Mechanism of Calf thymus DNA radioprotection by sucrose: A combined effect of scavenging action and altered water

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ABSTRACT

► *Original article*

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Background: Development of safe radioprotector is a challenging task. In this study radioprotective effect of sucrose has been demonstrated in calf thymus DNA (CtDNA). Sucrose is a free radical scavenger and also acts as osmolyte and therefore can influence the water activity around DNA and effects of radiation on DNA. Hoechst 33258 was used to probe the possible alteration in physicochemical properties due to altered hydration induced by sucrose in irradiated CtDNA. **Materials and Methods:** Calf thymus DNA (CtDNA), sucrose and Hoechst 33258 were obtained from Sigma Chemicals, USA. ^{60}Co Gamma source (Gamma5000, BRIT India) was used for irradiation. Radiation induced structural destabilization was monitored spectrophotometrically (Cary Bio 100, Varian, Australia) by measuring CtDNA melting temperature. Changes in physicochemical properties (altered water activity, minor groove binding characteristics) were investigated using absorption and fluorescence spectroscopic properties of Hoechst 33258-CtDNA interactions in presence of sucrose. **Results:** The CtDNA melting temperature data suggest that sucrose reduced the effect of γ-irradiation on CtDNA. Thermodynamics of binding interaction of Hoechst 33258 with irradiated CtDNA suggested favorable energetics accompanied by displacement of water molecules. Binding affinity in presence of sucrose was altered marginally at low concentration (<0.5Osm). The marginal changes in absorption and fluorescence spectroscopic properties of Hoechst 33258-CtDNA interactions at higher concentration of sucrose suggested unaltered functional capability of CtDNA. **Conclusion:** This study suggests that sucrose can provide structural protection in γ irradiated CtDNA. Both scavenging and altered water activities by sucrose contributed in the observed radioprotection. These physicochemical properties of sucrose can be considered for designing better radioprotector. The observations at low concentrations of sucrose appears important lead for further validation studies.

***Keywords:** Radioprotection, CtDNA, water activity, scavenging, sucrose.*

INTRODUCTION

Development of radioprotector for minimizing the effects of radiation exposure is a challenging task. Different approaches are at experimental stages in various laboratories and till now no safe radioprotector is available. DNA is the critical target of radiation because of direct and indirect interaction of radiation with this genetic material. Various types of damages

occur in DNA structure upon radiation exposure and the double strand breaks is considered lethal to cell survival. The damaging agents are free radicals generated during radiolysis of water as a result of deposition of energy from low energy gamma radiation. Molecules having ability to scavenge free radicals are considered as potential radioprotectors [1].

Development of mechanistic based approaches for radioprotector is aim of our studies. DNA minor groove binding ligand Hoechst 33258 and its derivatives have shown to reduce radiation induced single and double strand breaks in aqueous DNA solutions as well as in cells [2]. The radiochemical yields of scavenging free radicals by Hoechst 33258 are much better than many other experimental radioprotectors. Further studies carried out on this class of molecules in our laboratory revealed the important role of Hoechst 33258 induced structural stability in DNA as additional contributory factor in radioprotection [3, 4]. But their cytotoxicity to mammalian cells remains a matter of concern. In another study from our group by Athar *et al.* demonstrated radiosensitizing effects of Hoechst 33342 an an-alog of Hoechst 33258 in head and neck cancer cell lines [5] and thus cytotoxicity appears to be impending problems. Therefore, it will worth investigating mechanisms of relatively non toxic and biologically relevant molecules for developing radioprotector. In the present work we have focused on a small carbohydrate molecule, sucrose for investigating its role as radioprotector on CtDNA and the mechanism underlying radioprotection. The radioprotecting effects of sucrose was reported earlier by Yoshinaga et al and Ryu Hwa-Ha *et al.* [7, 8] in plasmid DNA and Escherichia coli cells with reference to ionizing radiation viz., γ and X-rays, and beta particles and also with UV radiation. No further studies were undertaken to validate the radioprotective ability of sucrose in other DNA models.

Sucrose is a common carbohydrate present in living system at varying concentrations inside the cells and therefore might not cause toxicity in cellular environment at low concentrations. In this study, we have chosen calf thymus DNA

(CtDNA) in aqueous solutions. Sucrose is also known to alter the water activity around DNA [9] and this was expected to influence DNA damage via indirect effect of γ- radiation. Because of its role as osmolyte, it can cause changes in water activity surrounding CtDNA and might alter its structure-function. In order to investigate the concentration dependent effect of sucrose vis a vis radiation effect on DNA structural stability was monitored by measuring thermal denaturation temperature. Further, we have used a well known DNA minor groove binding ligand Hoechst 33258 to probe the structural changes in CtDNA likely to be induced by sucrose. The results indicated that sucrose can reduce the effect of radiation on CtDNA even at a very low concentration of 0.031 Osm. In order to investigate the possible influence on binding characteristics of Hoechst 33258 with irradiated CtDNA in presence of sucrose, physicochemical parameters, viz. binding affinity, thermodynamics, change in water activity surrounding CtDNA were studied using absorption and fluorescence spectroscopic properties. The physicochemical characteristics of irradiated Hoechst 33258-CtDNA complex are not much affected by sucrose at low concentrations. Free radical scavenging and ability to lower water activity by sucrose contributed the observed radioprotection in irradiated CtDNA solution. These findings with sucrose are promising and likely to have implications in understanding and designing better approaches for development of radioprotective agent. The present findings also require further validation in other experimental models.

MATERIALS AND METHODS

Materials Calf Thymus DNA (CtDNA) (E.Merck, Germany) and Hoechst 33258 (2'-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole Sigma Chemical Co., U.S.A.) were used without further purification. All buff-er components and sucrose (Sigma chemicals) were used as received. Milli Q grade water obtained from Elix 3 Water Purification system

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(Millipore Corporation, USA) was used in all solution preparations.

Preparation of Stock solutions

The stock solution of CtDNA was prepared in the phosphate buffer by adding the 1 mg/ml of CtDNA in buffer (total volume was 10 ml) and the solution was slowly stirred overnight at room temperature. The stock solution of Hoechst 33258 was prepared in Milli Q water. The concentration of CtDNA was determined spectrophotometrically using molar extinction coefficient of 13,200 cm^{-1} M^{-1} (bp) at 260 nm [10]. The concentration of Hoechst 33258 was determined using the extinction coefficient 42,000 cm^{-1}M^{-1} at 340 nm [11]. Sucrose at the desired concentration was added directly to buffer, then required concentration of CtDNA and Hoechst 33258 was prepared and measurements were carried out after 30 minutes after mixing both CtDNA and Hoechst 33258. The osmolality of solutions were measured on freezing point depression based osmometer. The data reported in the result section is mean of two independent experiments and CtDNA concentration is expressed as molarity of base pairs.

Gamma irradiation of CtDNA in sucrose solutions

γ-irradiation of CtDNA solution was carried out in glass vials containing fixed volume i.e 3 ml of sample. The γ-irradiation was carried out in a Co^{60} gamma chamber GC 5000, Board of Radioactive Isotope Technology (BRIT), India at a dose rate of 2.4-2.6 kGy/hr. Hoechst 33258 at different concentrations were added prior to irradiation of CtDNA. Effect of sucrose on irradiated CtDNA and irradiated Hoechst 33258-CtDNA complex was compared by studying the different measured parameters in solutions without sucrose.

UV thermal denaturation and absorption spectral measurements

These measurements were carried out using double beam UV-Visible Spectrophotometer (Cary Bio 100, Varian, Australia) equipped with a 6x6 Peltier temperature controlled sample chamber. Thermal denaturation measurement of irradiated CtDNA (100μM) and Hoechst 33258 (1 and 10 μM)-CtDNA (100 μM) complex were carried out in the presence of sucrose. The solutions were kept in quartz cuvettes and heated from 45°C to 90°C at heating rate of 0.5°C/min and T$_m$ was calculated from the mid point of the plot of O.D. at 260 nm against temperature. The temperature was controlled by the programme provided in the instrument. Absorption spectra of irradiated and unirradiated Hoechst 33258-CtDNA complex at 10 μM concentration of Hoechst 33258 were recorded from 200 to 500 nm.

Fluorescence spectroscopy

Fluorescence spectra, anisotropy and excited state lifetime were measured by using an integrated steady state and the time resolved spectrofluorimeter (Model FS900/FL900CDT, Edinburgh Analytical Instruments, UK). Fluorescence anisotropy measurements were carried out using a pair of Glan–Thompson prisms fitted in the excitation and emission light path. The instrument details including the procedures for steady state measurement and excited state fluorescence lifetime measurements are mentioned in our earlier study [3].

In brief, the emission spectra of the irradiated Hoechst 33258-CtDNA complexes was measured with excitation wavelength at 354 nm and scanned from 375 to 650 nm. The measuring conditions for emission spectrum were: excitation and emission monochromator slits 1 mm; dwell time 0.2 ns and wavelength step 0.5 nm. For anisotropy measurement the polarizer was moved into the excitation and emission light paths in the sample compartment and emission spectra was recorded from 375 nm to 650 nm range in all the four directions. From the emission anisotropy spectra the value of anisotropy was calculated at corresponding spectral maximum position. Excited state fluorescence lifetime measurement of irradiated Hoechst 33258-CtDNA complex was carried out at 354 nm excitation and the intensity decay file at 480

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nm was acquired and for instrument response function, the scattering solution was Ludox and used for calculation of fluorescence lifetime.

Determination of Binding constant: Effect of sucrose and temperature

The association constant of Hoechst 33258 with irradiated CtDNA was determined by fluorescence titration method [3]. In brief, fixed concentration of Hoechst 33258 (1 μM) was taken in a cuvette and titrated with increasing concentration of CtDNA (50 μM) in phosphate buffer. The volume of Hoechst 33258 solution in cuvette was 2 mL. Data were transformed in the form of Scatchard plot of r/C_f versus r, where r is the ratio of bound ligand to the total CtDNA (base pair) concentration and C_f is the concentration of free ligand [3]. Similar titration was carried out in presence of different concentrations of sucrose (0-3 Osm). This binding titration was also carried out at different temperature ranging from 298 to 318 K to measure the binding forces from van't Hoff plot. Using the following van't Hoff equation:

$$\log K_o = -DH/2.303RT + DS/2.303R \qquad (1)$$

When the measured $\log K_o$ is plotted with respect to 1/T, where T is temperature in Kelvin, the thermodynamic parameters namely, the enthalpy change (ΔH) and the entropy change (ΔS) can be calculated from slope and y-intercept of the linear van't Hoff plot based on $\log K_o$ versus 1/T. In the equation, R denotes molar gas constant. The free energy change (ΔG) is estimated from the following relationship:

$$\Delta G = \Delta H - T\Delta S \qquad (2)$$

For understanding the effect of sucrose first the spectral parameters were obtained and then compared with sucrose containing solutions in buffer.

RESULTS

Thermal denaturation temperature and Physicochemical nature of interactions

UV thermal denaturation measurements of irradiated CtDNA in presence of sucrose (concentration between 0-3 Osm) was carried out to study the role of sucrose as a radioprotective agent in γ-radiation induced CtDNA damage. The T_m of CtDNA in phosphate buffer was 75 ºC and after irradiation (120 Gy) the T_m of CtDNA has reduced to 63 ºC (table 1). Presence of sucrose reduced the effect of irradiation at all concentrations. At higher concentration viz. 3 Osm, sucrose lowered the T_m of CtDNA by 5 ⁰C. In general, sucrose is able to reduce the effect of radiation (table 1), thus demonstrated the capability of protecting CtDNA from structural destabilization induced by radiation.

Hoechst 33258 though known as a radioprotector, but it is a well known DNA minor groove binding ligand. The physicochemical properties of interaction of Hoechst 33258 with DNA is well characterized. The techniques commonly used for such studies are absorption, fluorescence and NMR spectroscopy. Sucrose can affect the water activity in and around CtDNA as osmolyte and therefore can influence structure of CtDNA. Binding characteristics of Hoechst 33258 with CtDNA were studied in detail for investigating the effect of sucrose.

Hoechst 33258-CtDNA has been studied earlier in the perspective of structural stabilization in irradiated CtDNA [3]. The thermal denaturation measurement of Hoechst 33258-CtDNA complex showed that the structural stability of CtDNA remain unaffected when Hoechst 33258 was present prior to irradiation. The T_m of Hoechst 33258-CtDNA

Table 1. Comparison of the melting temperature T_m (ºC) of unirradiated and irradiated CtDNA in presence of sucrose.

Sucrose (Osm)	T_m (°C)		ΔT_m (°C)
	No irradiation	120 Gy	
0	75 (±0.0)	63 (±0.8)	-12
0.031	75 (±0.4)	68.0(±0.5)	-7
0.062	75 (±0.4)	67.6(±0.8)	-6.4
0.125	75(±0.7)	68.3(±0.7)	-6.7
0.25	75.0(±0.2)	74.0(±0.7)	-1.0
0.5	75.0(±0.2)	75.0(±0.5)	0
1	73.5(±0.7)	74(±0.7)	-1
2	72.5(±0.7)	73.2(±0.0)	-1.8
3	70.0 (±0.8)	72.0(±0.03)	-3.0

ΔT_m (°C) was calculated as T_m of CtDNA (0 Gy, 0 Osm sucrose) -T_m (at given radiation dose and sucrose concentration).

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complex was 83.5 and 79.6 ºC at 10 and 1 µM concentration of Hoechst 33258 respectively and after g-irradiation of the complex, the T_m of Hoechst 33258-CtDNA complex reduced to 81 ºC and 73 ºC at the respective concentrations of Hoechst 33258 (table 2). It is to be noted that in absence of Hoechst 33258, the T_m was lowered to 63 ºC.

In order to assess the effect of sucrose on stability of irradiated Hoechst 33258-CtDNA complex, the Hoechst 33258-CtDNA complex was prepared in phosphate buffer containing sucrose. Since at higher sucrose concentration (table 1) decrease in T_m indicates physicochemical effect of sucrose on CtDNA and therefore the subsequent studies were carried out only at higher concentration range of sucrose. T_m of the Hoechst 33258-CtDNA complex decreased slightly with increase in concentration of sucrose (table 2). Sucrose caused lowering of T_m almost to the similar extent at both the concentration of Hoechst 33258(1µM and 10µM) by about -5 °C (table 2). This is similar to the observation in table 1 where Hoechst 33258 was not present. Irradiation imparted measurable decrease in T_m in Hoechst 33258-CtDNA complex irrespective of the concentration of this ligand. The net decrease in T_m at 1 Osm sucrose was zero at both the concentrations of Hoechst 33258. At

Table 2. Combined effect of sucrose and irradiation on melting temperature T_m (ºC) of Hoechst 33258-CtDNA complex.

Hoechst 33258 (µM)	Sucrose [Osm]	T_m (°C)		ΔT_m (°C)
		No irradiation	120 Gy	
1	0	79.6(±1.1)	74.5(±2.1)	-5.1
	1	75.2(±1.3)	75.3(±1.2)	-4.3
	2	74.2 (±0.7)	71(±2.8)	-8.6
	3	73.4 (±0.6)	71(±4.2)	-8.6
10	0	83.5(±2.12)	82.2(±1.7)	-1.3
	1	80.5(±0.7)	79.5(±0.0)	-1
	2	79.6 (±0.6)	77.5(±0.7)	-6
	3	78.6(±0.9)	76.5(±1.4)	-7

ΔT_m (°C) was calculated as T_m of Hoechst 33258-CtDNA complex (0 Gy, 0 Osm sucrose) -T_m of Hoechst 33258-CtDNA complex (at given radiation dose and sucrose concentration)

higher concentrations of sucrose due to its osmolytic action cause hydration changes which is also intricately linked with induction of indirect effect of radiation and binding affinity of Hoechst 33258 and hence a matter of discussion for understanding the overall role of sucrose. The necessary data were generated by using binding affinity and thermodynamic measurements as described below.

Binding affinity and thermodynamics of Hoechst 33258-CtDNA interaction

Binding affinity measurements provide direct evidence of interaction of Hoechst 33258 with CtDNA. At first the binding affinity of Hoechst 33258 with unirradiated and irradiated CtDNA was measured by fluorescence binding titration method (table 3). The binding affinity of Hoechst 33258 with irradiated CtDNA was 2.8×10^7 M^{-1}, slightly less than unirradiated CtDNA 4.75×10^7 M^{-1}. The binding data suggests that the interaction of Hoechst 33258 is energetically feasible with irradiated CtDNA. Further, the binding of Hoechst 33258 with irradiated CtDNA is more entropically favorable as compared to enthalpic contribution. A comparison of thermodynamic parameters has been attempted on Hoechst 33258 interaction with unirradiated and irradiated CtDNA by using van't Hoff plot (figure 1). The temperature was varied from 298 to 318 K. The detailed information on binding energetics for the Hoechst 33258 interaction is summarized in table 3.

Effects on hydration changes by sucrose and Hoechst 33258-CtDNA interaction

During thermal denaturation measurement it was observed that sucrose reduced the effect of radiation on T_m of CtDNA (table 1). In order to investigate any possible changes in DNA structure specifically in the minor groove region, we have studied the effect on the binding affinity of Hoechst 33258 with CtDNA irradiated in presence of sucrose. A comparison of binding affinity of Hoechst 33258 with unirradiated and irradiated CtDNA is shown in table 4. The difference in binding affinity of Hoechst 33258 in presence of sucrose is clearly evident from the slope of plot of Sucrose (moles/Kg) Vs ln K_s/K_o

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(figure 2). The data show that as concentration of sucrose was increased from 0 to 3 Osm, the Hoechst 33258 binding affinity decreased. It is also possible to obtain the stoichiometry of water binding in the formation of Hoechst 33258-CtDNA complex from the slope of figure 2.

Assuming that there was no direct interaction of the sucrose with CtDNA or Hoechst 33258, the change in hydration is given by the equation:

$$\partial \ln(K_s/K_o)/\partial [Osm] = -\Delta n_w/55.5 \qquad (3)$$

where $\ln(K_s/K_o)$ is the change in binding free energy, Osm is the osmolality of the solution, Dn_w is the difference in the number of bound water molecules between the complex and the free reactants and 55.5 is the molarity of water. A positive sign for Δn_w signifies the uptake of water upon Hoechst 33258-CtDNA complexation, which indicates that the Hoechst 33258-CtDNA complex is more hydrated than the free Hoechst 33258 and CtDNA. The negative slope of fitted line (figure2) gives a positive value which indicates that additional water is bound upon complex formation. The Δn_w for Hoechst 33258-CtDNA complex was 30±1 in presence of sucrose while in irradiated Hoechst 33258-

Table 3. Comparison of the binding affinity and thermodynamic parameters of Hoechst 33258 binding with un-irradiated and irradiated CtDNA. The thermodynamic parameters were calculated by using van't Hoff equation. The concentration of Hoechst 33258 and CtDNA were 1 and 50 μM respectively.

Temp (Kelvin)	K_o ($10^7 M^{-1}$)		Thermodynamics parameters					
	0 Gy	120 Gy	ΔS (Jmole^{-1}K^{-1})		ΔG (kJmole^{-1})		ΔH (kJmole^{-1})	
			No irradiation	120 Gy	No irradiation	120 Gy	No irradiation	120 Gy
298	4.75	2.8			-43.8	-42.5		
303	4.75	2.7			-44.5	-43.2		
308	4.45	2.6	130.7(±3.5)	122(±5)	-45.1	-43.7	-4.8(±1.01)	-6.3(±1.5)
313	4.3	2.58			-45.8	-44.3		
318	4.2	2.6			-46.4	-44.9		

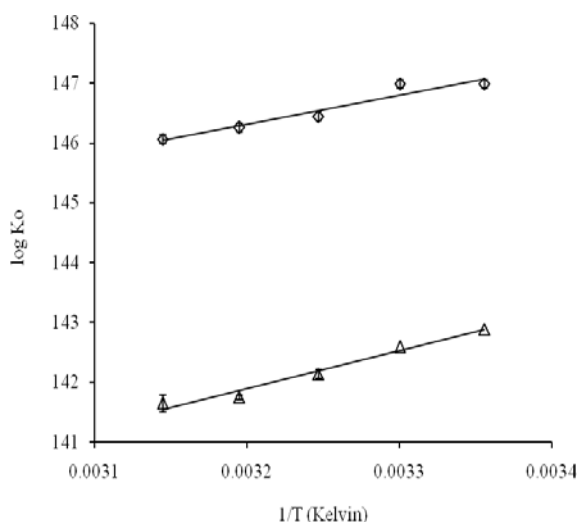**Figure 1.** van't Hoff plot for the interaction of Hoechst 33258 with un-irradiated (◊) and irradiated (Δ) CtDNA. The concentration of Hoechst 33258 and CtDNA was 1 and 50 μM respectively. The thermodynamic parameters were calculated by using equation 4 and 5. The radiation dose was 120 Gy.
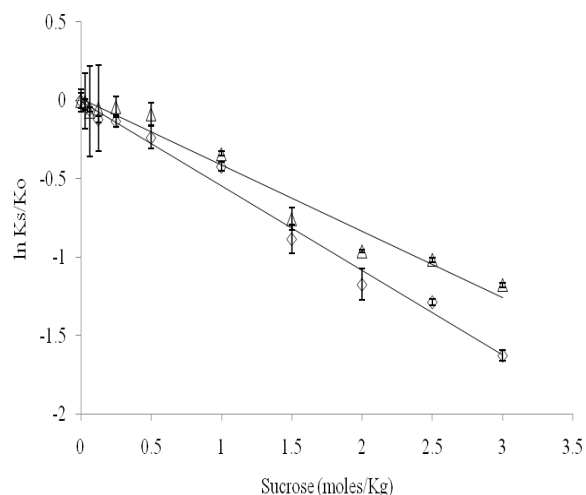

Figure 2. Plot of natural logarithm of the ratio of the binding affinity (K_s) at a given concentration of sucrose to the binding affinity (K_o) without sucrose. The sucrose concentration is represented on the abscissa. Linear least square fit using equation 6 gives changes in number of water molecule accompanying Hoechst 33258-CtDNA complexation Δn_w 30±1 for un-irradiated (◊) CtDNA and 22±2 for irradiated (Δ) CtDNA. CtDNA was irradiated at radiation dose of 120 Gy.

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CtDNA complex this has lowered to 22±2. In addition to this, it was also observed that the lower concentration of sucrose does not have measurable effect on the Hoechst 33258-CtDNA interaction (table 4) as well as on CtDNA stability (table 1) but sucrose reduced the effect of irradiation on CtDNA (table 1). The binding affinity showed strong dependency with sucrose concentration from 0.031 Osm to 3 Osm (table 4) and in irradiated solutions though this trend remained almost similar but the overall binding affinity was less. These observations suggested the additional the role sucrose i.e., alteration of water activity by sucrose which was expected to influence the binding affinity of Hoechst 33258.

Table 4. Comparison of the observed equilibrium constant for the binding of Hoechst 33258 with un-irradiated and irradiated CtDNA as a function of sucrose concentration ranging from 0 to 3 Osm. The concentration of Hoechst 33258 and CtDNA were 1 and 50 μM respectively.

Osmolyte conc. (moles/Kg)	Sucrose K_s ($10^7 M^{-1}$)	
	0 Gy	120 Gy
0	4.75 (±0.07)	2.8(±0.07)
0.031	4.65 (±0.1)	2.35(±0.05)
0.0625	4.48 (±0.12)	2.3(±0.03)
0.125	4.2 (±0.06)	2.3(±0)
0.25	4.14(±0.15)	2.2(±0.07)
0.5	3.75 (±0.07)	2(±0.07)
1	3.1 (±0.07)	1.59(±0.02)
1.5	2.0(±0.14)	1(±0.09)
2	1.4(±0)	0.86(±0.14)
2.5	1.3(±0.14)	0.81(±0.07)
3	0.93(±0.14)	0.69(±0.01)

Absorption and fluorescence spectral measurements

UV-visible absorption and fluorescence spectroscopic measurements provide insight to molecular properties of the complex. The absorption spectra of irradiated and unirradiated Hoechst 33258-CtDNA complex at 10 μM concentration of Hoechst 33258 are shown (figure 3 A and B). The concentration of CtDNA was 100 μM. The spectra were recorded in presence of 0-3 Osm sucrose. The absorbance at 260 nm has increased with concentration of sucrose and the extent of increase in presence of sucrose was relatively less irradiated CtDNA. This is correlated with the observation in T_m (table 1). The absorbance at 354 nm due to Hoechst 33258-CtDNA complex decreased with increase in concentration of sucrose without any spectral shift suggesting minor alterations in microenvironment at the binding site of Hoechst 33258.

Fluorescence properties of Hoechst 33258 show characteristic changes upon binding with DNA. Emission spectra of Hoechst 33258-CtDNA complex was recorded in presence of increasing concentration of sucrose from 0-3 Osm (figures 4 and 5). The fluorescence intensity of the complex decreased with increasing sucrose concentration (figures 4 and 5) along with red spectral shift in emission maxima. For example, in unirradiated Hoechst 33258 -CtDNA complex a red spectral shift from 466 to 482 nm, Δ_{nm} =16 nm (figure 5A) was observed while a slightly
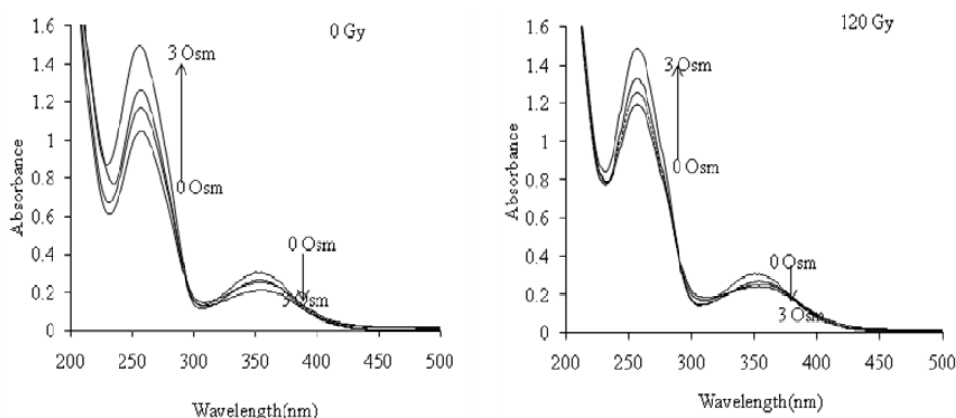

Figure 3. Absorption spectra of irradiated Hoechst 33258–CtDNA complex in presence of 0-3 Osm sucrose. Hoechst 33258 concentration was 10 μM. The concentration of CtDNA was 100 μM. The arrow in the figure indicates the changes in absorbance of the Hoechst 33258-CtDNA complex with gradual increase in sucrose concentration from 0 to 3 Osm.

lesser spectral shift, Δ_{nm} =11 nm (471 to 482 nm) was observed for the irradiated complex (figure 5B). Since fluorescence measurements are much sensitive as compared to absorption spectroscopy, a lower concentration of Hoechst 33258 i.e., 1µM was also considered for similar measurement (figures 4A & 4B), a nominal spectral shift of 7 nm and 6 nm respectively was observed. Therefore, absorption and fluorescence properties Hoechst 33258 bound to CtDNA showed changes due to altered hydration by sucrose.

Fluorescence anisotropy measurements provide information on orientation of bound Hoechst 33258. The anisotropy data of irradiated Hoechst 33258-CtDNA complex suggested that irradiation did not cause any measureable effect on binding orientation of Hoechst 33258. In presence of sucrose, the anisotropy value for the irradiated Hoechst 33258-CtDNA complex was 0.29 and did not change further with concentration of sucrose. This data suggest unaltered binding orientation of Hoechst 33258 with irradiated CtDNA in presence of sucrose (table 5).

Excited state fluorescence lifetimes provide information on relaxation dynamics of a fluorescent molecule. Fluorescence lifetime is an important excited state property and can provide insight to alterations caused by
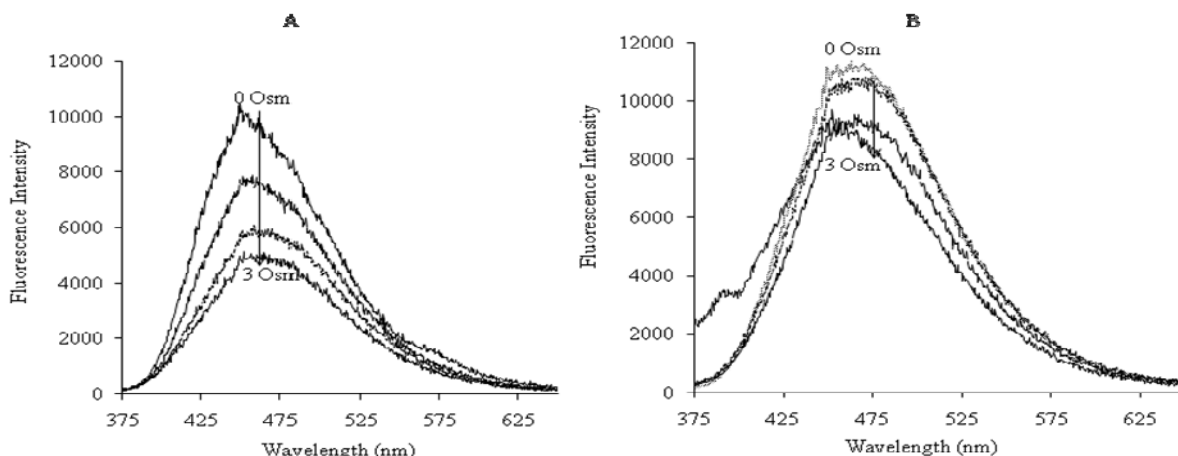

Figure 4. Emission spectra of unirradiated (A) and irradiated (B) Hoechst 33258–CtDNA complex in presence of 0-3 Osm sucrose. The concentration of Hoechst 33258 and CtDNA was 1 and 100 µM respectively. The arrow indicates the changes in fluorescence intensity of the Hoechst 33258-CtDNA complex with gradual increase in sucrose concentration from 0 to 3 Osm.

Figure 5. Changes in emission spectra of unirradiated (A) and irradiated (B) Hoechst 33258–CtDNA complex in presence of 0-3 Osm sucrose. The concentration of Hoechst 33258 and CtDNA was 10 and 100 µM respectively. The arrow indicates the changes in fluorescence intensity of the Hoechst 33258-CtDNA complex with gradual increase in sucrose concentration from 0 to 3 Osm.
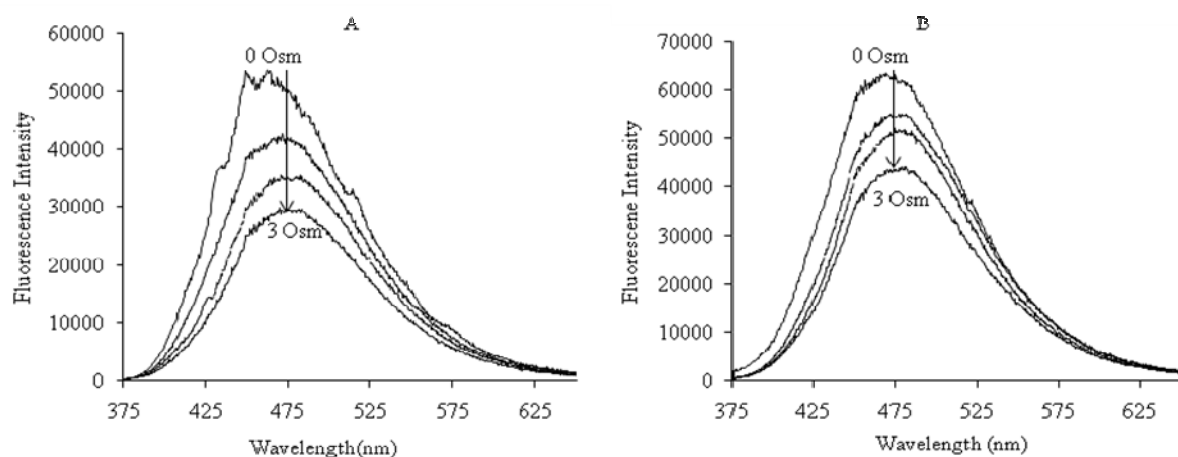
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Table 5. Excited State fluorescence lifetime and fluorescence anisotropy measurement of irradiated Hoechst 33258-CtDNA complex in the presence of different concentration of sucrose.

Hoechst (μM)	Sucrose [Osm]	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)	Ani
1	0	2.2	39	4.1	61	0.29
	3	2.4	49	4.5	51	0.3
10	0	1.9	29	4.1	71	0.26
	3	1.6	25	4.0	75	0.28

decrease in hydration by sucrose in Hoechst 33258-CtDNA. Fluorescence lifetime of Hoechst 33258 when bound to DNA is characterized by two exponentially decaying components viz, a short (t_1) and long (t_2) decay component at 2.2 ns and 4.1 ns respectively [3, 12]. The relative contribution from these components was 39% and 61% respectively (table 5). The numerical values of t_1 and t_2 and their relative contributions remained unaltered with sucrose suggesting similar excited state dynamics of Hoechst 33258-CtDNA complex. At higher concentration of Hoechst 33258 (10 mM) the relative contribution of these decay components viz. 1.9 ns and 4.1 ns were 29% and 71% respectively suggesting redistribution of different bound forms (table 5). No measureable changes could be observed in presence of sucrose.

DISCUSSION

Several chemical compounds have showed the ability to provide protection of DNA from the deleterious effects of ionizing radiation, but the desired safe radioprotector is yet to be made available. Among various other reasons, systemic toxicity is a matter of concern. Sucrose is a naturally occurring disaccharide and used in several biochemical preparation. Sucrose has ability to scavenge free radicals [7, 8]. In the present study we have focused on two aspects of sucrose viz., radical scavenging property and ability to change hydration on observed radioprotection on irradiated CtDNA in solution. The first aspect was studied by measuring DNA denaturation temperature (T_m) as decrease in T_m is correlated with DNA strand breaks [13] and effects on change in hydration and related prop-

erties were investigated by physicochemical tools using absorption and fluorescence spectroscopy. Minor groove binder Hoechst 33258 was used to probe the structural changes in CtDNA. Irradiation is known to cause structural destabilization of CtDNA (table 1). Sucrose is a known scavenger of free radicals [7, 8] and thus the observed effect was expected. The relative decrease in T_m in irradiated solutions of CtDNA in presence of sucrose is thus correlated with scavenging properties of sucrose. Further sucrose is also known to behave as osmolyte and therefore it will change the water activity in and around CtDNA, which is also intimately related to the effects of radiation on DNA. Thus, the focus of the discussion is on the two important aspects, first objective was the role of sucrose in radiation protection in CtDNA which is already demonstrated from DNA thermal denaturation studies and the second objective on the possible structural changes in CtDNA induced by sucrose and its consequences on physicochemical properties surrounding DNA using a well known DNA minor groove binder Hoechst 33258. The second objective is very important from the view point of understanding the mechanism of radioprotection. In addition, physicochemical basis of radioprotection by sucrose and other reported radioprotector molecules are not attempted and therefore this study assumes more importance.

The ability of sucrose to counter radiation effects by scavenging was already reported by Yoshinaga *et al.*, 1997 and Ryu, Hwa-Ha *et al.* 2002 [7, 8]. In another study by Morelli *et al.* [14] the free radical scavenging action of sucrose has been again demonstrated with a rate constant of 1.2×10^9 M^{-1} s^{-1}. These studies strongly support the fact that sucrose has free radical scavenging action. The present results on thermal denatura-

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tion of CtDNA demonstrated a concentration dependent effect of sucrose in irradiated CtDNA. Beyond 0.5 Osm concentration, sucrose caused lowering of T_m of CtDNA from 75 to 70 °C. This lowering in T_m indicated concern on structural alteration by sucrose alone and thus required detailed investigation. In this concentration range the change in water activity of DNA by sucrose is expected to be predominant [9].

Hoechst 33258 has been used as structural probe to investigate the changes in the binding sites arising out of conformation and sequence alteration in DNA [15]. Hoechst 33258 increase the stability of DNA and hence the T_m value (table 2) with strong affinity [3, 16]. Irradiation of Hoechst 33258-CtDNA complex in earlier study demonstrated this clear role [3]. In table 2 this data along with sucrose though demonstrated this effect but additional information were also reflected in terms of lowering of T_m in presence of sucrose. Because of these observations, we have first assessed the thermodynamics of Hoechst 33258–CtDNA interactions in both irradiated and unirradiated CtDNA solutions (table 3). Thermodynamics of Hoechst 33258-CtDNA interaction provide better understanding of the nature of binding forces involved in interaction process. The interaction forces between a small molecule like Hoechst 33258 and DNA primarily include several non-covalent forces like hydrophobic force, electrostatic interactions, vander Waals interactions, hydrogen bonds [17]. In the present study, the thermodynamic data suggest a favorable and strong binding of Hoechst 33258 with CtDNA. The ratio of $-T\Delta S/\Delta G$ was 0.88 correspond to minor groove binding of Hoechst 33258 with CtDNA [17]. The binding of Hoechst 33258 to irradiated CtDNA was also energetically favorable with ΔS 122 Jmole^{-1}K^{-1}, ΔH -6.3 kJmole^{-1} and ΔG - 42.5 kJmole^{-1} (table 3). The slight variation in thermodynamics between the Hoechst 33258 binding to unirradiated and irradiated CtDNA suggest that after irradiation the binding sites on the CtDNA become relatively more hydrated and thereby lowering the binding affinity of Hoechst 33258 with irradiated CtDNA. More importantly the ratio of $-T\Delta S/\Delta G$ was 0.85 [17] for the binding

of Hoechst 33258 to irradiated CtDNA revealed that the Hoechst 33258 still binds in the minor groove of irradiated CtDNA.

Our previous study on Hoechst 33258-CtDNA interaction [15] also elucidated the uptake of water molecule accompanying Hoechst 33258-CtDNA complexation in presence of sucrose. The present study also revealed uptake of water molecule for Hoechst 33258 binding with irradiated CtDNA in presence of sucrose. The binding affinity of Hoechst 33258 with irradiated CtDNA decreased with increase in concentration of the sucrose. The overall uptake of water molecules accompanying Hoechst 33258 binding with un-irradiated and irradiated CtDNA was calculated as 30±1 and 22±2 water molecules respectively. The lesser number of water uptake for the interaction of Hoechst 33258 with irradiated CtDNA is correlated with its weak binding affinity. This is also indicated in the thermodynamic study (table 3). Lower concentration of sucrose (0-0.5 Osm) is not expected to alter the water activity significantly and a very small change in binding affinity of Hoechst 33258 was observed. At higher concentration of sucrose (>0.5 Osm), a large decrease in binding affinity was observed and the decrease could be additionally attributed to alteration in water activity. This is closely linked with radiation induced damage in CtDNA. Hydration layers surrounding DNA play an important role in induction of damages in DNA [18-20]. The free radicals produced in surrounding water can interact immediately with the microenvironment and DNA [21], sucrose is thus able to counter radiation effect by changing the water activity.

Spectroscopic studies using Hoechst 33258 and CtDNA can provide insight into the microenvironment of the binding sites in CtDNA in presence of sucrose (1 to 3 Osm). UV-visible spectroscopy data reveals that as the concentration of sucrose increases the O.D. at 260 nm increased while absorbance at 354 nm displays a slight decrease. This characteristic of absorption spectral changes at 260 nm is correlated with T_m values measured at higher concentrations of sucrose (>1Osm). Similar effect was observed in irradiated solutions. In fluorescence spectral

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measurements, presence of sucrose caused decrease in fluorescence intensity (figures 4 and 5). In addition, a red spectral shift of ~8 nm was observed at low concentration of Hoechst 33258 in unirradiated and irradiated complex. At higher concentration of Hoechst 33258, a spectral shift of ~14 nm was observed for the Hoechst 33258-CtDNA complex. Jin and Breasslauer [22] showed that as the polarity of the microenvironment increases, the fluorescence characteristics of Hoechst 33258 are marked by decrease in fluorescence intensity with red spectral shift. A similar change in microenvironment might have occurred due to the presence of sucrose, indicating that Hoechst 33258-CtDNA complex is more hydrated in presence of sucrose. In anisotropy measurements, the unaltered values show that Hoechst 33258 remains in the bound form and remained unaffected by presence of sucrose and irradiation. Thus sucrose does not alter the binding orientation of Hoechst 33258.

Fluorescence excited state lifetime measurements provide valuable information on binding nature of Hoechst 33258 with DNA [3, 12, 16, 23]. In aqueous solution, Hoechst 33258 is known to exist in two conformers viz., planar and non planar [3, 12, 24]. Fluorescence lifetimes of these two conformers are different in free and bound state. For example, Hoechst 33258 in phosphate buffer has the two decay values at 0.14 ns (t_1) and 2.5 ns (t_2) corresponding to short and long components respectively [3]. The short and long decay components t_1 and t_2 correspond to non planar and planar conformers of Hoechst 33258. The numerical values and relative contribution of both the conformers of Hoechst 33258 change upon binding with CtDNA [3, 15]. For example, in phosphate buffer the t_1 and t_2 for irradiated Hoechst 33258 (1 μM) –CtDNA complex was 2.2 and 4.1 ns and at higher concentration of Hoechst 33258 (10 μM)-CtDNA complex the value of t_1 and t_2 are 1.9 and 4.1 ns (table 5). In presence of highest concentration viz., 3 Osm sucrose, t_1 and t_2 of irradiated Hoechst 33258 (10 μM)-CtDNA complex, the short and long decay constants become 1.6 and 4.0 ns without any significant alteration in their relative contribution (table 5) suggesting the bound form of

Hoechst 33258. Thus the two binding form of Hoechst 33258 continue to exist even in altered water activity induced by sucrose and remains unaffected from the effect of ionizing radiation. These findings suggested that though hydration is reduced by sucrose but the properties of altered water surrounding DNA remained unchanged.

CONCLUSIONS

Sucrose is a known free radical scavenger and able to reduce the radiation induced structural changes in DNA. At lower concentration (<0.5 Osm), sucrose reduces the effect of γ-radiation primarily by free radical scavenging and at higher concentration concentration (>0.5 Osm), an additional factor that is reduction of the water activity contributed in radioprotection. This combined mode of action of sucrose is an important finding for understanding the mechanism and designing better approaches for radioprotection. The detailed absorption and fluorescence spectral studies indicated minor changes (viz., water activity, and binding affinity) involved in the interaction of Hoechst 33258 with irradiated CtDNA at higher concentrations of sucrose. The overall nature of binding characteristics of Hoechst 33258 remains unaffected suggesting unaltered structural integrity of CtDNA in presence of sucrose. These results are expected to have implications in development of radioprotectors.

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