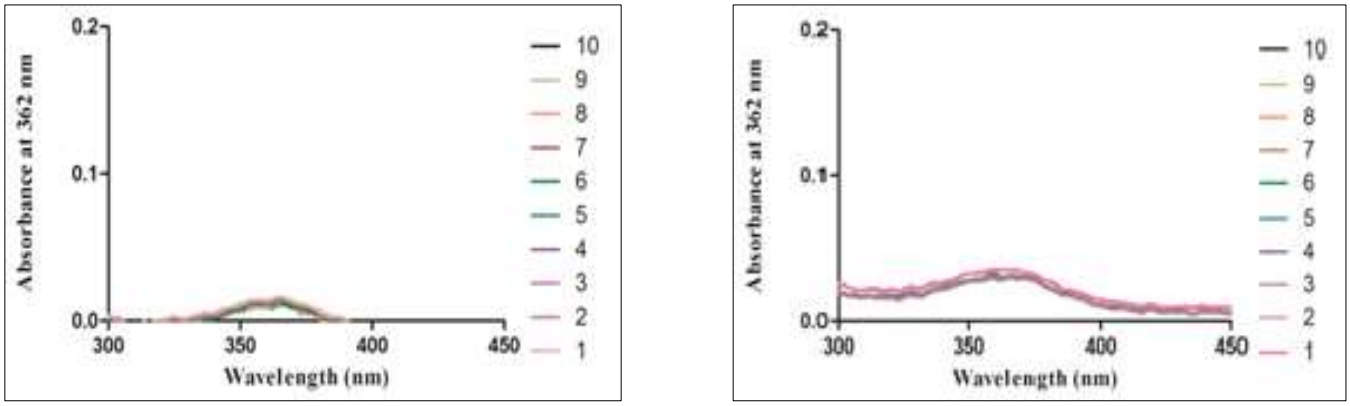


Fig 4: UV absorption properties of nitrated DNA with different concentration of peroxynitrite (50µM - 1000µM). Nitrated product of DNA was measured at 362nm. DNA nitration product increases dose dependently as the concentration of peroxynitrite increases from 50µM to 1000µM. (Y– axis and X- axis as Absorbance v/s Concentration).

Time scale study on Nitrated product of DNA and Guanine



(A) (B)

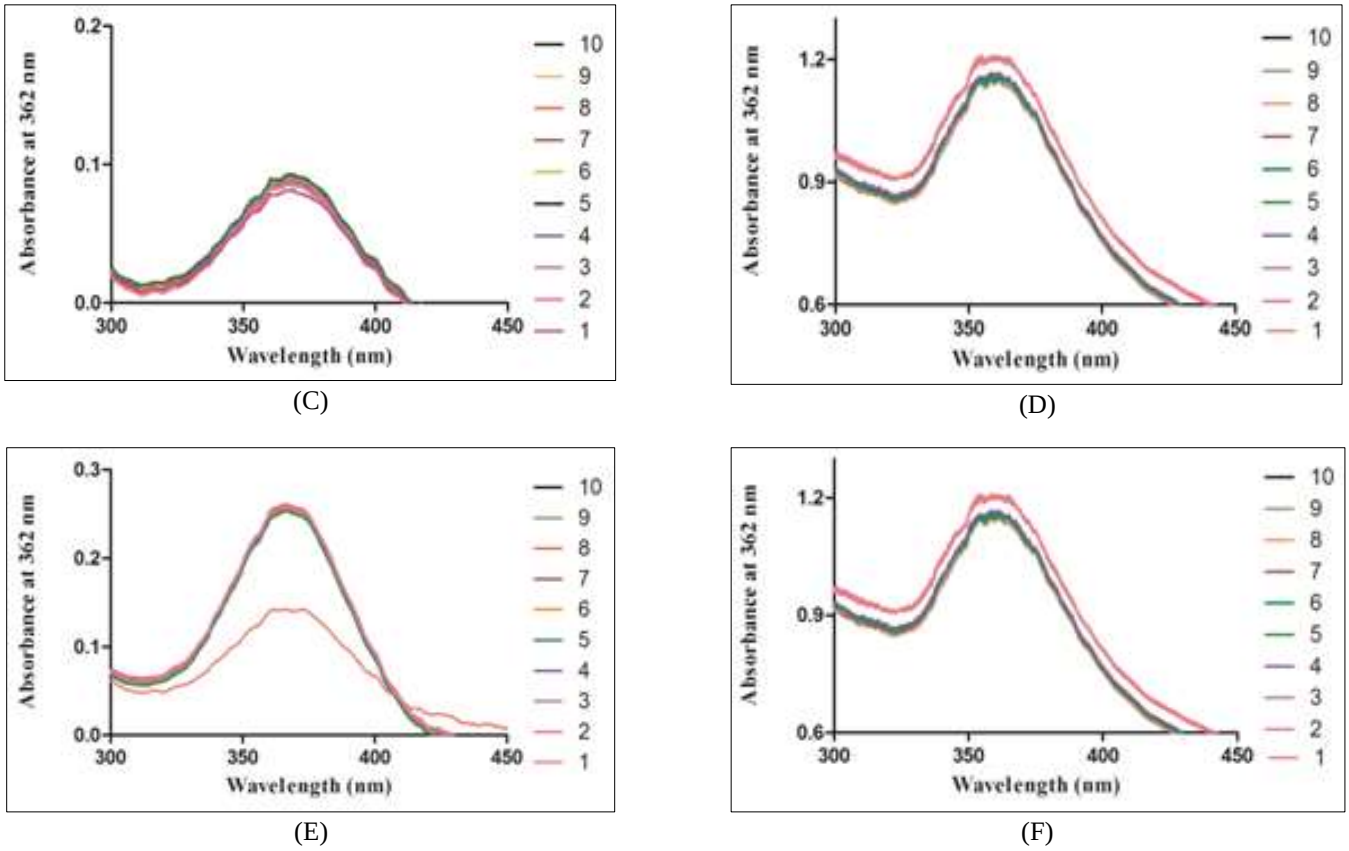


Fig 5: UV- Vis spectral analysis of nitrated DNA (A-E) and nitrated Guanine (F) with time (1-10 mins). Effect of time on different concentration of peroxynitrite treated DNA (A) 200µM (B) 300µM (C) 400µM (D) 500µM (E) 1mM and (F) Effect of time on PXN treated G. Effect of time on nitrated product of DNA and G respectively, shows that nitration product is stable till 10 mins. (Graph for 50µM and 100µM is not shown because of poor peak visibility).

The effect of time was studied on nitrated DNA and guanine. For DNA, effect of time for each concentration of PXN was studied separately. From 50µM to 400µM concentration of PXN, till 10 mins there is single peak at 362nm but the absorbance increases at 362nm from 50µM to 400µM (Fig. 5; A-C). For 500µM and 1mM, after 1 min second peak has been observed from 2 min till 10 mins at same wavelength and absorbance at second peak increases at 1mM (Fig. 5; D&E). It may be because nitration happens immediately after adding PXN i.e. in less than 1 min but for higher concentration of PXN, reaction continued till two mins. At lower concentration of PXN, DNA nitration is slow and constant but at higher concentration after 1 min DNA nitration due to PXN is very sever. DNA nitration product is stable till 10 mins.

Effect of time on product of nitrated G shows that, product remain stable till 10 mins. From 1 to 9 min absorbance is same but there is slight increase in absorbance after 9 min (Fig. 5; F). This may be because of the same reason as describe above for DNA nitration.

Temperature scale study on Nitrated product of DNA and Guanine

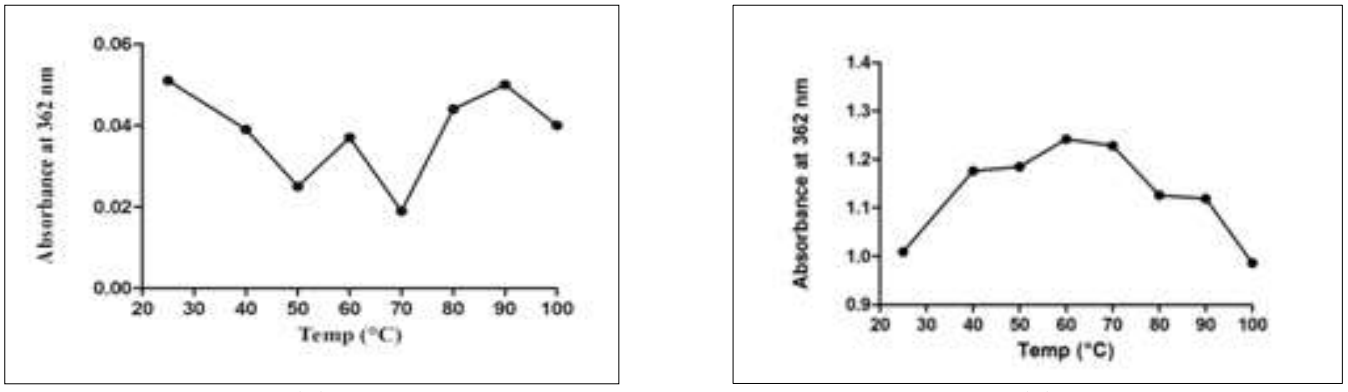


Fig. 6. UV- Vis spectral analysis of nitrated DNA (A) and nitrated Guanine (B) with different temperature (25 ˚C to 100 ˚C). Optimum temperature for DNA nitration product is 90 ˚C and nitrated product of DNA remain stable till 100 ˚C (A). At 60 ˚C and 70 ˚C, the nitration of guanine is highest and product was not degraded till 100 ˚C (B).

Effect of temperature on nitration product of DNA was studied (Fig.6; A). It has been observed that the optimum temperature for DNA nitration product is 90˚C and the nitration product of DNA due to PXN is stable till 100˚C. Effect of temperature on nitration product of guanine (Fig. 6; B) shows that concentration of nitration product increases till 70 ˚C. At 60 ˚C and 70 ˚C, the nitration of guanine is highest and product was not completely degraded till 100 ˚C. It means nitrated product of DNA and guanine is highly stable over a wide range of temperature.

Prolonged existence of these products may cause mutagenesis and carcinogenesis along with various other diseases such as neurological diseases (PD, AD, ALS) and biological ageing. The nitrated products are quite flexible to make diverse base pairings with other bases on the neighboring DNA strand. Occurrence of the ring-opened products of guanine in DNA is suggested to destabilize and distort the DNA duplex severely. These distortions may be sensed by repair enzymes to detect and repair the lesions to keep the genome error-free [5]. Hence, understanding the mechanism of DNA damage and the biological significance of the damaged products is of paramount importance in chemistry, biology, toxicology and medicine.

4. Conclusion

This study reported that, Guanine is the major site of nitration in PXN treated DNA and presence of Guanine at cleavage site in restriction site effect the activity of restriction endonucleases. DNA damage due to PXN treatment is mostly depend on the presence of G and hence DNA damage decrease in the order of Bam HI (5'- GGATCC-3')> EcoRI (5'-GAATTC-3')> HindIII (5'- AAGCTT-3')> Nde I (5'-CATATG-3'). DNA nitration product increases dose dependently as the concentration of peroxynitrite increases. So, the gene pool rich in G nucleotide has a greater chance to get damaged in the presence of PXN. Further the stability of nitrated product of DNA upon nitration with peroxynitrite. It is found that DNA nitration product was stable over a wide range of temperature and time.

The extent of damage caused by free radicals may be reduced through lifestyle modifications and dietary intervention, such as increasing the intake of vegetables, fruits and antioxidant supplements (e.g. beta-carotene, vitamins C and E) [28]. So to neutralize these nitration products further studies need to be done. This is because increased oxidative and nitrosative stress as well as DNA damage could be potential targets for therapeutic strategies for early management and prevention of various diseases.

5. Acknowledgement

The authors acknowledge the financial support and award of the INSPIRE fellowship to Anushree by Department of science and Technology (DST), New Delhi, India and Department of Microbiology and Biotechnology, Bangalore University, Bangalore, India for providing lab facility.

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