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Research Article

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Risk Associated with NAD (P) H: Quinone Oxidoreductase I (NQOI) C609T Polymorphism for Cigarette Smoke Induced Cardiovascular Disease (CVD): A Study on Male Current Smokers from Eastern India

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Abstract: NAD(P)H: quinone oxidoreductase I (NQO1) is a well-known enzyme for its protective role against cigarette smoke (CS) derived harmful quinones such as p-benzoquinone which is a major causative agent of CS-related cardiovascular disease (CVD). NQO1 C609T single nucleotide polymorphism has severe consequence on NQO1 function and its availability. We propose that the presence of the variant allele of this polymorphism exposes smokers towards a greater risk of developing CVD. For the verification of this hypothesis we perform a genotype analysis for this SNP in two groups of male current smokers (smokers with CVD, n=100 and smokers without CVD, n=200) by a PCR-RFLP method. Genotype distributions of both groups were in Hardy Weinberg equilibrium. The wild type (p) and the variant allele (q) frequencies were different for these two groups (in smokers without CVD: p=67.3%, q=32.7%; in smokers with CVD: p=59%, q=41%). The genotype frequencies were significantly different between the two smoker groups (in smoker without CVD: CC=43.5%, CT=47.5% and TT=9%; in smokers with CVD: CC=32%, CT=54% and TT=14%). Cochran-Armitage-linear Trend Test indicated that the 'proportions of smokers with CVD' have a linear trend with the number of variant alleles of NQO1 C609T SNP (CA Test $\chi 2$= 4.329, $p<0.05$). The variation of genotype distribution between the two smoker groups was analysed by different genetic test model. Significant variation was observed in additive model ($p<0.05$). The odds ratios (OR) for the heterozygous (CT) and the homozygous (TT) were greater than one (for CT: OR=1.55, 95%CI: 0.91-2.61; for TT: OR=2.11, 95%CI: 0.94-4.74).Thus, the study strongly indicated that the variant allele of the NQO1 C609T SNP is associated with greater risk for developing CS-related CVD in cigarette smokers.

Keywords: NAD(P)H: quinone oxidoreductase I (NQO1), Single Nucleotide Polymorphism (SNP), Cigarette Smoke (CS), Cardiovascular Disease (CVD), Polymerase Chain Reaction (PCR), Restriction Fragment Length Polymorphism (RFLP).

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1. INTRODUCTION

NAD(P)H: quinone oxidoreductase 1 (NQO1) is one of the key components of cellular defence mechanism against quinone mediated oxidative damage and cellular toxicity. It catalyses the two-electron reduction of benzoquinones to hydroquinones which are lesser harmful for human body.[1,2] Apart from this, NQO1 has several other additional protective activities, such as p53 stabilization, balancing of cytosolic antioxidants and scavenging of superoxide.[1] The deleterious effects of several cigarette smoke (CS) derived harmful quinones, such as p-benzoquinone and benzo (a)pyrene 3,6-quinone, are opposed by cellular NQO1 activity.[3,4] Para-benzoquinone (p-BQ) from cigarette smoke is a major causative factor for CS-induced pathological cardiovascular events and myocardial injury.[5] The expression of NQO1 protein in the epithelial cells of the cardiovascular system strongly indicates the protective role of NQO1 against p-BQ induced cardiovascular damage.[5,6] NQO1 gene is located on chromosome 16 of human genome.[7,8] NQO1 C609T polymorphism (found at 609 position of NQO1 cDNA) is a single nucleotide polymorphism (SNP) of this gene with major structural and functional influence on NQO1 protein.[9,10] It also dictates the stability of NQO1 protein. Due to the phenotypic gene-dose effect of this SNP, individuals with homozygous variant type of NQO1 gene are deprived of full scale NQO1 activity.[11,12] Cigarette smoking is one of the primary risk factors for developing cardiovascular diseases (CVD). CS-induced acute thrombosis and atherosclerosis eventually lead to deadly pathological cardiovascular events.[13] CVD is the leading cause of global mortality.[14] Cigarette smoking contribute at least 10% of CVD-related mortality.[15] Cardiovascular tissues and cells express a high level of NQO1 and activation of this protein reduces atherogenesis in the animal model.[16] In an epidemiological study, it was found that patients with *NQO1* C609T polymorphism acquire more atherosclerotic plaques than those not having this polymorphism in their carotid artery.[17] Another study revealed that the patients with *NQO1* C609T polymorphism develop higher inflammatory response measured by serum interleukin-6 levels.[18] This finding was supported by the fact that the CHD patients with low level of NQO1 activity express a high level of C-reactive protein which is a general biomarker of inflammation leading to cardiovascular damage.[19] All of these facts strongly suggest the protective role of NQO1 in pathological cardiovascular events and against the deleterious effect of CS-derived harmful components (such as p-BQ) responsible for cardiovascular damage in smokers. Thus, we hypothesized that cigarette smokers with NQO1 C609T polymorphism would be at greater risk for developing CVD. In this present study we performed a genotype distribution analysis of NQO1 C609T polymorphism between two groups of male current cigarette smokers (with and without CVD) to test the validity of our hypothesis.

2. MATERIALS AND METHODS

2.1 Study Design

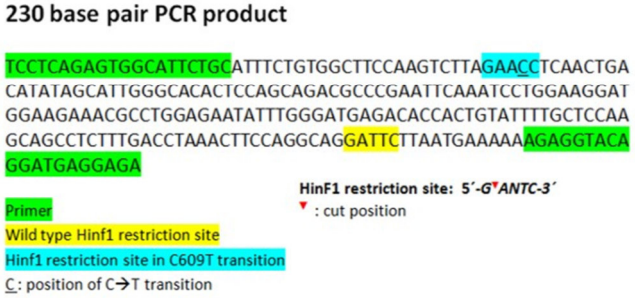
For the evaluation of the risk of developing CS-induced CVD associated with NQO1 C609T SNP, comparative analysis of the genotype distribution between smokers with and without CVD was performed. 200 smokers without CVD (also without CS-related other diseases such as lung cancer and COPD) and 100 smokers with CVD (without other CS-related diseases) were included in the study. All smokers of both groups were male having age between 45 to75 years. From Mallikpur Block Hospital (West Bengal, India), the patients with CVD were recruited in the study. Individuals suffering from any kind of infection, malnutrition and uncontrolled diabetes were excluded. Information about the age and attributes related to cigarette smoking (the average number of cigarettes smoked per day and years of smoking) were collected from the participants. Samples were collected over five years (2012-2017). This study was ethically approved by 'Institutional Bioethics Committee for Human Research Studies' of University of Calcutta. From all participating individuals of this study, informed consents were obtained prior to collection of blood sample.

2.2 Genomic DNA Isolation

One millilitre of venous blood was taken in an EDTA vial for the purpose of genomic DNA isolation. QIAGEN's Blood Mini DNA Isolation Kit was used to isolate human genomic DNA following the manufacturer's protocol. Human genomic DNA was stored in -20°C until further use.

2.3 NQO1 Genotyping by PCR-RFLP

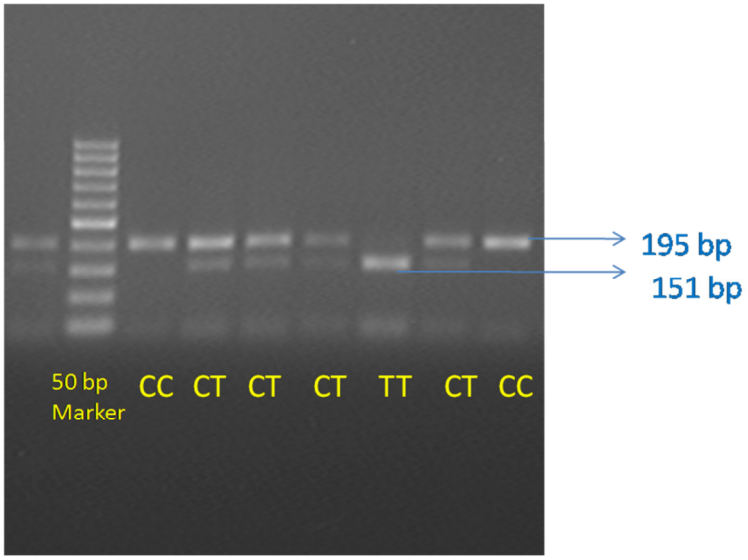
A PCR product of 230 bp size was produced for each sample of genomic DNA using Fast Cycling PCR kit (QIAGEN) and these following primers: - 5'-TCCTCAGAGTGGCATTCTGC-3' (sense primer) and 5'-TCTCCTCATCCTGTACCTCT-3' (antisense primer). This 230 bp long PCR product consists of part of exon 5 and exon 6 of NQO1 gene and includes NOQ1 C609T polymorphism (Figure 1). There is a single Hinf1 restriction site (5'-GANTC-3') in the PCR product originated from wild type NQO1 allele, whereas the PCR product originated from NQO1 allele having C609T polymorphism has two Hinf1 restriction site (generated from a C→T conversion).



The regions highlighted in green represent the positions of primers, the region highlighted in yellow represents the wild type Hinf1 restriction site, and the region highlighted in blue represents the position of the Hinf1 restriction site generated in NQO1 C609T SNP by C to T transition. The red downward arrow within the restriction sequence represents the restriction cut position.

Fig 1.The DNA sequence of 230 bp long PCR product of NQO1 gene segment of interest

For PCR (in Thermal Cycler, BIORAD), 30ng of genomic DNA was used in a total volume of 20 µl PCR reaction. The mixture was consisted of 10 µl of PCR master mix (Qiagen) and 0.5 µl of each primer (initial concentration:20 µM). PCR reaction steps were programmed following the manufacturer's protocol. Briefly, after the initial activation of DNA polymerase (HOTstarTaq Plus) at 95°C for 5 minutes, the 28 cycles of amplification were done. Each cycle consisted of the following 3 steps: i) denaturation (5s at 96°C), ii) annealing (5s at 58°C) and iii) extension (7s at 68°C). A final extension step of 1minute at 72°C was used. PCR purification kit (QIAGEN) was used for the purification of the amplified DNA product following the manufacturer's protocol. The purified product was processed for restriction digestion without delay. Restriction enzyme Hinf1 (from Sib enzyme) of volume 5 µl was used for restriction digestion of 40 µl of the purified PCR product at 37° C for 30 min following the manufacturer's protocol. Two fragments of oligonucleotide (195bp and 35bp) are generated from the restriction digestion of wild type PCR product, whereas three fragments of oligonucleotides (151bp, 44bp, and 35 bp) are generated from the restriction digestion of the PCR product having the C609T polymorphism. Digested product of purified PCR product was loaded immediately on 2% agarose gel (containing 0.5µg/ml of Ethidium bromide) in TAE buffer. The gel electrophoresis was performed at 100 V constant voltages until the dye had migrated to the bottom and the bands were distinctly separated. The DNA gel bands of 195bp and 151 bp oligonucleotides are clearly visible under UV light. Presence of these bands indicates the genotype status of the individual for NQO1 C609T polymorphism (case 1: presence of only 195 bp gel band = homozygous wild type; case 2: presence of both 195 bp and 151bp gel bands= heterozygous; case 3: presence of only 151 bp gel band= homozygous variant type). This unique feature of restriction digested PCR products in agarose gel is the key for the NQO1 C609T genotyping by PCR-RFLP method (Figure 2).



The 2nd lane contained 50bp molecular weight marker; lane 3 and 9 contained homozygous wild type (CC); lane 1,4,5,6 and 8 contained heterozygous type (CT); lane 7 contained homozygous variant type (TT).

Fig 2. Representative image of Agarose gel showing three different genotypes for NQO1 C609T polymorphism (analysed by PCR-RFLP).

2.4 Statistical Analysis for *NQO1* Genotyping

Hardy–Weinberg equilibrium test was performed for both groups of smokers. Genotype distribution of NQO1 C609T polymorphism was compared between 'smokers without CVD' and 'smokers with CVD' groups using the Pearson's Chi-square test and Cochran Armitage (CA) Trend test. Additive, dominant and recessive genetic models of genotype distribution were analysed. Evaluation of the risk for developing CS-related CVD associated with NQO1 C609T SNP was performed form Odds Ratio, Relative Risk and their 95% confidence interval. Linear trend of genotype distribution in different genetic model (additive, dominant and recessive model) were assessed by Cochran Armitage (CA) Trend test. The XLSTAT and MedCalc software were used for the statistical analysis. Mann-Whitney U test (SPSS software) was used for the general comparison of attributes (age, pack-year) between the groups.

3. RESULT

3.1 Comparison of Age and Pack-years of Smoking

NQO1 C609T genotyping was performed in the following two groups of male current smokers: - 200 smokers without CVD and 100 smokers with CVD. Individuals were included in the study following the general inclusion criteria discussed in the 'materials & method' section, all having age between 45 to 75 years. The Shapiro–Wilk test for normality revealed that the distribution of the attributes (age and pack-years of smoking) of the individuals included in NQO1 C609T study, significantly ($p<0.05$) varied from that of the normal curve. Thus nonparametric Mann Whitney U test was performed for the comparison of the general attributes .The mean (±SD) ages of the two groups were 58.7±7.9 (smokers without CVD) and 62.5±7.8 (smokers with CVD). The mean rank of age for smokers with CVD (319.00) was greater than that of the smokers without CVD individuals (247.09) (Table 1). Mann Whitney U test revealed that there was significant difference ($p<0.05$) of age between these two groups. The

mean (±SD) pack-years of smoking of the two groups were 38.4±17.7 (smokers without CVD) and 35.5±15.2 (smokers with CVD). The mean rank of pack-years of smoking for smokers with CVD (244.60) was smaller than that of the smokers without CVD individuals (269.32) (Table 1). However, Mann Whitney U test revealed that there was no significant difference (p>0.05) of pack-years of smoking between these two groups.

3.2 *NQO1* C609T Genotype Frequency

The wild-type and C609T allele frequencies (p & q respectively) of NQO1 were 67.3% and 32.7% respectively in smokers without CS-related CVD. The distribution of *NQO1* C609T genotypes of these 200 smokers was in Hardy Weinberg equilibrium (χ2 value=1.23, p>0.05). The individual numbers in the three genotypes of this group were as follows: - 87(CC), 95 (CT) and 18 (TT) (Table 1). Frequency of these three genotypes were as follows: - 43.5% (CC), 47.5% (CT) and 9% (TT) (Table 1). In smokers with CS-related CVD, the allele frequencies were p=59% and q=41%. The genotype distribution of this group was also in Hardy Weinberg equilibrium (χ2 value=1.35, p>0.05). The numbers of the three genotypes in the disease group were as follows: - 32 (CC), 54 (CT) and 14 (TT) (Table 1). Frequency of three genotypes were as follows: - 32% (CC), 54% (CT) and 14% (TT) (Table 1).

Table 1: NQO1 C609T genotype distribution among smokers with or without CVD.

Type	N	*NQO1* C609T SNP genotype			Allele frequency		HWE Test (χ 2 value, p)	Age (years) Mean ±SD [mean rank]	Pack-years of smoking Mean ±SD [mean rank]
		CC [n, (%)]	CT [n,(%)]	TT [n,(%)]	p	q			
Smokers without CVD	200	87 (43.5)	95(47.5)	18(9)	67.3	32.7	χ2=1.23, *p*>0.05	58.7±7.9 [247.09]	38.4±17.7 [269.32]
Smokers with CVD	100	32(32)	54(54)	14(14)	59	41	χ2=1.35, *p*>0.05	62.5±7.8 [319.00]	35.5±15.2 [244.60]

N, number of subjects in each group. CC, homozygous wild type; CT, heterozygous; TT, homozygous variant type. n , numbers of individuals in each genotype. (%), genotype frequency in each smoker group. The p= allele frequency of wild-type C609C, the q= allele frequency of the variant C609T allele. HWE TEST, Hardy-Weinberg equilibrium test. p, probability. SD, standard deviation; [mean rank], mean rank from Mann Whitney U test.

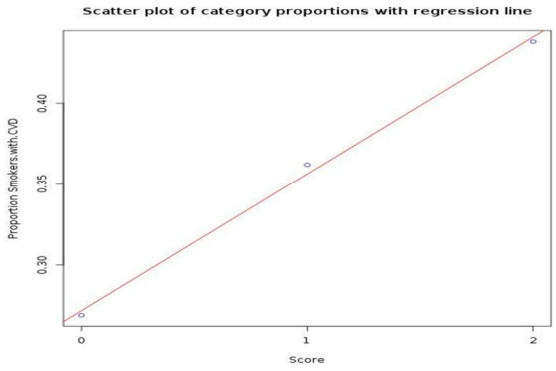
3.3 Cochran-Armitage-linear Trend Test

The linear trend of the developing CS-related CVD in male current smokers was evaluated by Cochran-Armitage-linear trend-test (CA trend test). For this analysis genotypes were assigned score according to the number of polymorphic alleles in that particular genotype. As for example, CC genotype was assigned score zero due to the complete absence of the polymorphic allele. Similarly, CT and TT genotypes were assigned score 1 and 2 respectively (Table 2). The analysis clearly indicated that the outcome (i.e. the CS-related CVD of smokers) followed a linear trend with the presence of variant (T) allele (CA test linearity χ2= 4.329, p<0.05). Figure 3 shows the scatter plot of category proportions of three genotypes with regression line generated from CA linear trend test.

Table 2: Data Summary of Chi-square test for linear trend in CS-related CVD associated with variant allele of NQO1 C609T SNP.

Genotype	Score	Smokers with CVD	Smokers without CVD	Total	Proportion of Smokers with CVD
CC	0	32	87	119	0.269
CT	1	54	95	149	0.362
TT	2	14	18	32	0.438
Total		100	200	300	0.333

CC, homozygous wild type; CT, heterozygous; TT, homozygous variant genotypes. Scores: 0, no of T allele=0; 1, no of T allele=1; 2, no of T allele=2



The Y-axis represents the proportions of 'smokers with CVD' against the corresponding score (the number of variant T allele present in a genotype, i.e. CC=0, CT=1 and TT=2). Associated CA test linearity χ2= 4.329, degree of freedom(df)=1 and significance (p)=0.037.

Fig 3. *Scatter plot of category proportions with regression line from the Cochran-Armitage-linear Trend Test analysis.*

3.4 Verification of Relevant Genetic Test Models

For further evaluation of the association between the genotypes (CC,CT and TT) and the outcome (presence or absence of CS-related CVD), the variation of genotype distribution between two groups was analysed following the 1) dominant [(TT+CT) vs CC], 2) recessive [TT vs (CT+CC)] and additive [TT vs CT vs CC] genotypic test models by Cochran-Armitage test (Table 3). Significant variation was observed in additive model (p=0.037, z-observed value >2.0). In the dominant model analysis, the variation of genotype distribution between two groups indicated a clear tendency to significance (Pearson $\chi 2$= 3.672, df=1, p (two-tailed) =0.055, z-observed value:1.919).

Table 3 : Evaluation (by Cochran – Armitage test) of the three genetic test model based on the variation of genotype distribution in two groups

	Statistical parameters	Additive Model	Dominant model	Recessive model
Smoker with CVD	z-observed value	2.081	1.919	1.323
	z-critical value	1.960	1.960	1.960
	Significance level (*p*-two tailed)	0.037*	0.055#	0.186

p, probability; *, statistically significant, #, tends to significance

3.5 Risk Evaluation

For each of the genotype with the variant T allele (i.e. CT and TT), the risk of CS-related CVD was assessed. The odds ratio (OR) for the CT and TT genotypes were 1.545 (95%CI:0.914-2.613) and 2.115 (95%CI:0.943-4.741), respectively (Table 4). The risk of CS-related CVD associated with NQO1 C609T SNP was also evaluated in dominant genetic model. The odds ratio and relative risk (RR) were as follows: OR=1.636 [95%CI:0.988-2.711, z-statistics:1.912, p=0.06] and RR=1.397 [95%CI:0.984-1.984, z-statistics: 1.869, p=0.06].

Table 4: Risk of CS-related CVD associated with NQO1 C609T variant genotypes in male current smokers.

Statistical parameters		Variant genotypes for NQO1 C609T SNP	
		Heterozygous (CT)	Homozygous variant (TT)
Odds Ratio	OR	1.5454	2.1146
	95% CI	0.9140-2.6128	0.9431-4.7414
	z-statistics	1.625	1.818
	p	0.1043	0.0691
Relative Risk	RR	1.3477	1.6270
	95% CI	0.9357-1.9412	0.9947-2.6612
	z-statistics	1.603	1.939
	p	0.1089	0.0525

OR, odds ratio; RR, relative risk; p, probability; CI, confidence interval.

4. DISCUSSION

Different types of cardiovascular disease such as chronic heart disease (CHD), peripheral arterial disease (PAD) and cerebrovascular disease has cigarette smoking as common risk factor. CS-induced inflammation, apoptosis, prothrombotic effect and endothelial dysfunction all contribute in the development of atherosclerosis and acute thrombosis. These two events ultimately lead to CVD in smokers. Cigarette smoke, which is a complex mixture of more than 4000 compounds, have several pathogenic agents for oxidative damage of vascular system mostly by inducing inflammatory effects. Para-benzoquinone (p-BQ) derived from CS, is one of such prominent compounds with significant contribution towards CS -related CVD.[20] The wild type NQO1 protein completely detoxify p-BQ. Whereas absence of fully functional NQO1 allows para-benzoquinone to initiate its deleterious effects on cardiovascular system. In the present study, we evaluated the risk associated with the variant NQO1 allele by comparing the NQO1 genotype distribution between male cigarette smokers of eastern India with or without CVD. In the present study, the genotype distributions of both groups were in Hardy Weinberg equilibrium (p>0.05, i.e. any deviation from Hardy Weinberg equilibrium was insignificant). The NQO1 genotype distribution of general population vary greatly among ethnicities.[21] Again, it also varies among different regions of India.[22-25] Our study revealed the genotypic distribution of NQO1 C609T polymorphism in a general population (i.e. in smokers without CS-related diseases, n=200) from eastern India. The genotype frequencies of homozygous wild type (CC), heterozygous (CT) and homozygous variant (TT) with respect to NQO1 C609T SNP were as follows: 43.5% (CC), 47.5% (CT) and 9% (TT). In smokers with CVD, the allele frequency of the variant allele is much higher than that of the smokers without CVD (41% vs 32.7%). To evaluate the risk of variant NQO1 C609T allele (T), we performed Cochran-Armitage-linear trend-test. The three genotypes were assigned score (CC=0, CT=1 and TT=2) with respect to the number of the variant allele in that particular genotype. As the 'outcome effect', the 'proportions of smokers with CVD' were assayed for linearity trend against the three scores. The analysis indicated that the 'proportions of smokers with CVD' for the three genotypes followed a clear trend of linearity (p<0.05, i.e. deviation from the non-linearity was significant). Proportion of smokers with CVD increases in the following order: CC<CT<TT (CC:0.269; CT:0.362; TT:0.438). Clearly this result suggests towards the additive genetic model of genotype (for NQO1 C609T SNP) and phenotype (i.e. development of CS-related CVD) relation. The variation of genotype distribution was analysed in 1) additive, 2) dominant and 3) recessive model. The variation of distribution did not follow the recessive model (p>0.05). The variation shows a marginal trend towards significance when assessed in dominant genetic model (p=0.055). As expected from the test of linearity, the variation of genotype

distribution clearly followed the additive model ($p<0.05$). As per our knowledge, our study is the first of its kind where, NQO1 C609T polymorphism was assessed as a risk factor for CS related CVD in India. We observed that both of the odds ratios for TT and CT genotypes were greater than one [for TT: 2.12 (95%CI:0.94-4.74) and for CT:1.55 (95%CI:0.91-2.61)].This clearly indicated the risk of CS-related CVD in the male current smokers of eastern India in presence of NQO1 C609T variant allele. Further, the odds ratio of developing CS- related CVD was 1.636 [95%CI:0.988-2.711] in dominant genetic model. We suggest that similar studies from different parts of India, with larger sample size are needed for further evaluation of the true strength of NQO1 C609T SNP as the potential risk factor for CS-related CVD.

5. CONCLUSION

In this study we tested our hypothesis that the C609T single nucleotide polymorphism of NQO1 protein is a risk factor for CS-related CVD in smokers. We observed that the variant allele of NQO1 is associated with greater risk of CVD in smokers following a linear trend. This particular type of study might actually identify more genetic predisposition factors in smokers for CS-related diseases and also would help to understand the molecular pathological events induced by cigarette smoke for disease development.

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7. AUTHOR CONTRIBUTION STATEMENT

Dr.Santanu Banerjee designed and performed the experiments, wrote the manuscript and reviewed it.

8. CONFLICT OF INTEREST

Conflict of interest to declare none.

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