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Binding of circulating autoantibodies in breast cancer to native and peroxynitrite-modified RNA

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Abstract: Peroxynitrite ($ONOO^-$) is a powerful oxidant and nitrosative agent and has in vivo existence. The half life of $ONOO^-$ at physiological pH is less than 1 s. It can react with nucleic acids, proteins, lipoproteins, saccharides, cardiolipin, etc., and can modify their native structures. Action of $ONOO^-$, synthesized in the authors' laboratory by a rapid quenched flow process, on structural changes of commercially available RNA was studied by ultraviolet (UV), fluorescence, and agarose gel electrophoresis. Compared to native RNA, the $ONOO^-$-modified RNA showed hyperchromicity at 260 nm. Furthermore, the ethidium bromide (EtBr) assisted emission intensities of $ONOO^-$-modified RNA samples were found to be lower than the emission intensity of native RNA-EtBr complex. Agarose gel electrophoresis of $ONOO^-$-modified RNA showed a gradual decrease in band intensities compared to native RNA, an observation clearly due to the poor intercalation of EtBr with $ONOO^-$-modified RNA. Native and $ONOO^-$-modified RNA samples were used as an antigen to detect autoantibodies in sera of patients with clinically defined breast cancer. Both direct binding and inhibition enzyme-linked immunosorbent assay (ELISA) confirmed the prevalence of native and 0.8 mmol/L $ONOO^-$-modified RNA specific autoantibodies in breast cancer patients. Moreover, the progressive retardation in the mobility of immune complexes formed with native or 0.8 mmol/L $ONOO^-$-modified RNA and affinity purified immunoglobulin G (IgG) from sera of breast cancer patients supports the findings of the direct binding and inhibition ELISAs. The peroxynitrite treatment to RNA at a higher concentration appears to have damaged or destroyed the typical epitopes on RNA and thus there was a sharp decrease in autoantibodies binding to 1.4 mmol/L $ONOO^-$-modified RNA. It may be interpreted that cellular nitrosative stress can modify and confer immunogenicity on RNA molecules. Higher concentrations of nitrogen reactive species can be detrimental to RNA. However, the emergence of native as well as 0.8 mmol/L $ONOO^-$-modified RNA as a novel antigen/substrate for autoantibodies in breast cancer patients indicates that, in future, these molecules might find a place on the panel of antigens for early diagnosis of breast cancer.

Key words: RNA, Peroxynitrite, ELISA, Breast cancer, Band shift assay
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1 Introduction

Peroxynitrite ($ONOO^-$) is formed in biological systems when superoxide reacts with nitric oxide. Peroxynitrite reacts with a variety of cellular targets yielding strand breaks and 8-oxoguanine in nucleic acids, protein sulfoxidation, nitration and hydroxylation, peroxidation of lipids and low density lipoproteins, oxidation of monohydroascorbate and triphosphopyridine nucleotide (NADPH), etc. (Pacher *et al*., 2007). With a pKa of approximately 6.6 (Goldstein *et al*., 2005), peroxynitrite is protonated to its conjugate peroxynitrous acid (HOONO) at physiological conditions. However, the acid itself is very unstable and the half life of peroxynitrite is less than 1 s (Denicola *et al*., 1998).

Peroxynitrite can damage DNA by introducing oxidative modifications in nucleobases as well as sugar phosphate backbone (Burney *et al*., 1999). Of the four

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bases, guanine is the hot spot for the reaction due to its low reduction potential (Yu *et al*., 2005; Niles *et al*., 2006). The major product of guanine oxidation is 8-oxoguanine, which further reacts with peroxynitrite and forms cyanuric acid, oxazolone, spiroiminodihydantoin, and guanidinohydantoin (Niles *et al*., 2006). In addition, 8-nitroguanine and 5-guanidino-4-nitroimidazole may also appear as a consequence of guanine nitration. It has been shown that reactive nitrogen species also introduces formation of 8-nitroguanosine in calf liver RNA, which is relatively more stable as compared to 8-nitroguanine in DNA and thus can be a good marker of cellular damage mediated by peroxynitrite (Masuda *et al*., 2002). Ultimately, guanine oxidation by peroxynitrite results in guanine fragmentation, a critical step towards mutagenesis and carcinogenesis. The genotoxic potential of peroxynitrite detected in cell culture studies and purified DNA supports the contention that intense and prolonged peroxynitrite dependent on oxidative and nitrosative stresses prevailing under inflammatory conditions might foster the development of cancer (Pacher *et al*., 2007).

Immune system produces a humoral response to cancer derived surface antigen and induces antibody formation. Wasserman *et al*. (1975) observed a higher incidence of smooth muscle autoantibodies (SMA) and antinuclear antibodies (ANA) in breast cancer patients at mastectomy than in matched controls. Breast cancer is the second most common type of cancer and the fifth most common cause of death (Jin and Zangar, 2009). Like many cancers, breast cancer is also the result of multiple environmental and hereditary factors. Chapman *et al*. (2007) assessed the diagnostic potential of autoantibodies to multiple known tumor associated proteins. Sera from normal control (n=94), primary breast cancer patients (n=97), and patients with ductal carcinoma (n=40) investigated for autoantibodies to p53, cellular-myc (c-myc), human epidermal growth factor receptor 2 (HER2), New York esophageal cell carcinoma-1 (NY-ESO-1), breast cancer type-1 and type-2 (BRCA1), BRCA2, and MUC1 (also known as cancer antigen 15-3 (CA15-3)) antigens by enzyme-linked immunosorbent assay (ELISA) showed elevated levels of autoantibodies against one of the above antigens in 64% of primary breast cancer and 45% of ductal carcinoma sera. It was suggested that the use of a panel of antigens might be helpful in the early diagnosis of breast cancer.

Chronic inflammation is important constituents of the local environment of tumors and has long been recognized with increased risk of cancer (Mantovani *et al*., 2008; Sing *et al*., 2011). Furthermore, chronic inflammation can induce persistent generation of peroxynitrite which can cause potential destruction of host constituents, possibly through induction of oxidative, nitrative, and nitrosative damages to nucleic acids, proteins, and lipids. Reactive nitrogen species have been implicated as a cause of diverse pathophysiological conditions including cancer (Masuda *et al*., 2002). Therefore, the present study was designed to analyze the structural changes in RNA under the influence of peroxynitrite. This was followed by evaluation of the binding profile and specificity of breast cancer autoantibodies to RNA and its peroxynitrite-modified forms. We hypothesize that a serum autoantibody assay against a panel of antigens including RNA and/or peroxynitrite-modified RNA may be a step forward towards early diagnosis of breast cancer.

2 Materials and methods

2.1 Materials

Yeast RNA and tetramethylethylenediamine (TEMED) were purchased from BDH (Poole, England). Diethylene penta-acetic acid (DTPA), bovine serum albumin, para-nitrophenyl phosphate, ethidium bromide, Coomassie brilliant blue, Tween-20, agarose, and dialysis tubing were purchased from Sigma Chemical Company, USA. Acrylamide, bisacrylamide, and sodium dodecyl sulphate (SDS) were purchased from Sisco Research Laboratories (SRL), India. Flat bottom ELISA modules were obtained from NUNC, Denmark. Protein A-agarose matrix was from Genei, India. All other chemicals used in the study were of highest analytical grade available.

2.2 Blood samples

Blood samples were collected from 30 female patients admitted in the radiotherapy ward of the Jawaharlal Nehru Medical College, Aligarh Muslim University, having direct and indirect signs and symptoms of breast cancer. The diagnosis was made on the basis of radiological imaging, clinical symptoms, and

fine needle aspiration cytology (FNAC) findings typical of breast cancer. Control blood samples (n=30) were obtained from apparently healthy individuals who were not suffering from any acute or chronic disease(s) and not taking any medication. Collected blood samples were allowed to clot at room temperature and sera was separated after centrifugation at 2000 r/min for 10 min. All serum samples were decomplemented by heating at 56 °C for 30 min and stored at −80 °C until further analysis.

2.3 Isolation of serum immunoglobulin G

Immunoglobulin G (IgG) was isolated from serum samples of breast cancer patients and healthy subjects using a protein A-agarose column (Goding, 1978). The concentration of isolated IgG was determined using 1.4 optical density at 280 nm (OD_{280})=1.0 mg IgG/ml. The IgG was dialyzed against phosphate buffered saline (PBS) and stored at −20 °C with 0.1% (1 g/L) sodium azide.

2.4 Synthesis of peroxynitrite

Peroxynitrite was synthesized in the authors' laboratory by a rapid quenched flow process (Koppenol *et al*., 1996). The synthesized $ONOO^-$ was kept in 1.2 mol/L sodium hydroxide (NaOH) and stored at −20 °C. The concentration of stored $ONOO^-$ was evaluated before use by recording its absorbance at 302 nm using a molar extinction coefficient of 1670 $(mol/L)^{-1}\cdot cm^{-1}$ (Hughes and Nicklin, 1968).

2.5 Modification of RNA by peroxynitrite

RNA (0.2 mmol/L) was dissolved in 10 mmol/L phosphate buffer (pH 7.4) and treated with 10 mmol/L DTPA to chelate ions. $ONOO^-$ at varying concentrations (0.8 mmol/L, 1.4 mmol/L, and 2.0 mmol/L) was then added to DTPA-treated RNA solution. The assay tubes were thoroughly mixed and reaction was continued for 30 min at 37 °C.

2.6 Spectroscopic studies of native and $ONOO^-$-modified RNA

Absorption profile of yeast RNA and its $ONOO^-$-modified counterparts were recorded in the wavelength range of 220–450 nm using quartz cuvette of 1 cm path length. Fluorescence emission spectra of native and modified-RNA samples were recorded in the presence of ethidium bromide (EtBr). Equal amounts of native/$ONOO^-$-modified RNA samples were excited at 310 nm and the loss in emission intensity was recorded. The difference in emission intensities of native and $ONOO^-$-modified RNA samples was calculated to analyze the effect of $ONOO^-$ on RNA structure.

2.7 Agarose gel electrophoresis

Native and $ONOO^-$-modified RNA samples were mixed with 1/10 volume of sample buffer (0.125% (1.25 g/L) bromophenol blue, 30% Ficoll-400, 5 mmol/L ethylenediaminetetraacetic acid (EDTA) in 10× electrophoresis buffer). Electrophoresis was carried out for 2 h at 30 mA constant current. Gels were stained with EtBr (0.5 μg/ml in distilled water) and visualized under ultraviolet (UV) light.

2.8 ELISA

ELISA was carried out on flat bottom polystyrene modules as described earlier (Ali and Alam, 2002). Briefly, microtitre wells were coated with 100 μl of RNA and/or $ONOO^-$-modified RNA antigens (10 μg/ml in tricine-buffered-saline (TBS), pH 7.4) and incubated for 2 h at 37 °C and then overnight at 4 °C. Each sample was coated in duplicate and half of the plate served as control, devoid of antigen only. The antigen coated wells were emptied and washed thrice with TBS-Tween 20 (T) to remove unbound antigen. Unoccupied sites were blocked with 150 μl of 1.5% (15 g/L) non-fat dry milk (in TBS, pH 7.4) for 4–5 h at 4 °C and wells were washed once. In direct binding ELISA antibodies were directly added into antigen-coated wells and incubated for the desired time period at 37 and 4 °C, respectively. The wells were emptied and extensively washed with TBS-T. Anti-immunoglobulin alkaline phosphatase conjugate (diluted as per manufacturer's instructions) was added to each well. The conjugate was incubated at 37 °C for 2 h and then the plates were washed four times with TBS-T and three times with distilled water. Paranitrophenyl phosphate was added and developed color was read at 410 nm in a microplate reader. The results were expressed as mean of difference of absorbance values in test and control wells (A_{test}–$A_{control}$).

2.9 Inhibition ELISA

The antigenic specificity of antibodies was determined by inhibition ELISA. Varying amounts of

inhibitors (0–20 μg/ml) were mixed with a constant amount of IgG purified from the sera of breast cancer patients. The mixture was incubated at room temperature for 2 h and overnight at 4 °C. The immune complex thus formed was coated in the wells instead of antibodies. The remaining steps were the same as in direct binding ELISA. Percent inhibition (I) was calculated using the formula ($A_{\text{inhibited}}$: absorbance value of inhibited; $A_{\text{uninhibited}}$: absorbance value of uninhibited):

$$I = \left(1 - A_{\text{inhibited}} / A_{\text{uninhibited}}\right) \times 100\%.$$

2.10 Band shift assay

Antigen-antibody interaction was visualized by gel retardation assay (Dixit *et al*., 2011). Immune complexes were prepared by incubating varying amounts of affinity purified breast cancer IgG (10–50 μg) with constant amount of antigen (native, 0.8 mmol/L, and 0.4 mmol/L $ONOO^-$-modified RNA) for 2 h at 37 °C and overnight at 4 °C. The immune complex thus formed was electrophoresed on 0.8% (8 g/L) agarose gel for 2 h at 30 mA. The gel was visualized under UV light after staining with ethidium bromide.

3 Results

3.1 UV characteristics of $ONOO^-$-modified RNA

Generation of peroxynitrite was confirmed by the formation of yellow color and characteristic peak at 302 nm. Treatment of RNA with peroxynitrite produced hyperchromicity. Furthermore, the hyperchromicity showed an increasing trend with an increase in peroxynitrite. The UV characteristics of RNA and $ONOO^-$-modified RNA samples have been summarized in Table 1.

Table 1 UV characteristics of RNA and $ONOO^-$-modified RNA

Sample	Absorbance at 260 nm	Hyperchromicity (%)
RNA	0.149	
0.8 mmol/L $ONOO^-$-RNA	0.327	54.4
1.4 mmol/L $ONOO^-$-RNA	0.776	80.7
2.0 mmol/L $ONOO^-$-RNA	1.856	91.9

3.2 Fluorescence spectroscopy of $ONOO^-$-modified RNA

Emission profiles of samples (Native RNA and its $ONOO^-$-modified counterparts) were recorded after excitation at 310 nm. As shown in Fig. 1, EtBr gave an emission peak at around 585 nm. Interaction of EtBr with native RNA produced a tremendous increase in fluorescence intensity at 585 nm. None of the three $ONOO^-$-modified RNA samples showed matching fluorescence intensity with EtBr-RNA. Furthermore, we observed that the EtBr-assisted fluorescence of $ONOO^-$-modified RNA samples was gradually decreased to 4.7%, 22.0%, and 41.9%, respectively with increase in peroxynitrite concentration (Table 2).

3.3 Agarose gel electrophoresis

Native and $ONOO^-$-modified RNA samples were subjected to electrophoresis in agarose gel to

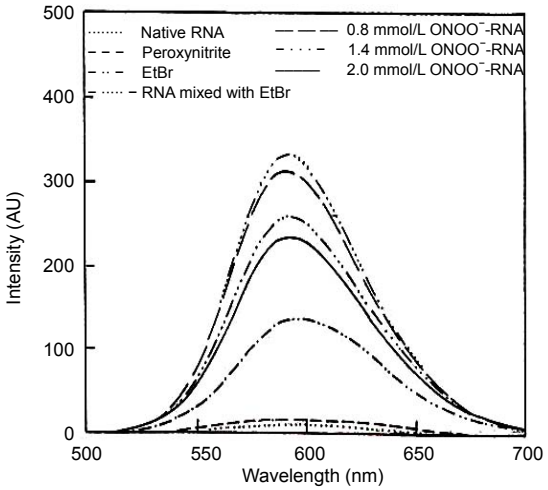

Fig. 1 Emission profile of native RNA, $ONOO^-$, ethidium bromide (EtBr), RNA mixed with EtBr and RNA samples treated with 0.8, 1.4, and 2.0 mmol/L $ONOO^-$

Table 2 EtBr-assisted fluorescence characteristics of RNA and $ONOO^-$-modified RNA

Sample	FI	Percent quenching of FI (%)
RNA	335	
0.8 mmol/L $ONOO^-$-RNA	319	4.7
1.4 mmol/L $ONOO^-$-RNA	261	22.0
2.0 mmol/L $ONOO^-$-RNA	236	41.9

FI: fluorescence intensity

study the effect of peroxynitrite on migration and EtBr staining properties of RNA. Native RNA moved as single broad band (Fig. 2). Under identical conditions, the banding pattern of RNA treated with 0.8 mmol/L, 1.4 mmol/L, and 2.0 mmol/L $ONOO^-$ showed a gradual decrease in fluorescence intensity. This shows that, native RNA molecules have secondary structure which favors EtBr intercalation. Moreover, peroxynitrite induced modification of RNA appears to have destroyed the secondary structures in yeast RNA. To demonstrate that the yeast RNA indeed possesses secondary structures, we performed agarose gel electrophoresis of native and heat denatured RNA. The agarose gel electrophoresis of heat denatured RNA did not show any fluorescence when stained with EtBr.

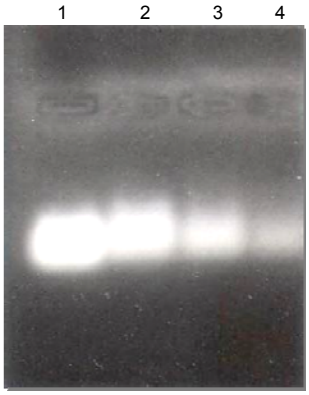

Fig. 2 Agarose gel electrophoresis of native RNA and RNA modified by different amounts of peroxynitrite
Lane 1: native RNA; Lanes 2, 3, and 4: 0.8, 1.4, and 2.0 mmol/L $ONOO^-$-RNA

3.4 ELISA and gel retardation assay

Direct binding ELISA of autoantibodies in breast cancer patients suggests that native RNA and 0.8 mmol/L $ONOO^-$-modified RNA are the preferred antigens (Fig. 3). Binding specificity of breast cancer IgG antibodies as well as IgG isolated from normal subjects was evaluated by inhibition ELISA using native RNA and $ONOO^-$-modified RNA as inhibitors (Table 3). Negligible inhibition was observed in IgG isolated from healthy subjects. Inhibition studies showed that native and 0.8 mmol/L $ONOO^-$-modified RNA emerged as the most effective inhibitors of IgG antibodies derived from sera of breast cancer patients. In other words, epitopes of native and RNA modified with lower concentration of $ONOO^-$ were specifically recognized by IgG autoantibodies of breast cancer

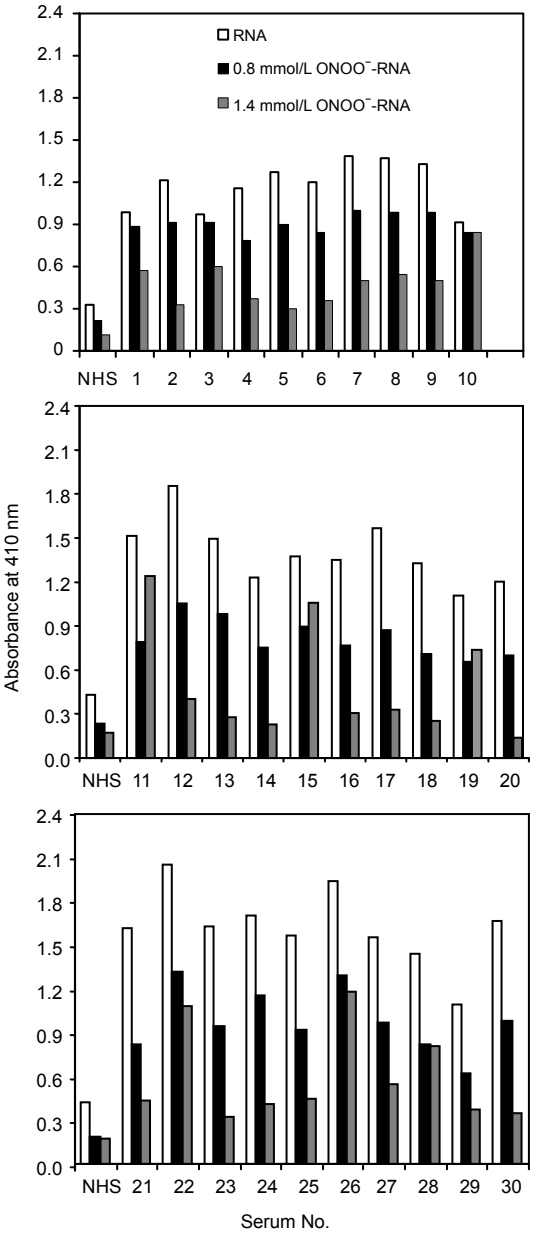

Fig. 3 Direct binding ELISA of breast cancer sera (1:100 dilution) with native RNA, 0.8 and 1.4 mmol/L $ONOO^-$-modified RNA
Microtitre wells were coated with respective antigens. NHS: normal human serum

Table 3 ELISA of breast cancer IgG with native and $ONOO^-$-modified RNA

Inhibitor	I (%)*
RNA	82.2
0.8 mmol/L $ONOO^-$-RNA	70.4
1.4 mmol/L $ONOO^-$-RNA	55.0

* Maximum percent inhibition (I) at 20 μg/ml. Microtiter wells were coated with 100 μl of native and $ONOO^-$-modified RNAs (10 μg/ml)

patients. A similar observation was made in the gel retardation assay (Fig. 4).

4 Discussion

Peroxynitrite is a cytotoxic reactive nitrogen species generated from the reaction of superoxide radical and nitric oxide and has in vivo existence. Like nitric oxide, peroxynitrite has both deleterious and beneficial effects. Both neutrophils and macrophages produce peroxynitrite by a rapid biradical reaction generated simultaneously from within the cells (Ischiropoulos *et al*., 1992). An advantage of the oxidant mediated deleterious effect of peroxynitrite is that it contributes to the host defense response to bacterial invasion. The toxicity of peroxynitrite has been attributed to its ability to oxidize thiols, lipids, proteins, enzymes, nucleic acids (Pacher *et al*., 2007) and cardiolipin (Pope *et al*., 2008).

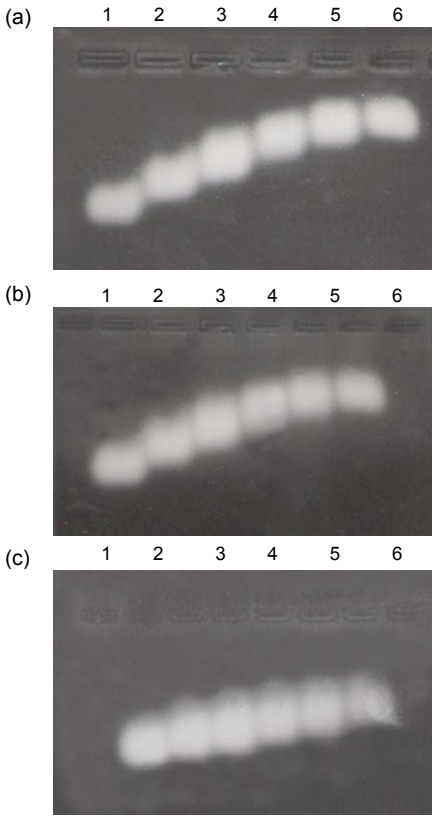

Fig. 4 Mobility shift assay of native RNA (a), 0.8 mmol/L $ONOO^-$-RNA (b), and 1.4 mmol/L $ONOO^-$-RNA (c) in presence of varying amounts of breast cancer IgG

4 μg of RNA or 0.8/1.4 mol/L $ONOO^-$-RNA (Lane 1) was mixed with 10, 20, 30, 40, and 50 μg of IgG (Lanes 2 to 6) and electrophoresed on 0.8% agarose gel after necessary incubation

In our study, treatment of yeast RNA by different doses of peroxynitrite produced hyperchromicity at 260 nm. In the environment of increasing peroxynitrite, the hydrogen bonds of the paired bases appear to have undergone gradual destruction due to both oxidation and nitration by peroxynitrite. It has also been reported earlier by Khan *et al*. (2009) that peroxynitrite induced modification of H_2A histone caused hyperchromicity at 276 nm, which could be attributed to peroxynitrite induced denaturation, oxidation, and nitration. Finally, it may be said that the nitration effect of peroxynitrite has been masked by the hyperchromicity produced due to its oxidizing action. Further support in favor of existence of base paired structures in yeast RNA come from a pronounced increase in fluorescence intensity of RNA-EtBr mixture when excited at 310 nm. The presence of a base paired structure in yeast RNA appears to have favored EtBr intercalation and thus an increase in fluorescence intensity. Once the hydrogen bonds of the paired bases were broken, the room for EtBr intercalation decreased, which might be responsible for the decrease in fluorescence intensities of all species of $ONOO^-$-modified RNA as compared to fluorescence intensity of native RNA. In addition to the above, the destruction of base paired hydrogen bonds in yeast RNA might also be caused by nitration of bases. In agarose gel electrophoresis the decrease in fluorescence intensities of $ONOO^-$-modified RNA samples substantiates our results of UV and fluorescence studies, and the heat denatured RNA could not be stained with EtBr, which reiterates the existence of hydrogen bonded base paired structures which were destroyed by the application of heat.

Antigen binding and specificity of autoantibodies in breast cancer sera were evaluated by direct binding and inhibition ELISA using RNA and $ONOO^-$-modified RNA as antigens. Results of direct binding ELISA conclusively demonstrated that native as well as 0.8 mmol/L $ONOO^-$-modified RNA is the preferred antigen for autoantibodies in breast cancer patients. Poor binding of paitents autoantibodies to 1.4 mmol/L $ONOO^-$-modified RNA could be attributed to loss of native epitopes due to peroxynitrite mediated oxidation and nitration. Results of inhibition ELISA further reiterated that RNA and

0.8 mmol/L $ONOO^-$-modified RNA are effective inhibitors. The above findings were further confirmed by gel retardation assay. It may be interpreted that cellular nitrosative stress at higher concentration of $ONOO^-$ is detrimental to RNA. However, the emergence of RNA and 0.8 mmol/L $ONOO^-$-modified RNA as a novel antigen/substrate for autoantibodies in breast cancer indicates that, in future, these vital molecules might find a place on the panel of antigens for early diagnosis of breast cancer. The real clinical significance of autoantibodies for screening and early diagnosis of breast cancer lies in the use of a panel of native and altered/modified autoantigens to detect the full range of heterogeneous autoantibodies in breast cancer patients. However, these autoantibodies are either absent or present in very low titer in healthy individuals (Tan and Zang, 2008).

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