Applied Biological Research 24(3): 317-326; (2022)
DOI: 10.5958/0974-4517.2022.00038.6

BIOPHYSICAL AND IMMUNOLOGICAL CHARACTERIZATION OF CARBAMYLATED-DNA: SERUM AUTOANTIBODIES OF DIABETIC NEPHROPATHY PATIENTS EXHIBIT STRONG BINDING WITH CARBAMYLATED-DNA

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(Receive d 15 May, 2022; accepted 16 July, 2022)

ABSTRACT

Isocyanates (ICN) have drawn considerable attention in recent past as they can react with a variety of nucleophiles including DNA, proteins and cause damage(s). However, pathophysiological implications resulting from the occupational and accidental exposures of ICN are yet too elusive. It may be produced in high concentrations in the conditions of chronic renal failure and chronic inflammatory diseases (diabetes mellitus, rheumatoid arthritis, etc.). In this study, mammalian dsDNA was modified with ICN and characterized by UV, fluorescence, FT-IR and thermal melting methods. ICN mainly reacts with exocyclic nitrogen of DNA bases. Antibodies against carbamylated-DNA were raised in rabbits and characterized by direct binding and inhibition ELISA. The presence of anti-carbamylated-DNA autoantibodies in the sera of diabetic nephropathy patients was evaluated by ELISA. Carbamylated-DNA exhibited hyperchromicity at 260 nm and depicted increase in ethidium bromide-assisted fluorescence as compared to the native DNA. Furthermore, the appearance of prominent new peaks in FT-IR profile at different wave numbers suggested carbamoylation-induced changes in the vibration of sugar-phosphate backbone, base(s) etc. Carbamylated-DNA was highly immunogenic as compared to the native DNA. Experimental induction of antibodies against carbamylated-DNA and presence of autoantibodies against carbamylated-DNA in the sera of diabetic nephropathic patients point towards the significance of carbamylated-DNA in diabetic nephropathy.

Keywords: Autoantibodies, carbamylated-DNA, diabetic nephropathy. isocyanates

INTRODUCTION

Isocyanates (ICN; R-N=C=O), are highly reactive electrophilic chemicals that react with a variety of nucleophiles including DNA and proteins (Vandenabeele-Trambouze *et al*., 2001; Beyerbach *et al*., 2006). They are extensively used in the manufacture of polyurethane foam, rubber, adhesives, pesticides, and many other products (Heath, 2017). ICN can also be formed *in vivo* by urea deamination, by myeloperoxidase mediated catabolism of thiocyanate (Delanghe *et al*., 2017), or through the metabolism of hydroxamic acids (Wang, 2009; Smith, 2011). ICN act as direct acylating agents on the primary amino groups of both DNA nucleobases and proteins in a reaction called carbamoylation (Beyerbach *et al*., 2006; Delanghe *et al*., 2017). Unlike most other classes of

carcinogens and mutagens, ICN do not require metabolic activation to form covalent bonds with the DNA nucleobases (Liljenberg *et al.*, 2020). Recently, Liljenberg *et al.* (2020) have developed computational protocols suitable for investigating the reactivity and mechanism of the reaction between an ICN and a primary amine, and used it to investigate several possible reaction pathways of carbamoylation of nucleobases.

The hazardous effects of ICN include occupational asthma, other lung problems, and severe irritation of eyes, nose, throat, and skin; and are known to cause genotoxic, mutagenic, and carcinogenic effects (Beyerbach *et al*., 2006; Benigni and Bossa, 2011; Delanghe *et al*., 2017). Further, methyl isocyanate (MIC) is a reactive industrial byproduct and probably one of the most toxic ICN. It exerts detrimental effects on numerous organ systems and immunity (Worthy, 1985). *In vitro* studies on MIC and its reaction products have revealed the evidences of mutagenicity evoking probable clastogenic activity (Caspary and Myhr, 1986). The MIC also reacts with exocyclic amino group of dNTPs to produce carbamylated products (Segal *et al*., 1989; Tamura *et al*., 1990); producing DNA cross-links which**,** in turn, contribute towards cytotoxicity (Baumann, 2005; Mishra *et al*., 2008; Liljenberg *et al*., 2020). These researches implicate that occupational and accidental exposure to MIC might possibly increase the susceptibility of individuals by eliciting hypersensitivity reactions, causing autoimmunity or immune suppression. The objectives of present study was to i) modify mammalian dsDNA with N-succinimidyl N-methylcarbamate (NSNM) and study the changes in NSNM-modified DNA by physicochemical techniques like UV, fluorescence and FT-IR spectroscopy, agarose gel electrophoresis and thermal melting; ii) evaluate the immunogenicity of NSNM-modified DNA in experimental animals; and iii) use the NSNM-modified DNA as an antigen for detection of autoantibodies against NSNM-modified DNA in the sera of diabetic nephropathy patients and thereby propose a potential role for NSNM-modified DNA in T2DM nephropathy.

MATERIALS AND METHODS

Materials

Highly polymerized calf thymus DNA, N-succinimidyl N-methylcarbamate (NSNM), Freund's adjuvant (complete and incomplete), tween-20, ethidium bromide (EtBr), methylated BSA, anti-human- and anti-rabbit-IgG alkaline phosphatase conjugates, and dialysis tubing were purchased from Sigma-Aldrich, USA. Disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride and ethylenediaminetetraacetic acid (EDTA) were purchased from Qualigens/Thermo-Fischer, India. Para-nitrophenyl phosphate (PNPP) was procured from SRL, India. Dimethyl sulfoxide (DMSO) was obtained from J.T. Baker Chemical Company, USA. All other reagents and chemicals used were of highest analytical grade available.

Collection and processing of animal and human blood samples

The work protocol was duly approved by the Institutional Ethical Committee vide letter D. No. 307/FM dated 28-05-2014. Rabbits (3-6 months old and weighing 1.0-1.5 kg) were housed in the cages with wide square mesh at the bottom and placed in a well-ventilated room with 12 h light and 12 h dark cycle, maintained at 28±2°C temperature and 50% humidity. All animals had free access to clean water and food for the entire duration of the experiment. Blood was withdrawn from the marginal ear veins of rabbits before and after the immunization and transferred to plain glass vials. The blood was left to clot and sera were separated. All sera were heat decomplemented at 56ºC for 30 min and stored at -20ºC.

A total of 30 patients of diabetic nephropathy (17 males and 13 females) and equal number of healthy subjects (16 males and 14 females) were included in this study. All the patients and healthy

subjects consented for providing their blood samples. Furthermore, the patients of diabetic nephropathy fulfilled the 2013 revised criteria of the American Diabetes Association.

Modification of dsDNA by N-succinimidyl N-methylcarbate

The commercially obtained calf thymus DNA was purified free of proteins, RNA and single stranded regions (Tantry *et al*., 2018). DNA (25µg mL^{-1}) was mixed with NSNM (0.19 and 0.58 mM) in 10 mM PBS (pH, 7.4) and incubated at 25ºC for 2 h in capped sterile glass tubes. The samples were then extensively dialyzed against PBS to remove excessive NSNM. Solutions of NSNM and DNA, dissolved separately in the same buffer, kept under identical conditions served as control.

Absorbance spectroscopy

UV profiles of native and NSNM-modified DNA were recorded on spectrophotometer (Shimadzu, Japan; UV-1700) using quartz cuvette of 1 cm path length. The hyperchromicity was calculated by the following formula:

$$\% \text{ Hyperchromicity at 260 nm} = \left(\frac{\text{OD modified sample} - \text{OD native sample}}{\text{OD native sample}} \right) \times 100$$

Fluorescence studies

Fluorescence measurements were carried out on spectrofluorophotometer (Shimadzu, Japan; RF-5301PC) in a cell of 1 cm path length. Native DNA and NSNM-modified DNA samples were slowly mixed with ethidium bromide (5 mg mL^{-1}) and incubated at 25ºC for 20 min in dark. The samples were then excited at 325 nm and emission spectra obtained in the range of 500 to 700 nm. The loss in fluorescence intensity (FI) was calculated from the following equation:

$$\% \text{ Loss in FI} = \left(\frac{\text{FI native sample} - \text{FI modified sample}}{\text{FI modified sample}} \right) \times 100$$

Fourier- transform infrared (FT-IR) spectroscopy

Ten microlitres each of native and NSNM-modified DNA samples were placed on the attenuated total reflection (ATR) and FT-IR scans were obtained in wave number range of 800 to 1700 cm^{-1} to cover vibrations of the characteristic bands in dsDNA.

Thermal denaturation studies

Thermal denaturation of native and NSNM-modified DNA sample was carried out on Peltier system attached with spectrophotometer [PerkinElmer UV/Vis spectrophotometer (Lambda-25) coupled with a temperature programmer; PTP-1+1 Peltier system and controller assembly, Julabo, USA]. The temperature was raised from 30 to 95ºC at a rate of 1ºC min^{-1} and change in the absorbance at 260 nm was taken as a measure of helix denaturation. The process was characterized by determining the percent DNA in denatured state as a function of temperature using the following equation:

$$\% \text{ Denaturation} = \left(\frac{A_T - A_{30}}{A_{max} - A_{30}} \right) \times 100$$

where, A_T = Absorbance at temperature (ºC); A_{max} = Final absorbance on the completion of denaturation (95ºC); A_{30} = Initial absorbance at 30ºC

Enzyme-linked immunosorbent assay (ELISA)

Direct binding ELISA*:* Direct binding ELISA was performed on flat bottom maxisorp plates as described (Talha *et al*., 2021). Briefly, the wells were filled with 100 µL antigen (native or NSNM-modified DNA) prepared in antigen coating buffer and incubated at 37ºC for 2 h followed by overnight incubation at 4ºC. Each sample was coated in duplicate and half of the plate, devoid of antigen, served as control. Following the overnight incubation, the plates were washed thrice with TBS-T (20 mM tris, 2.68 mM KCl 150 mM NaCl, pH 7.4 containing 0.05% tween-20) and unoccupied sites were blocked by 150 µL of 1.5% fat-free skimmed milk in TBS (10 mM tris, 150 mM NaCl, pH 7.4) for 4-6 h at 37ºC, followed by 3-4 washing with TBS-T. Thereafter, 100 µL sera

(serially diluted in TBS) was added to each well and plates placed for 2 h at 37ºC and overnight at 4ºC. The plates were then washed 4 times with TBS-T and bound antibodies assayed with anti-rabbit alkaline phosphatase conjugate (diluted in TBS). This was followed by 2 h incubation at 37ºC and 3-4 washing with TBS-T. Finally, the plates were washed with distilled water. Para-nitrophenyl phosphate was added and after 45 min incubation at 37ºC. The color was read at 405 nm on an automatic microplate reader (Bio-Rad, USA). The results were expressed as the mean of A_{test} - $A_{control}$.

Inhibition ELISA

Inhibition ELISA (Alam and Jabeen, 2007) was performed for specificity of antibodies. The plates were coated with 100 µL antigen (native or NSNM-modified DNA, 2.5 µg mL^{-1}) for 2 h at 37ºC and overnight at 4ºC. Varying amounts of inhibitors (0–20 µg mL^{-1}) were mixed with a constant amount of antiserum or affinity purified IgG. The mixtures were incubated at 37ºC for 2 h and overnight at 4ºC. The immune complex was coated in wells instead of serum. Rest of the steps were same as mentioned above in direct binding ELISA. Percent inhibition was calculated using the following equation:

$$\text{Percent Inhibition} = 1 - \left(\frac{A_{\text{inhibited}}}{A_{\text{uninhibited}}}\right) \times 100$$

Statistical analysis

Data were expressed as mean ± standard deviation (SD). The data were found to be normally distributed when analyzed by Kolmogorov-Smirnov test of normality. Furthermore, the statistical significance of the results was determined by Student's t-test; a *p* value of <0.05 was considered as significant. Figures labeled with star (*) indicate p-values ≤ 0.05 with respect to the control.

RESULTS AND DISCUSSION

Isocyanates are highly reactive compounds and have potential to cause structural changes in macromolecules. These compounds form covalent adducts with critical macromolecules such as nucleic acids, resulting in a series of biotransformation that initiate the generation of reactive intermediates (Shelby *et al*., 1987; Marczynski and Shapiro, 1992). DNA damage leading to the cellular demise in mammalian cells upon treatment with ICN has previously been reported (Beyerback *et al*., 2006).

Methyl isocyanate, one of the most toxic ICN exerts immunological, mutagenic and genotoxic effects (Deo *et al*., 1987; Saxena *et al*., 1988). Previous studies conducted on ICN suggest that they are capable of inducing apoptosis (Chen *et al*., 2003). Although the exact mechanism of such induction is largely unknown, most studies have reported the involvement of mitochondria-mediated pathway, i.e., release of cytochrome C into the cytoplasm and activation of caspase 9 and caspase 3 (Hu *et al*., 2003; Zhang *et al*., 2003; Tang and Zhang, 2005). ICN derivatives (carbamates) may also enhance the levels of inflammatory cytokines such as IL-6, TNF-α, and IL-1 β (East *et al*., 1992). In present study, calf thymus dsDNA was carbamylated with N-succinimidyl N-methylcarbamate. Modifications in DNA were characterized by multiple physico-chemical techniques (like UV-visible, fluorescence and FT-IR spectroscopy, thermal melting, etc.) and enzyme immunoassay.

Spectroscopic analysis of native and NSNM-modified DNA

DNA (25 µg mL^{-1}) treated with varying concentrations of NSNM showed hyperchromicity at 260 nm as compared to the native DNA (Fig. 1). Significant increase in absorbance was observed between 256 and 292 nm in all the modified samples as compared to the native DNA. This might be due to the generation of single stranded breaks in DNA helix inter alia unstacking of bases leading to enhanced absorption of UV light (Alam *et al*., 1993; Tantry *et al*., 2018).

Fluorescence spectroscopy

The formation of NSNM-DNA adduct was probed by ethidium bromide (EtBr). The samples were excited at 325 nm and the excitation wavelength of EtBr and emission intensities were recorded at 595 nm. EtBr gave an emission peak at 595 nm. Interaction of DNA with EtBr showed increase in fluorescence intensity (FI). Furthermore, the FI of NSNM-modified DNA incubated with EtBr increased with increase in NSNM concentration. The increase in FI of NSNM-modified DNA was calculated to be 7.8 and 10.7% at 0.19 and 0.58 mM NSNM, respectively, as compared to the native DNA (Fig. 2).

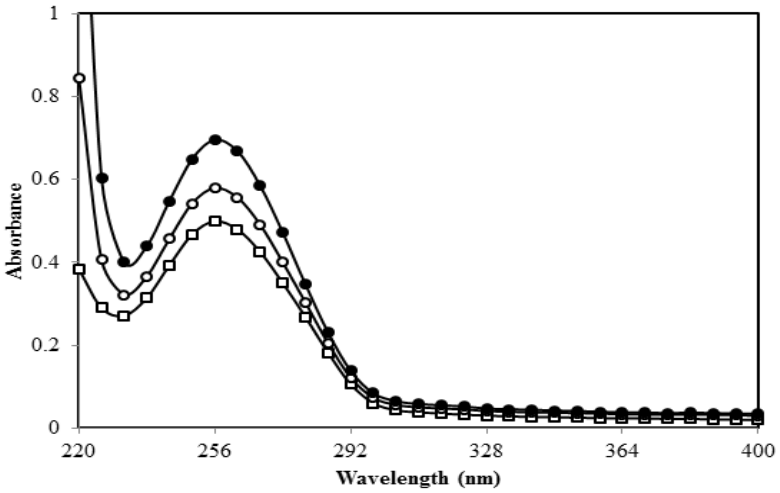


Fig. 1: UV absorption spectra of native DNA (-□-) modified with 0.19 mM (-○-) and 0.58 mM (-●-) NSNM

FT–IR spectroscopy

FT-IR spectroscopy is a sensitive tool to study DNA damage (Dovbeshko *et al*., 2000; Pijanka *et al*., 2009). Band fitting was performed in the spectral region of 800–1800 cm^{-1}. Earlier studies have suggested that the bands falling in the regions of 980-1149 and 1151-1350 cm^{-1} correspond to symmetric and asymmetric phosphate stretching (Polak *et al*., 2006). Further, the symmetric stretching of P–O–C bond and ribose-phosphate skeletal motion has been observed at 1110 and 970 cm^{-1}, respectively (Ugenskiene *et al*., 2007). In this study, the appearance of prominent new bands at different wave numbers suggests the carbamoylation-induced changes in the vibration of sugar-phosphate, base(s) etc. In other words, the adduction of carbamoyl group with base(s) in DNA is bound to cause stretching or bending of bonds vis-à-vis generation of a new signals at different wave numbers.

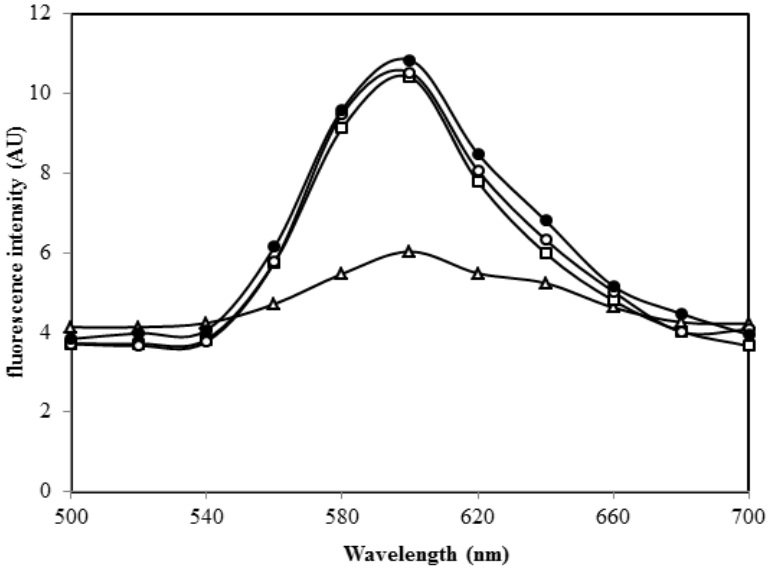


Fig. 2: Ethidium bromide (-△-) assisted emission profile of native DNA (-○-) modified with 0.19 mM (-●-) and 0.58 mM (-□-) NSNM. The samples were excited at 325 nm

FT-IR spectra of native and NSNM-modified DNA preparations were recorded in the region of 700–1800 cm^{-1}. When NSNM was added to the DNA, the guanine peak at 1400 cm^{-1} shifted to 1530 cm^{-1}. The peak at 1230 cm^{-1} gradually shifted to 1237 cm^{-1} while the peak at 1082 cm^{-1} shifted to 1088 cm^{-1} on the addition of NSNM. The percent transmittance of NSNM-modified DNA was significantly reduced as compared to the native DNA (Fig. 3).

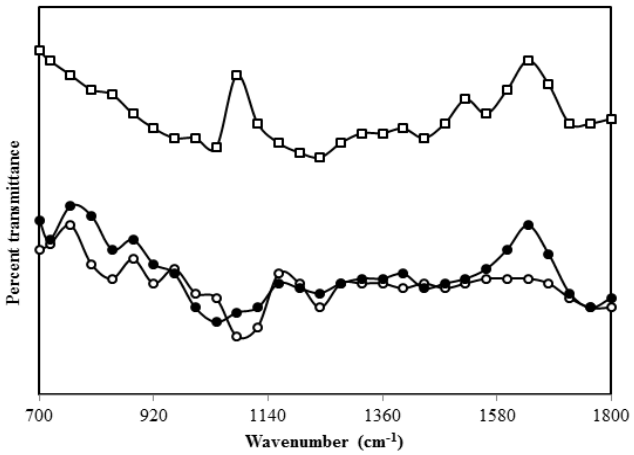


Fig. 3: FT–IR spectra of native DNA (- □ -) modified with 0.19 mM (-○ -) and 0.58 mM (-● -) NSNM

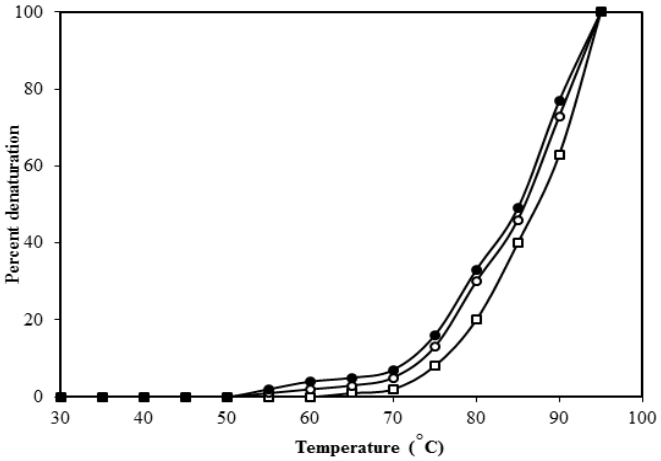


Fig. 4: Melting profile of native DNA (- □ -) modified with 0.19 mM (-○-) and 0.58 mM (-● -) NSNM

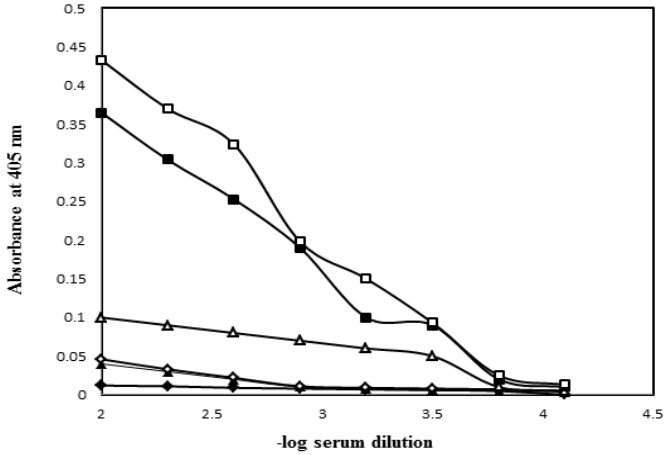


Fig. 5: Direct binding ELISA of native and NSNM modified-DNA from animal sera (immune and pre-immune). Pre-immune native (-◆-), modified1 (-▲ -) and modified2 (-◇-) DNA. Immune native (- △ -), modified1 (-■-) and modified2 (-□ -)

Thermal denaturation of NSNM-modified DNA

The effect of carbamoylation on the thermal stability of DNA was studied by recording heat-induced changes in absorbance at 260 nm. Both native and NSNM-modified DNA showed increase in absorbance from 75ºC onwards. The mid-point melting temperature (T_m) was calculated to be 87.5, 86.0 and 85.0ºC for native DNA and DNA modified with 0.19 and 0.58 mM NSNM, respectively. The observed decrease in *Tm* of NSNM-modified DNA samples suggest that hydrogen bonding between bases have changed during adduction of carbamoyl group. This may cause thermal destabilization in DNA (Alam *et al*., 1993). The thermal denaturation profiles of native and NSNM-modified DNA is depicted in Fig. 4.

Immunogenicity of native and NSNM-modified DNA

The carbamoylation-induced immunological changes in DNA were also probed by inducing antibodies in the experimental animals. Antisera were subjected to direct binding ELISA on maxisorp microtitre modules coated with respective immunogens. Antigen binding specificity of the induced antibodies was ascertained by inhibition ELISA

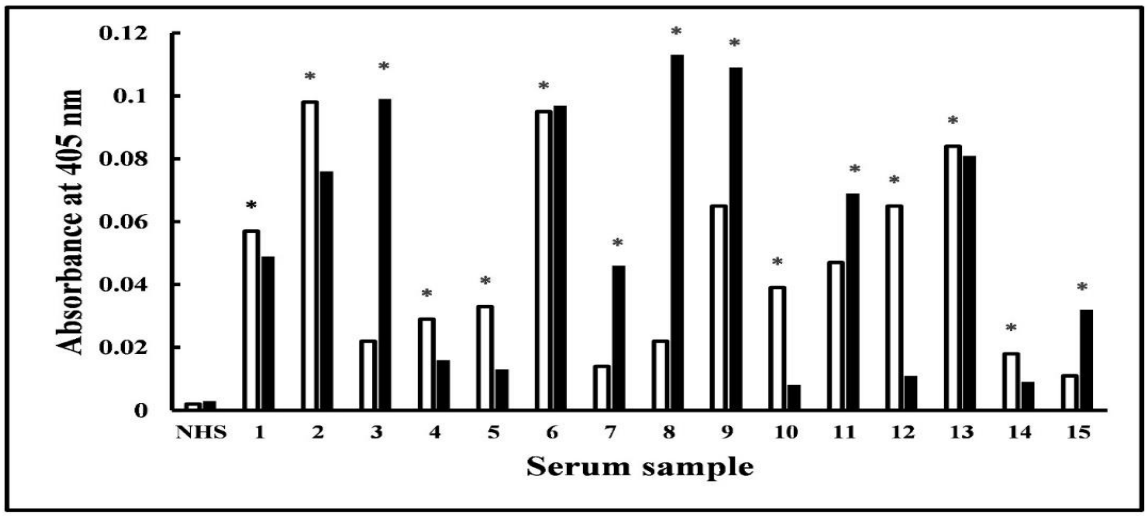
Antibody induction against native and NSNM-modified DNA

Rabbits immunized with native and NSNM-modified DNA induced low to moderate antibody response with a titre of <1:400 to >1:3200 as revealed by direct binding ELISA (Fig. 5). Pre-immune serum, included as control, showed negligible binding with the immunogens. A maximum of 14.7, 62.2 and 61.1% inhibition were observed with n DNA, 0.19 mM NSNM-DNA and 0.58 mM NSNM-DNA, respectively. Native DNA injected into rabbits did not elicit significant immune response. But NSNM-modified DNA

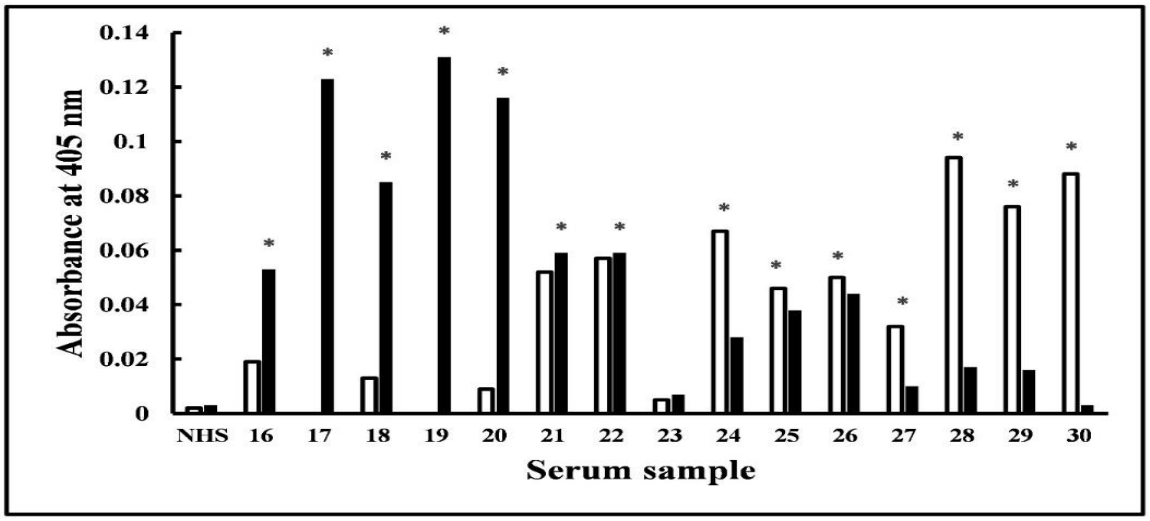
produced antibodies of low to moderate titre. This suggests the generation of immunogenic epitopes during the course of carbamoylation. Many studies have also reported the induction of antibodies against modified-DNA (Alam *et al*., 1993; Waris and Alam, 2004; Ahmad *et al*., 2014).

Detection of autoantibodies against NSNM-modified DNA in the sera of diabetic nephropathy patients

The study was extended to find out the clinical relevance of NSNM-modified DNA in diabetic nephropathy. This study included 30 patients of diabetic nephropathy (DN) and 30 healthy human subjects (HHS). These HHS served as control and were age- and sex-matched. Thirty sera each of diabetic nephropathy patients and healthy humans were diluted (1:100) and direct binding ELISA was performed on microtitre plates coated with native and NSNM-modified DNA. The autoantibodies in diabetic nephropathy sera showed enhanced binding with NSNM-modified DNA as compared to native DNA (Fig. 6a,b). Direct binding ELISA gave a strong clue that NSNM-modified DNA is better recognized by serum autoantibodies in DN patients.



(a)



(b)

Fig. 6: Direct binding ELISA of 1:100 diluted diabetic nephropathy sera [a) sample 1 to 15; b) sample 16 to 30] to native DNA (-□-) and 0.58 mM NSNM-DNA (-■-). Normal human sera (NHS) served as negative control. The ELISA plate was coated with the respective antigens (2.5 µg mL^{-1}). * indicates p-values ≤ 0.05 with respect to control.

Inhibition ELISA of patient's sera

Inhibition ELISA was performed to check the specificity of circulating autoantibodies towards NSNM-modified DNA. There was high inhibition of DN serum autoantibodies with NSNM-modified DNA as compared to the native DNA. The binding specificity of autoantibodies in diabetic nephropathy patient's sera with native and NSNM-modified DNA was assessed by inhibition ELISA. Patient's sera (No. 8, 9, 17, 18, 19 and 20) exhibiting high binding with NSNM-modified DNA in direct binding binding ELISA were selected for the inhibition studies. Mean percent inhibition was found to be 26.98 ± 6.18, 43.25 ± 3.28 and 45.1 ± 3.60% with nDNA, 0.19 mM and 0.58 mM NSNM-DNA, respectively (Table 1). This suggests true binding of autoantibodies with NSNM-modified DNA. Thus, the autoantibodies exhibited preference for NSNM-modified DNA. These autoantibodies might cross react with organ tissues, causing damage to them. Therefore, regular monitoring of serum urea level in diabetes mellitus seems important to prevent kidney disease.

Table 1: Inhibition of diabetic nephropathy serum autoantibodies binding by nDNA, 0.19 mM NSNM-DNA and 0.58 mM NSNM-DNA

Serum sample	Maximum percent inhibition at 20 µg mL^{-1}		
	nDNA	0.19 mM NSNM-DNA	0.58 mM NSNM-DNA
8	21.5	40.5	40.9
9	33.5	39.4	43.8
17	24.3	41.7	41.7
18	22.1	46.8	47.6
19	24.5	47.3	46.4
20	36.0	43.8	50.2
Mean ± SD	26.98 ± 6.18	43.25 ± 3.28	45.1 ± 3.60

Microtitre plates were coated with 0.58 mM NSNM-DNA

REFERENCES

Ahmad, S., Uddin, M., Habib, S., Shahab, U., Alam, K. and Ali, A. 2014. Autoimmune response to AGE modified human DNA: Implications in type 1 Diabetes mellitus. *Journal of Clinical and Translational Endocrinology*, 1: 66-72.

Alam, K. and Jabeen, S. 2007. Immunogenicity of mitochondrial DNA modified by hydroxyl radical. *Cellular Immunology*, **247**: 12-17.

Alam, K., Ali, A. and Ali, R. 1993. The effect of hydroxyl radical on the antigenicity of native DNA. *FEBS Letters*, **319**: 66-70.

Baumann, R.P., Seow, H.A., Shyam, K., Penketh, P.G. and Sartorelli, A.C. 2005. The antineoplastic efficacy of the prodrug Cloretazine TM is produced by the synergistic interaction of carbamoylating and alkylating products of its activation. *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics*, **15**: 313-325.

Benigni, R. and Bossa, C. 2011. Mechanisms of chemical carcinogenicity and mutagenicity: a review with implications for predictive toxicology. *Chemical Reviews*, **111**: 2507-2536.

Beyerbach, A., Farmer, P.B. and Sabbioni, G. 2006. Biomarkers for isocyanate exposure: Synthesis of isocyanate DNA adducts. *Chemical Research in Toxicology*, **19**: 1611-1618.

Caspary, W.J. and Myhr, B. 1986. Mutagenicity of methylisocyanate and its reaction products to cultured mammalian cells. *Mutation Research Letters*, **174**: 285-293.

Chen, C.N., Porubleva, L., Shearer, G., Svrakic, M., Holden, L.G., Dover, J.L., Johnston, M., Chitnis, P.R. and Kohl, D.H. 2003. Associating protein activities with their genes: rapid identification of a gene encoding a methylglyoxal reductase in the yeast Saccharomyces cerevisiae. *Yeast*, **20**: 545-554.

Delanghe, S., Delanghe, J.R., Speeckaert, R., Van Biesen, W. and Speeckaert, M.M. 2017. Mechanisms and consequences of carbamoylation. *Nature Reviews Nephrology*, **13**: 580-593.

Deo, M.G., Gangal, S., Bhisey, A., Somasundaram, R., Balsara, B., Gulwani, B., Darbari, B.S., Bhide, S. and Maru, G.B. 1987. Immunological, mutagenic and genotoxic investigations in gas exposed population of Bhopal. *Indian Journal of Medical Research*, **86**: 63-76.

Dovbeshko, G.I., Gridina, N.Y., Kruglova, E.B. and Pashchuk, O.P. 2000. FTIR spectroscopy studies of nucleic acid damage. *Talanta*, **53**: 233-246.

East, C.J., Abboud, C.N. and Borch, R.F. 1992. Diethyldithiocarbamate induction of cytokine release in human long-term bone marrow cultures. *Blood*, **80**: 1172-117,

Heath, R. 2017. Isocyanate-based polymers: polyurethanes, polyureas, polyisocyanurates, and their copolymers. pp. 799-835. **In**: *Brydson's Plastics Materials 8th edn*. (ed. M. Gilbert). Elsevier, Oxford, UK.

Hu, R., Kim, B.R., Chen, C., Hebbar, V. and Kong, A.N.T.2003. The roles of JNK and apoptotic signalling pathways in PEITC-mediated responses in human HT-29 colon adenocarcinoma cells. *Carcinogenesis*, **24**: 1361-1367.

Liljenberg, M., Ripa, L. and Shamovsky, I. 2020. Theoretical Studies of the Mechanism of Carbamoylation of Nucleobases by Isocyanates. *Chemical Research in Toxicology*, **33**: 2845-2853.

Marczynski, G.T. and Shapiro, L. 1992. Cell-cycle control of a cloned chromosomal origin of replication from *Caulobacter crescentus*. *Journal of Molecular Biology*, **226**: 959-977.

Mishra, P.K., Panwar, H., Bhargava, A., Gorantla, V.R., Jain, S.K., Banerjee, S. and Maudar, K.K., 2008. Isocyanates induces DNA damage, apoptosis, oxidative stress, and inflammation in cultured human lymphocytes. *Journal of Biochemical and Molecular Toxicology*, **22**: 429-440.

Pijanka, J.K., Kohler, A., Yang, Y., Dumas, P., Chio-Srichan, S., Manfait, M., Sockalingum, G.D. and Sulé-Suso, J. 2009. Spectroscopic signatures of single, isolated cancer cell nuclei using synchrotron infrared microscopy. *Analyst*, **134**: 1176-1181.

Polak, W., Lekki, J., Veselov, O., Stachura, Z. and Styczen, J. 2006. Single proton hit facility at the IFJ PAN in Cracow. *Acta Physica Polonica - Series A General Physics*, **109**: 417-420.

Saxena, A.K., Singh, K.P., Nagle, S.L., Gupta, B.N., Ray, P., Srivastav, R.K., Tewari, S.P. and Singh, R. 1988. Effect of exposure to toxic gas on the population of Bhopal: Part IV--Immunological and chromosomal studies. *Indian Journal of Experimental Biology*, **26**: 173-176.

Segal, A., Solomon, J.J. and Li, F. 1989. Isolation of methylcarbamoyl-adducts of adenine and cytosine following in vitro reaction of methyl isocyanate with calf thymus DNA. *Chemico-Biological Interactions*, **69**: 359-372.

Shelby, M.D., Allen, J.W., Caspary, W.J., Haworth, S., Ivett, J., Kligerman, A., Luke, C.A., Mason, J.M., Myhr, B., Tice, R.R. and Valencia, R. 1987. Results of in vitro and in vivo genetic toxicity tests on methyl isocyanate. *Environmental Health Perspectives*, **72**: 183-187.

Smith, G.F. 2011. Designing drugs to avoid toxicity. *Progress in Medicinal Chemistry*, **50**: *1-47*.

Talha, M., Mir, A.R., Habib, S., Abidi, M., Warsi, M.S. and Islam, S. 2021. Hydroxyl radical induced structural perturbations make insulin highly immunogenic and generate an auto-immune response in type 2 diabetes mellitus. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, **255**: 1-14.

Tamura, N., Aoki, K. and Lee, M.S. 1990. Characterization and genotoxicity of DNA adducts caused by 2-naphthyl isocyanate. *Carcinogenesis*, **11**: 2009-2014.

Tamura, N., Aoki, K. and Lee, M.S. 1992. Selective reactivities of isocyanates towards DNA bases and genotoxicity of methyl carbamoylation of DNA. *Mutation Research Letters*, **283**: 97-106.

Tang, L. and Zhang, Y. 2005. Mitochondria are the primary target in isothiocyanate-induced apoptosis in human bladder cancer cells. *Molecular Cancer Therapeutics*, **4**: 1250-1259.

Tantry, I.Q., Waris, S., Habib, S., Khan, R.H., Mahmood, R. and Ali, A., 2018. Hypochlorous acid induced structural and conformational modifications in human DNA: a multi-spectroscopic study. *International Journal of Biological Macromolecules*, ***106***: 551-558.

Ugenskiene, R., Lekki, J., Polak, W., Prise, K.M., Folkard, M., Veselov, O., Stachura, Z., Kwiatek, W.M., Zazula, M. and Stachura, J.2007. Double strand break formation as a response to X-ray and targeted proton-irradiation. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms*, **260**: 159-163.

Vandenabeele-Trambouze, O., Mion, L., Garrelly, L. and Commeyras, A. 2001. Reactivity of organic isocyanates with nucleophilic compounds: amines; alcohols; thiols; oximes; and phenols in dilute organic solutions. *Advances in Environmental Research*, **6**: 45-55.

Wang, Z. 2009. Comprehensive Organic Name Reactions and Reagents, Hoboken, NJ, John Wiley.

Waris, G. and Alam, K. 2004. Immunogenicity of superoxide radical modified-DNA: studies on induced antibodies and SLE anti-DNA autoantibodies. *Life Sciences*, **75**: 2633-2642.

Worthy, W.1985. Methyl isocyanate-The chemistry of a hazard. *Chemical and Engineering News*, **63**: 27-33.

Zhang, Y., Tang, L. and Gonzalez, V. 2003. Selected isothiocyanates rapidly induce growth inhibition of cancer cells. *Molecular Cancer Therapeutics*, **2**: 1045-1052.