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Study of Methylene Tetrahydrofolate Reductase (MTHFR) Gene Polymorphisms in Personnel Prolonged Exposure to Trace Quantities of Anaesthetic Gases in Operation Theatres

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Abstract: *Nitrous oxide first synthesized by Joseph Priestly in 1722. Its pschychotropic effects were first appreciated by Humbphrey Davy. Its association with Haematologic disease was reported by Larsen et tal in 1956.Methylene Tetrahydrofolate Reductase is one of the main regulatory enzyme in metabolism of Homocysteine. Mutation in MTHR gene led to reduced activity of enzyme and hyperhomo cysteinemia. C677T and A 1298C mutations are two most common mutations. Here we evaluated prevalence of MTHR C677T and A 1298C mutation in Operation Theatre exposed personnel and compared with Non OT exposed healthy controls. A total of 87 personnel exposed to anaestetic gases for more than 3 years were registered to study various epi8dimiological parameters and to screen for C667 and C1298 polymorphism of MTHR gene. Also 150 controls who are not exposed to OT anaesthetic gases are selected at random to compare with data generated on subjects exposed to OT anesthetic gases. 5 ml of blood was collected in EDTA vaccutainers from all subjects. Isolation of DNA was done from whole blood using rapid non-=enzymatic method. Restriction diagestion analysis was done by PCR amplication Restirction digestion and electrophoresis. Based on number of bands and fragment sises visualized samples were interpreted and genotyped as homozygous normal, homozygous mutant and as hetero zygotes if all three bands were present. SNP status (http://bioinfo.inconcologia.net/index.php?module=snapshots) was usedto determine odds ratio (Ors), 95% confidence intervals (Cls) and P values. Under models (codominant, dominant, recessive, overdominant, and longadditive) were employed to anayze the data. On the whole our data has indicated statistically significant difference in prevalence of A1298C mutation in sex wise distribution in OT exposed personnel.*

Key words: *methlene tetrahydrofolate reductaise : anaesthetic gases: C677 : A128C mutation.*

I. Introduction

Nitrous Oxide, first Synthesized by Joseph Priestly in 1722. Its Psychotropic effects were first appreciate by Humphrey Davy from the time that nitrous oxide was first used for analgesia by Horace wells in 1844 until 1956, nitrous oxide was considered to be completely benign. The first clear association of nitrous oxide and hematologic disease came in a report by Lassen, et al in the Lancet in 1956. They studied it prospectively and found than Granulocytopenia developed on the fourth day (with 50% N2O), The N2O was discontinued and thrombocytopenia followed within several days. A bonemarrow biopsy was consistent with pernicious anaemia with megaloblasic changes.In 1978, Sahenk reported a case of polyneuropathy from recreational nitrous oxide use and Layzer reported on dentists who developed polyneuropathy and it was linked to the deficiency of vitamin B12.

Nitrous oxide irreversibly Oxidizes the cobalt atom of vitamin B12, inactivating it which is a co-factor for methionine synthase. Methionine is an essential aminoacid that serves as a methyl donor (via its activated from s-adenosyl methionine) in hundreds of biological reactions. The end product of methylation is catalyzed by the vit B12 dependent enzyme methionine synthase.

Methylenetetrahydrofolate reductase (MTHFR) is one of the main regulatory enzymes in the metabolism of homocysteine that catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate [7]. Mutations in MTHFR gene lead to decreased activity of enzyme and hyperhomocysteinemia, which induces platelet aggregation through promotion of endothelial oxidative damage [8]. Although several mutations within the MTHFR gene were described, C677T and A1298C mutations are the two most common mutations .C677T transition is a missense mutation in the exon 4 of this gene, which converts an alanine to a valine codon (at codon 222) in the N-terminal catalytic domain of the protein leading to a thermolabile protein, with decreased enzymatic activity .The second mutation is MTHFR A1298C, that is, associated with decreased activity of enzyme, but not with thermolability. A1298C transversion is a point mutation in exon 7, characterized by a glutamate to alanine substitution (at codon 429) within the C-terminal regulatory domain of the protein .Numerous investigations have been performed on the incidence of MTHFR C677T and A1298C mutations in in OT EXPOSED PERSONNEL. Some of these studies have demonstrated a relationship between these mutations whereas others have been unable to confirm these results .Thus, the role of MTHFR C677T and A1298C mutations in RSA is still controversial. Here, we evaluated the prevalence of

MTHFR C677T and A1298C mutations in OT EXPOSED PERSONNEL in Gandhi hospital secunderabad&osmania general hospital Hyderabad,and compared with non OT exposed healthy controls. By using PCR-restriction fragment length polymorphism (PCR-RFLP).

II. Materials And Methods

Subjects

A total of 87 personnel exposed to anaesthetic gases in operation theatres of more than 3 years who gave consent to participate in the study were registered to study various epidemiological parameters and to screen for c677&c1298 polymorphism of MTHFR gene. These subjects are selected from Gandhi Hospital,Secundeabad & Osmania General Hospital, Hyderabad. Subjects with history of systemic disease are excluded from the study.

Also 150 controls who are not exposed to anaesthetic gases are selected at random to compare with the data generated on the subjects exposed to OT anaesthetic gases.

Collection of data: From all the cases(AN) &controls(CT)detailed information pertaining to various epidemiological parameters such as sex,age,history of exposure,other comorbidities was collected using a special proforma.

Collection Of Blood Samples: 5ml of blood was collected in EDTA vaccutainers from all the subjects who gave their consent to participate in study after explaining to them the purose of the study

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Isolation Of Dna:

Sequence of primers, PCR product size, restriction enzymes, and size of digested fragments that are used for screening by PCR-RFLP

Mutation	Sequence of primers	PCR product(bp)	Restrictionenzyme	Wild type	Heterozygote	Mutant
MTHFR C677T	F-TGAAGGAGAAGGTGTCTGCGGGA R-AGGACGGTGCGGTGAGAGTG	198	Hinf1	198	198/175/23	175/23
MTHFR A1298C	F-CTTTGGGGAGCTGAAGGACTACTAC R-CACTTTGTGACCATTCCGGTTTG	163	MboII	56/31/30/28/18	84/56/31/30/28/18	84/31/30/18

DNA was isolated from the whole blood using rapid non-enzymatic method (Lahiri and Nurnberger, 1991) with minor modifications. Non-enzymatic method of DNA isolation involves separation of the DNA from blood by salting out the cellular proteins by dehydration and precipitation with saturated sodium chloride solution and finally extraction by absolute alcohol.

Equipment: Centrifuge, Water bath, Micro centrifuge, Incubator, Submarine Gel Electrophoresis Unit, DC power pack, UV transilluminator and Spectrophotometer.

Reagents: TKM 1 Buffer: Tris-HCL (pH 7.6) – 10 mM, KCL – 10mM, $Mgcl_2$. 10 mM, EDTA - 2 mM

TKM 2 Buffer: Tris-HCL (pH 7.6) - 10 mM, KCL-10 mM, $Mgcl_2$ - 10 mM, EDTA-2 mM, NaCl-0.4 Mm

Triton-X, 10% SDS, 6 M NaCl, Tris-EDTS (TE) Buffer: Tris HCL - 10 mM, EDTA : 1 mM

Procedure:

- 5 ml of whole blood collected in a vacutainer tubes containing 100µl of 15% EDTA were transferred to 15ml of centrifuge tubes. Equal volumes of TKM 1 buffer (low salt buffer) and 125µl of Triton-X (detergent) were added and mixed well by inverting the tubes several times to lyse the RBCs.
- The contents in the 15ml centrifuge tubes were then centrifuged at 10,000rpm for 10 minutes in a Remi T8 table top centrifuge which separated lysed RBCs as supernatant leaving the nuclear pellet as precipitate at the bottom.
- The supernatant was discarded and the pellet was washed in 5ml of TKM1 buffer and centrifuged as above in order to remove the left over RBCs.

- Again the supernatant was discarded and to the white pellet 0.8ml of TKM2 buffer was added and gently mixed till the pellet was dislodged and then 125µl of 10% SDS was added, mixed gently again. The tubes were then incubated at 55^{o} C in water bath for 10-20 minutes to lyse the WBC and release the DNA
- The contents were then transferred into sterile eppendorf tubes and 300µl of 6M NaCl was added, mixed well and micro-centrifuged at 10,000rpm for 5 minutes to precipitate the proteins.
- The supernatant containing DNA were transferred into fresh 15ml centrifuge tubes and 2 volumes of absolute ethanol was added carefully through the wall of the centrifuge tubes and the tubes were swirled slowly several times till the strands of DNA developed as coil.
- The precipitated high molecular DNA was then spooled out with sterile glass rod and washed with chilled 70% ethanol in an eppendorf tube and microfuged at 10,000rpm for 5 minutes.
- The supernatant was discarded and the pellet was dried in incubator at 37^{o} C till it became transparent and was then dissolved in 500µl of TE buffer and incubated at 55^{o} C for 30 minutes.
- The quantity of DNA was determined by running on 1% agarose and quantified using spectrophotometer.

Rflp

Restriction Fragment Length Polymorphism (RFLP) is a process where a variation in the DNA sequence can be detected by cutting the DNA molecule into smaller fragments with specific restriction enzymes. RFLP method is a vital tool in genome mapping and in the analysis of association of a variant with genetic disease. RFLP analysis was also the basis for early methods of Genetic Fingerprinting, which helps in the identification of samples retrieved from crime scenes, in the determination of paternity and in characterization of genetic diversity or breeding patterns in animal populations.

Equipment: Thermocycler, Waterbath, Horizontal gel electrophoresis unit, Vertical gel electrophoresis unit, DC power supply, Gel documentation system

Reagents:

For PCR: (PCR mix for 10 samples): 10X PCR buffer - 10µl, 200µM of each dNTP's-8µl (mix of all 4 dNTPs), Taq DNA polymerase-0.5µl (2.5 units), Primers (Forward and Reverse)- 25 picomoles each, Deionised triple distilled water-80µl

Steps Involved In Restriction Digestion Analysis

i) PCR amplification
ii) Restriction Digestion
iii) Electrophoresis – a) Agarose c.2756 A-G & c.677C-T
b) PAGE c.1298A-C

PCR Amplification of C677T:

Amplification was performed for the polymorphisms selected as given below with 50ng of genomic DNA mixed with 9µl of PCR master mix (prepared as mentioned above) using the PCR conditions:

Stage/ Phase	C677T
Initial denaturation	94^{o}C 5min's
Denaturation	94^{o}C 45sec
Annealing	55^{o}C 50sec
Extension	72^{o}C 50sec
Final extension	72^{o}C 7min's
No. of Cycles	30

Restriction Digestion

To 5µl of the PCR product 1 unit of Hinf 1 enzyme was added and incubated overnight at 37^{o} C.

Genotyping By Agarose Gel Electrophoresis:

Agarose gel electrophoresis is a widely used method in molecular biology to separate DNA, RNA and Protein molecules of different sizes. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis).

In the present study, agarose gel electrophoresis was used to check the a) quality of DNA b) amplification of PCR products and c) for genotyping few of the polymorphisms mentioned above.

Equipment:

Agarose (Horizontal) gel electrophoresis unit, DC power supply

Reagents: Agarose , 1 X TBE buffer, Loading dye, Ethidium Bromide

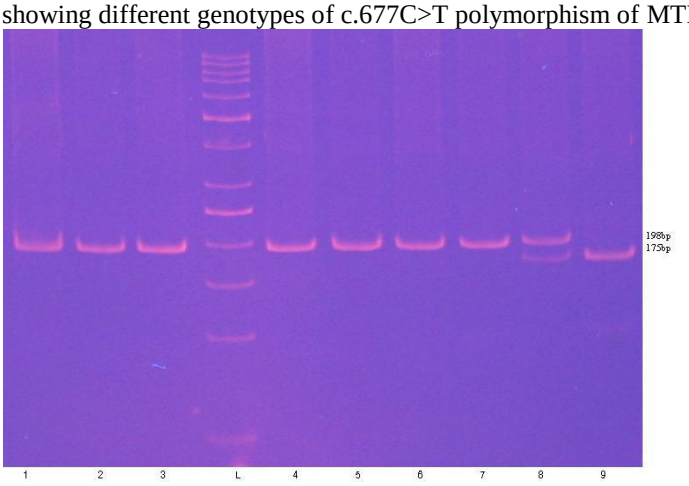
Procedure:

- 3% agarose gel was prepared by dissolving required amount of agarose in 1XTBE buffer.
- The solution was boiled for few minutes and agarose was dissolved by swirling the flask until it became transparent.
- The solution was then cooled to 50^{o} C and 1µl of ethidium bromide (10mg/ml) was added and poured into the gel casting mould (sealed with tape on either sides) avoiding any air bubbles. The comb was placed about 1cm from one end of the gel for the formation of the wells and the gel was allowed to set at room temperature for 15-20 minutes.
- After gelling, tape and comb were removed and the mould was placed in the horizontal submarine electrophoretic unit filled with 1X TBE buffer and pre-run was carried out for 5minutes to clear the wells of any gel particles.
- 5µl of the PCR products were mixed with 1µl of 6X loading dye and loaded in to the wells. 100bp ladder used as marker was also loaded in one of the wells to compare the size of the PCR products.
- Electrophoresis was carried out at 100volts for 15-20 minutes and the gel was viewed under UV transilluminator and photographed using gel documentation system

Interpretation:

Based on the number of bands and fragment sizes visualized, the samples were interpreted and genotyped as homozygous normal (198bp), homozygous mutant (175bp&23bp) and as heterozygotes if all the three bands were present.

Fig.1: Gel showing different genotypes of c.677C>T polymorphism of MTHFR gene



Lane 1, 2, 3, 4, 5, 6	:	Homozygous normal (CC)
Lane 8	:	Heterozygous (CT)
Lane 9	:	Homozygous mutant (TT)
Lane 7	:	Uncut
Lane L	:	50bp ladder

Polyacrylamide Gel Electrophoresis(Page) polyacrylamide ge l(8%) electrophoresis was carried out to determine the type of the digested PCR products for mutations of A1298C.

Procedure:

The vertical gel gel electrophoresis unit along with glass plates,spacers and combs were cleaned with mild liquid detergent followed by distilled water and then ethanol. The glass plates along with spacers were assembled on the gel casting stand and the gel was prepared as follows.

30% acrylamide stock solution	10.6 ml
5XTBE	8.0 ml
Double distilled water	21.0 ml
10% ammonium per sulphate	280micro l
TEMED	14 micro l
Final volume	40 ml

The above reagents are mixed in a beaker and were immediately poured with a sterile syringe into the gap between the two glass plates and comb was inserted carefully avoiding air bubbles.

The gel was then layered with water to avoid contact with air and to ensure that the wells are formed with uniform surface. The was allowed to polymerize for 1 hour and then the comb was gently removed.The wells were washed with distilled water to remove gel particles if any.

Then the plates were mounted onto electrophoresis set up and anode & cathode tanks were filled with 1X TBE buffer Pre run was carried out for 15 minutes at 200 volts.

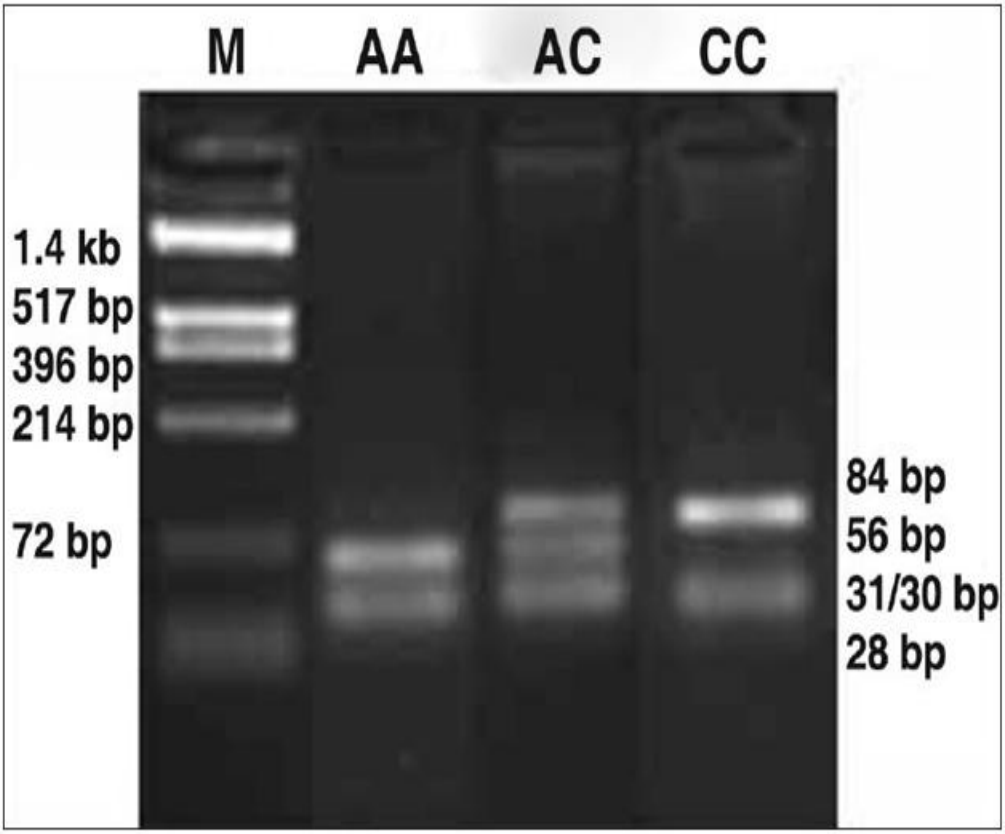
5micro litre PCR product was mixed with 1 micro litre of 6X loading dye and loaded into the wells along with a standard 100bp DNA marker. Then the electrophoresis was carried out at 200 volts for 2 hours the bromophenol blue of loading dye reached the edge of the gel. The gel was then removed from the electrophoresis unit and transferred to aclean tray for staining.

The gel was stained with 10 micro litre of ethidium bromide (10mg/ml) in 1X TBE and the bands were visualized under UV transilluminator and photographed using gel doc.

Interpretation:

Based on the number of bands and fragment sizes visualized, the samples were interpreted and genotyped as homozygous normal (56/31/30/28/18bp), homozygous mutant (84/31/30/18bp) and as heterozygotes if all the three bands were present.

Fig.1: Gel showing different genotypes of A1298C polymorphism of MTHFR gene



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Lane 1, 4	:	Homozygous normal (AA)
Lane 3, 5	:	Heterozygous (AC)
Lane 2	:	Homozygous mutant (CC)
Lane 6	:	Uncut
Lane L		50bp ladder

Statistical Analysis:.

SNP status (http://bioinfo.iconcologia.net/index.php?module=snapshots) was usedto determine odds ratio(ORs),95%confidence intervals(CIs)and P values under models(codominant, dominant, recessive, overdominant, and longadditive)were employed to analyze the data. The genotype distributions of each mutation, the frequency of heterozygous and homozygous were compared between patients and controls with Pearson's Chi-square test. A P value of <0.05 was regarded as significant. The homozygote and heterozygote genotypes of each group were unified as a new group and then odds ratios and 95% confidence intervals were calculated.

III. Results And Observations

Table 1: Baseline haracteristics of OT EXPOSED and CONTROL subjects studied

	AN	CT
Total	87	150
M	30 (34.5%)	90 (60.0%)
F	57 (65.5%)	60 (40.0%)
BMI	24.0+ 0.25	24.14+ 0.39
Age range	23-64yrs	19-58yrs
Duration of exposure to nitrous oxide (in yrs)		
3-10	51(58.6%)	--
11-20	25 (28.7%)	--
21-30	10 (11.5%)	--
31-40	1(1.2%)	--
BMI	24.0+ 0.25	24.14+ 0.39
Age range	23-64yrs	19-58yrs

Demography of OT exposed personnel:

A total of 87 OT exposed personnel are participated in the study of them 30(34.5%) males & 57(65.5%) females present.

These subject's mean BMI in the range of 24.0+ 0.25

Age range 23-64yrs

These subjects duration of exposure to OT anaesthetic gases(nitrous oxide):

3-10 years-51(58.6%)
11-20years-25(28.7%)
21-30years-10(11.5%)
31-40 years-1(1.2%)

Demography of control group:

A total of 150 controls who were not exposed to anaesthetic gases were selected randomly.
Of these 90(60%)males& 60(40%) females are participated.

Their mean BMI : 24.14+ 0.39 these people age range between 19-58yrs

Table 2a: Genotype frequencies of c.677C>T polymorphism of MTHFR gene

	AN		CT	
Genotype	**N**	**Freq**	**N**	**Freq**
C/C	61	0.8	103	0.82
C/T	14	0.18	22	0.17
T/T	1	0.01	1	0.01

Table2b: Allele frequencies of c.677C>T polymorphism of MTHFR gene

	AN		CT	
Allele	**N**	**Freq**	**N**	**Freq**
C	136	0.89	228	0.9
T	16	0.11	24	0.1

HWE for cases:p-value-0.048; Controls-0.00037

Table 2c: Risk of c.677C>T genotypes under different models

	Genotype	AN	CT	OR (95% CI)	P-value
Codominant	C/C	61 (80.3%)	103 (81.8%)	1.00	0.93
	C/T	14 (18.4%)	22 (17.5%)	1.04 (0.48-2.23)	
	T/T	1 (1.3%)	1 (0.8%)	0.57 (0.03-10.19)	
Dominant	C/C	61 (80.3%)	103 (81.8%)	1.00	0.99
	C/T-T/T	15 (19.7%)	23 (18.2%)	1.01 (0.48-2.12)	
Recessive	C/C-C/T	75 (98.7%)	125 (99.2%)	1.00	0.7
	T/T	1 (1.3%)	1 (0.8%)	0.57 (0.03-10.09)	
Overdominant	C/C-T/T	62 (81.6%)	104 (82.5%)	1.00	0.91
	C/T	14 (18.4%)	22 (17.5%)	1.05 (0.49-2.24)	
Log-additive	---	---	---	0.97 (0.49-1.94)	0.94

Table2d: Sex wise genotypic distribution of c.677C>T polymorphism among subjects exposed to OT anaesthetic gases and controls

F		AN	CT	OR (95% CI)	p-value
	C/C	39(76.4%)	43(79.6%)	1.00	
	C/T	11(21.5%)	11(20.3%)	0.91 (0.35-2.33)	0.83
	T/T	1(0.019%)	0(0%)	0.00	0.97

M		AN	CT	OR (95% CI)	
	C/C	22(88%)	60(83.3%)	1.00	
	C/T	3(12%)	11(15.2%)	1.34 (0.34-5.27)	0.92
	T/T	0(0%)	1(0.13%)	---	0.00

Genotype Distribution of C677T MTHFR Gene Mutations in Case and Control Groups

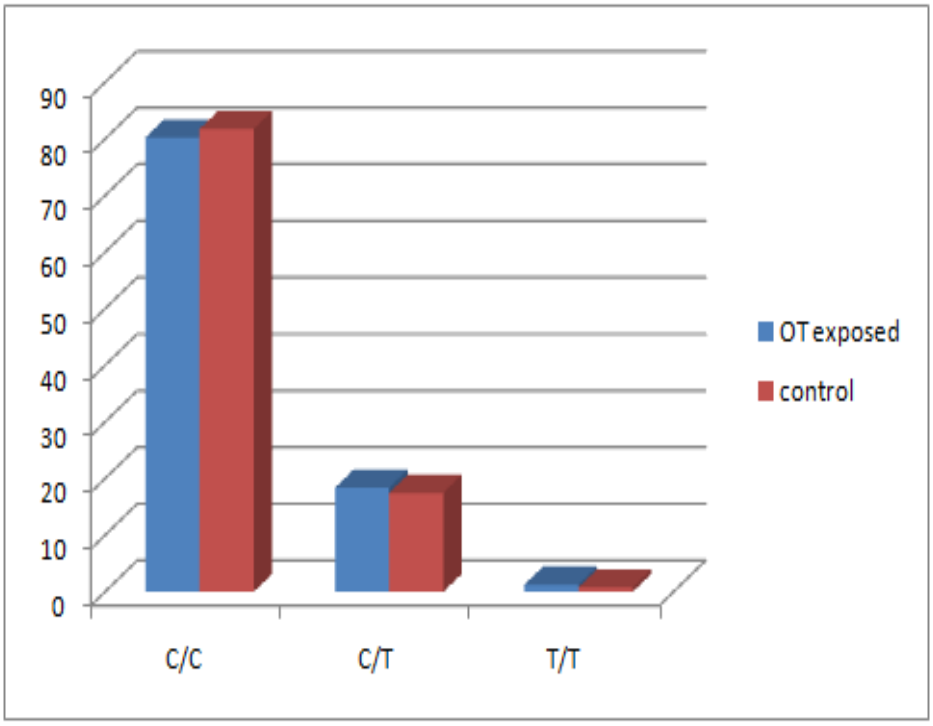

FIG:3,genotype frequency in codominant model of c677t MTHFR gene

In co dominant model The frequency of **677C/T** genotype MTHFR gene was18.4%in patients and 17.5% in controls. The odds ratios (ORs) of the MTHFR **677C/T** (OR = 1.04; 95% confidence interval (CI) = (0.48-2.23) while the frequency of **677T/T** genotype was 1.3% in patients and 0.8%in controls. The odds ratios (ORs) of the MTHFR **677T/T** (OR = 1.04; 95% confidence interval (CI) = (0.48-2.23) The frequency of **677C/C**genotype MTHFR gene was80.3%in patients and 81.8% in controls. . The odds ratios (ORs) of the MTHFR **677C/C** (OR = 1.00;).The **P-value** for codomonant model 0.93.

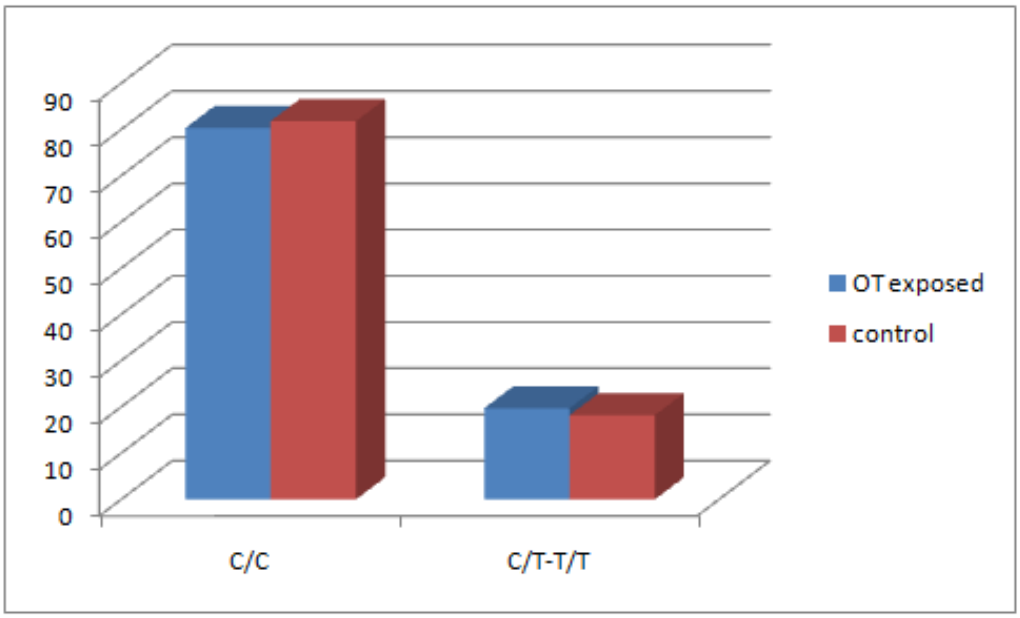

FIG:4, genotype frequency in dominant model c677t MTHFR gene

In Dominant Model The frequency of **677C/C** genotype MTHFR gene was80.3%in patients and 81.8% in controls. The odds ratios (ORs) of the MTHFR **677C/T** (OR = 1.00); while the frequencyof **677C/T-T/T** genotype was 19.7% in patients and 18.2%in controls. The odds ratios (ORs) of the MTHFR **677T/T** (OR = 1.01; 95% Confidence interval (CI) = (0.48-2.12). The **P-value** for domonant model 0.99.

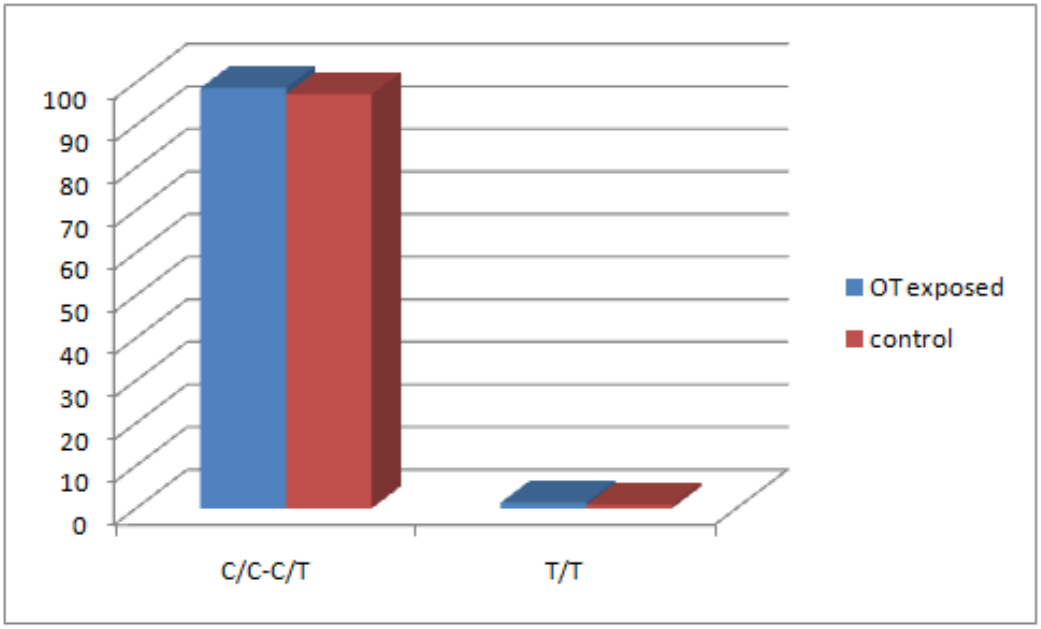

FIG:5, genotype frequency in RECESSIVE model c677t MTHFR gene

In Recessive Model The frequency of **677C/C-C/T** genotype MTHFR gene was98.7%in patients and 99.2% in controls. The odds ratios (ORs) of the MTHFR **677C/T** (OR = 1.00); while the frequencyof **677T/T** genotype was 19.7% in patients and 18.2%in controls. The odds ratios (ORs) of the MTHFR **677T/T** (OR = 0.57 (0.03-10.09); 95% confidence interval (CI) = (0.03-10.09). The **P-value** for recessive model 0.7.

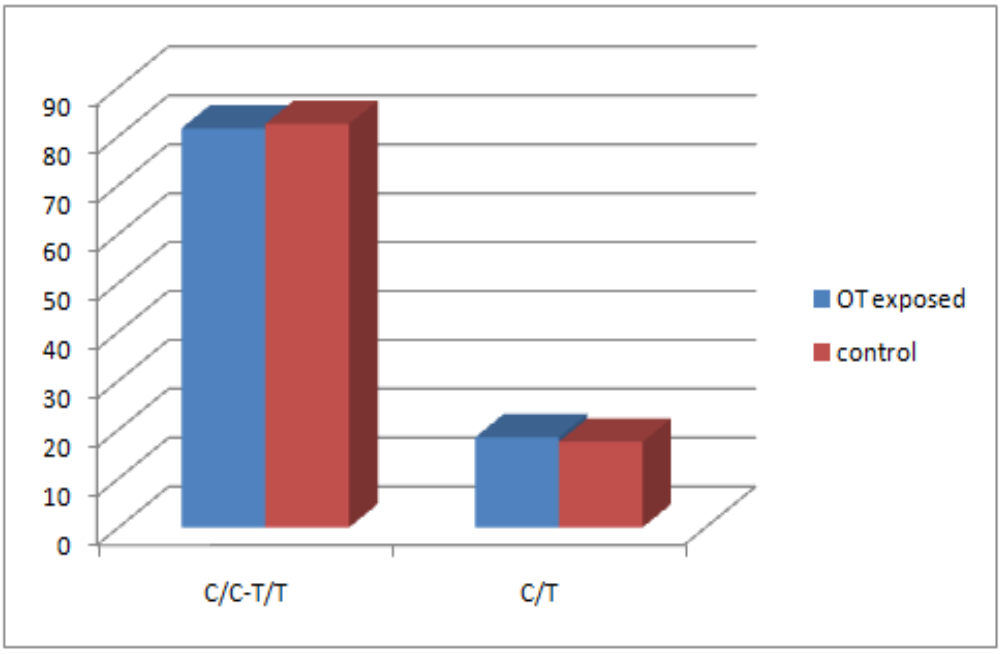

FIG:6, genotype frequency in over dominant model c677t MTHFR gene

In **Over Dominant Model** The frequency of **677C/C-T/T** genotype MTHFR gene was81.6%in patients and 82.5% in controls. The odds ratios (ORs) of the MTHFR **677C/C-T/T** (OR = 1.00); while the frequency of **677C/T** genotype was 18.4% in patients and 17.5%in controls. The odds ratios (ORs) of the MTHFR **677T/T** (OR = 1.05; 95% confidence interval (CI) = (0.49-2.24). The **P-value** for over domonant model 0.91.IN **Long Additive Model** MTHFR C677T genotype odds ratios(OR=0.97;confidence interval(CI)=(0.49-1.94). The **P-value** for LONG-ADDITIVE model 0.94.

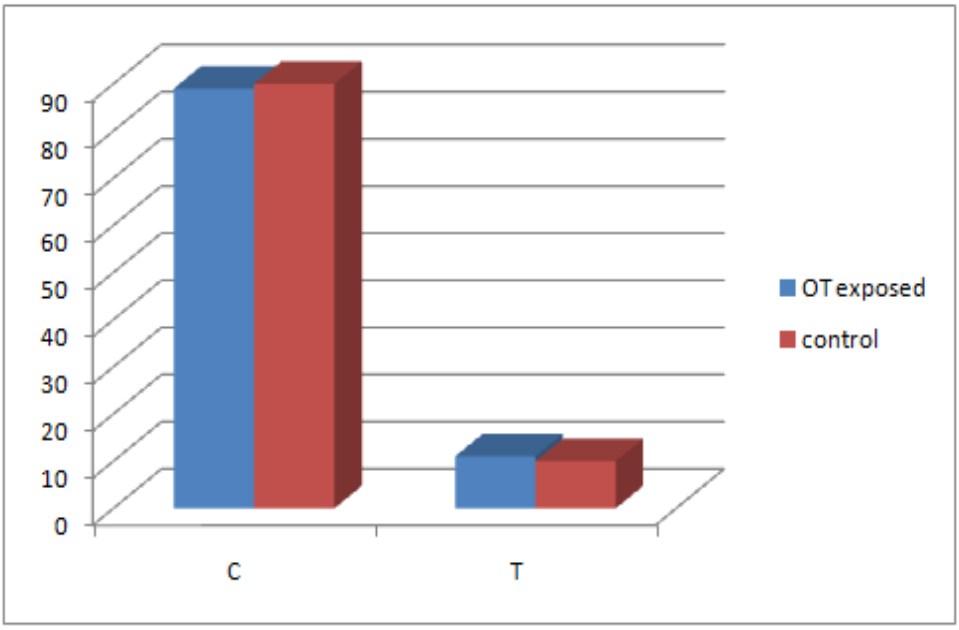

FIG:7, Allele frequencies of c.677C>T polymorphism of MTHFR gene

The frequency of 677C MTHFR allele was 89% in patients and 90% in controls The frequency of 677T allele was 11% in patients and 10% in controls (Figure 3).There is no significant difference in the prevalence of 677T/T genotype among OT exposed personnel and controls. HWE for cases:p-value-0.048; Controls-0.00037

SEX wise genotypic distribution of c.677C>T polymorphism among subjects exposed to OT anaesthetic gases and controls

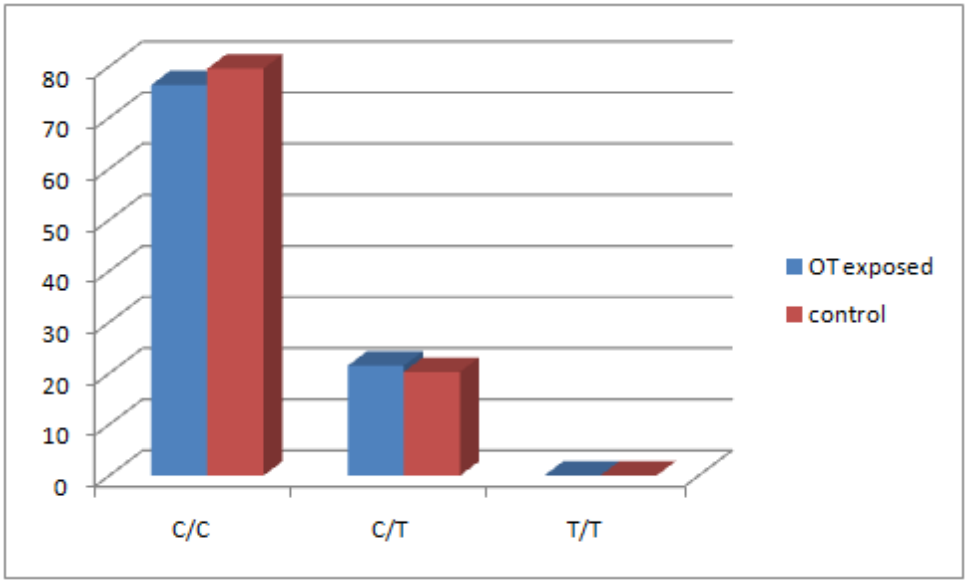

FIG:8,genotype frequency in females c677t MTHFR gene

The frequency of **677C/C** genotype MTHFR gene in females was76.4%in patients and 79.6% in controls. The odds ratios (ORs) of the MTHFR **677C/C** (OR = 1.00); while the frequencyof **677C/T** genotype in females was 21.5% in patients and 20.3%in controls. The odds ratios (ORs) of the MTHFR **677C/T** (OR = 0.91; 95% confidence interval (CI) = (0.35-2.33). The **P-value** is 0.83.The frequencyof **677T/T** genotype in females was 0.019% in patients and 0%in controls. The odds ratios (ORs) of the MTHFR **677C/T** (OR = 0.00; The **P-value** is 0.97.

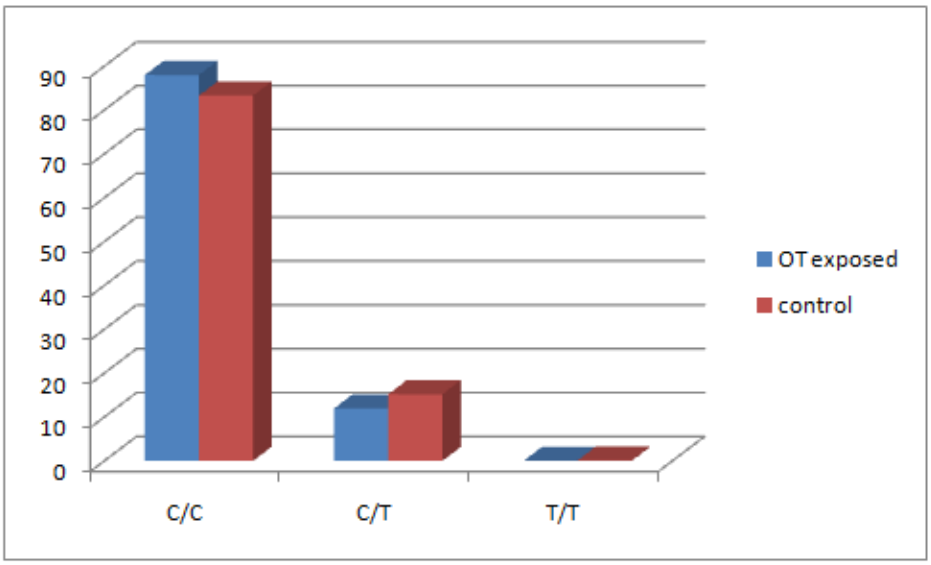

FIG:8,genotype frequency in females c677t MTHFR gene

The frequency of **677C/C** genotype MTHFR gene in males was88%in patients and 83.3% in controls. The odds ratios (ORs) of the MTHFR **677C/C** (OR = 1.00); while the frequencyof **677C/T** genotype in males was 12% in patients and 15.2%in controls. The odds ratios (ORs) of the MTHFR **677C/T** (OR = 1.34; 95% confidence interval (CI) = (0.34-5.27). The **P-value** is 0.92.The frequencyof **677T/T** genotype in males was 0.00% in patients and 0.13%in controls. The odds ratios (ORs) of the MTHFR **677C/T** (OR = 0.00; The **P-value** is 0.00.

Table 3a: Genotype frequencies of c.1298A>C polymorphism of MTHFR gene

	AN (76)		CT(126)	
Genotype	**N**	**Freq**	**N**	**Freq**
A/A	34	0.45	55	0.44
A/C	27	0.36	41	0.33
C/C	15	0.2	30	0.24

Table 3b: Allele frequencies of c.1298A>C polymorphism of MTHFR gene

	AN		CT	
Allele	**N**	**Freq**	**N**	**Freq**
A	95	0.62	151	0.6
C	57	0.38	101	0.4

HWE for cases:p-value-0.048; Controls-0.00037

Table 3c: Risk of c.1298A>C genotypes under different models

Model	Genotype	AN	CT	OR (95% CI)	P-value
Codominant	A/A	34 (44.7%)	55 (43.6%)	1.00	0.9
	A/C	27 (35.5%)	41 (32.5%)	1.14 (0.58-2.23)	
	C/C	15 (19.7%)	30 (23.8%)	1.16 (0.53-2.52)	
Dominant	A/A	34 (44.7%)	55 (43.6%)	1.00	0.65
	A/C-C/C	42 (55.3%)	71 (56.4%)	1.15 (0.63-2.07)	
Recessive	A/A-A/C	61 (80.3%)	96 (76.2%)	1.00	0.8
	C/C	15 (19.7%)	30 (23.8%)	1.10 (0.53-2.26)	
Overdominant	A/A-C/C	49 (64.5%)	85 (67.5%)	1.00	0.8
	A/C	27 (35.5%)	41 (32.5%)	1.08 (0.58-2.03)	
Log-additive	---	---	---	1.08 (0.74-1.58)	0.67

Table 3d: Sex wise Genotypic distribution of c.1298A>C polymorphism among subjects exposed to OTand controls

		AN	CT	OR (95% CI)	p-value
F					
	A/A	25(49%)	17(31.4%)	1.00	
	A/C	17(33%)	28(51.8%)	**2.42 (1.02-5.73)***	**0.04**
	C/C	9(17%)	9(16.6%)	1.47 (0.48-4.46)	0.49
M		**AN**	**CT**	**OR (95% CI)**	
	A/A	9(36%)	38(52.7%)	1.00	
	A/C	10(40%)	13(18%)	**0.31 (0.10-0.92)***	**0.03**
	C/C	6(24%)	21(29.1%)	0.83 (0.26-2.65)	0.75

Genotype Distribution of A1298C MTHFRGene Mutations in Case and Control Groups. The genotypedistribution of each MTHFR mutations in patients and controls are Shown in (Figure9).

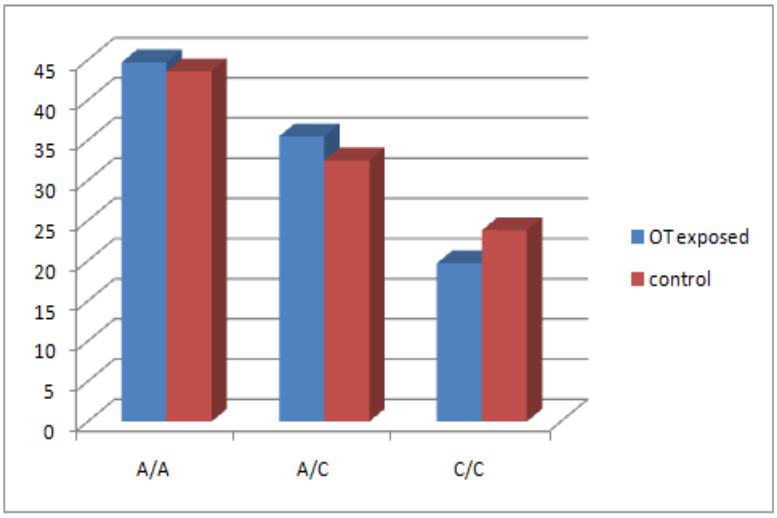

FIG:9,.genotype frequency in codominant model of a1298c MTHFR gene

In co dominant model The frequency of **1298A/A** genotype MTHFR gene was44.7%in patients and 43.6% in controls. The odds ratios (ORs) of the MTHFR **1298A/A** (OR = 1.00; while the frequencyof **1298A/C** genotype was 35.5% in patients and 32.5%in controls. The odds ratios (ORs) of the MTHFR **1298A/C** (OR = 1.14; 95% confidence interval (CI) = (0.58-2.23) The frequency of **1298C/C**genotype MTHFR gene was19.7%in patients and 23.8% in controls. . The odds ratios (ORs) of the MTHFR **1298C/C** (OR = 1.16; 95%confidence interval (CI)=(0.53-2.52).The **P-value** for codominant model 0.9.

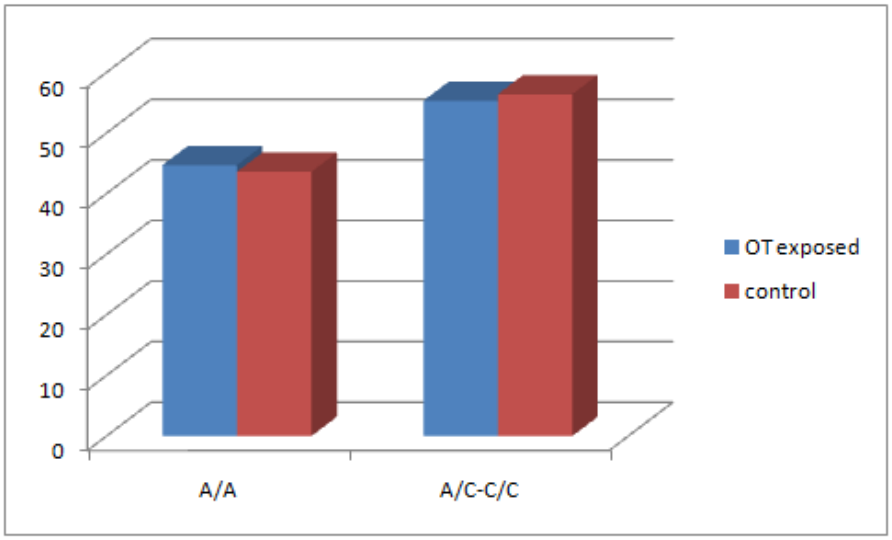

FIG:10,.genotype frequency in codominant model of a1298c MTHFR gene

In Dominant Model The frequency of **1298A/A**genotype MTHFR gene was 44.7%in patients and 43.6% in controls. The odds ratios (ORs) of the MTHFR **1298A/A** (OR = 1.00); while the frequencyof **1298A/C-C/C** genotype was 55.3% in patients and 56.4%in controls. The odds ratios (ORs) of the MTHFR **1298A/C-C/C** (OR = 1.15; 95% confidence interval (CI) = (0.63-2.07). The **P-value** for dominant model 0.65.

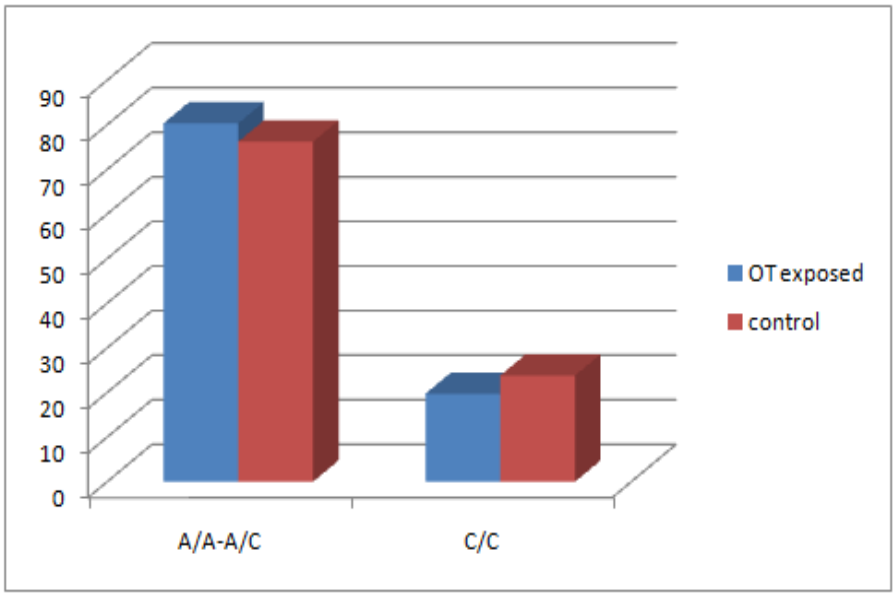

FIG:11,.genotype frequency in codominant model of a1298c MTHFR gene

In Recessive Model The frequency of **1298A/A-A/C** genotype MTHFR gene was80.3%in patients and 76.2% in controls. The odds ratios (ORs) of the MTHFR **1298A/A-A/C** (OR = 1.00); while the frequencyof **1298C/C** genotype was 19.7% in patients and 23.8%in controls. The odds ratios (ORs) of the MTHFR **1298C/C** (OR = 1.10; 95%confidence interval (CI) =(0.53-2.26).The **P-value** for recessive model 0.8.

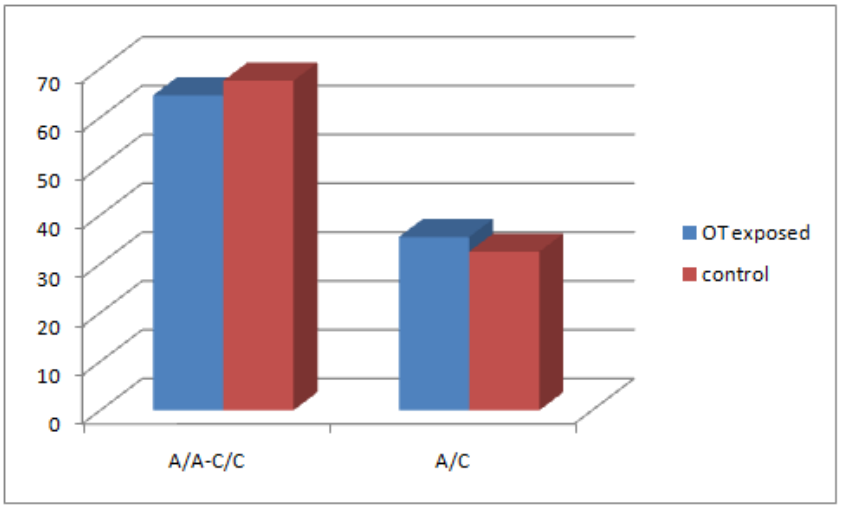

FIG:12,.genotype frequency in over dominant model of a1298c MTHFR gene

In Over Dominant Model The frequency of **1298A/A-C/C** genotype MTHFR gene was64.5%in patients and 67.5% in controls. The odds ratios (ORs) of the MTHFR **1298A/A-C/C** (OR = 1.00); while the frequency of **1298A/C** genotype was 35.5% in patients and 32.5%in controls. The odds ratios (ORs) of the MTHFR **1298A/C** (OR = 1.08; 95% confidence interval (CI) = (0.58-2.03). The **P-value** for over dominant model 0.8.

In Long Additive Model MTHFR A1298C genotype odds ratios(OR=1.08;confidence interval (CI) = (0.74-1.58). The **P-value** for LONG-ADDITIVE model 0.67.

Allele frequencies of A1298C polymorphism of MTHFR gene

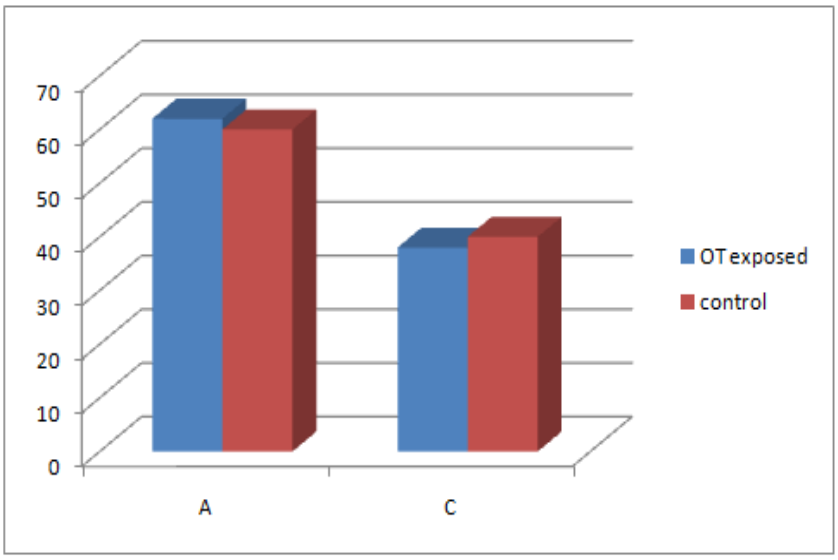

FIG:13,.allele frequency of a1298c MTHFR gene

The frequency of 1298A MTHFR allele was 62% in patients and 60% in controls The frequency of 1298C allele was 38% in patients and 40% in controls (Figure 3).There is no significant difference in the prevalence of 677T/T genotype among OT exposed personnel and controls. HWE for cases:p-value-0.048; Controls-0.00037

SEX wise genotypic distribution of A1298C polymorphism among subjects exposed to OT anaesthetic gases and controls

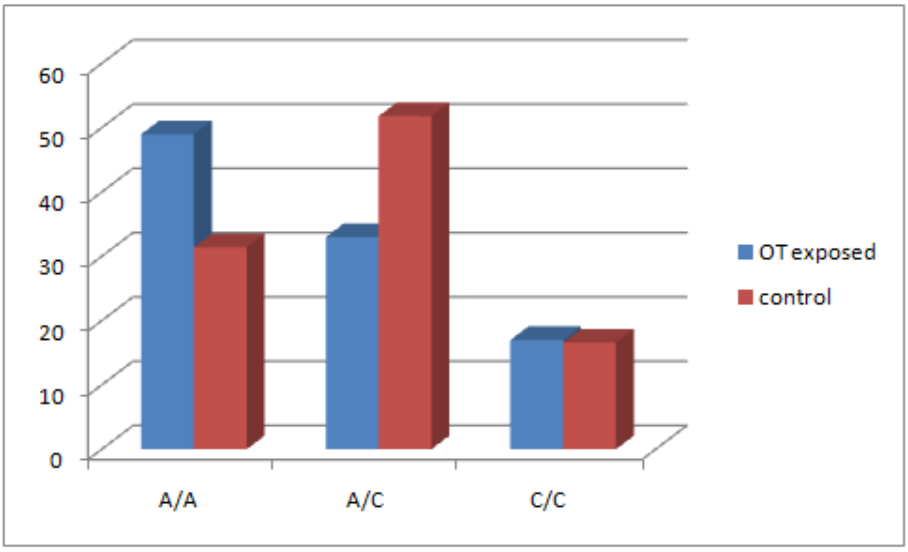

FIG:14,.genotype frequency in females of a1298c MTHFR gene

The frequency of **1298A/A** genotype MTHFR gene in females was49%in patients and 31.4% in controls. The odds ratios (ORs) of the MTHFR **1298A/A** (OR = 1.00); while the frequencyof **1298A/C** genotype in females was 33% in patients and 51.8%in controls. The odds ratios (ORs) of the MTHFR **1298A/C** (OR = 2.42; 95% confidence interval (CI) = (1.02-5.73). The **P-value** is 0.04.The frequencyof **1298C/C** genotype in females was 17% in patients and16.6%in controls. The odds ratios (ORs) of the MTHFR **1298C/C** (OR = 1.47;95% confidence interval (CI) =(0.48-4.46).The **P-value** is 0.49.

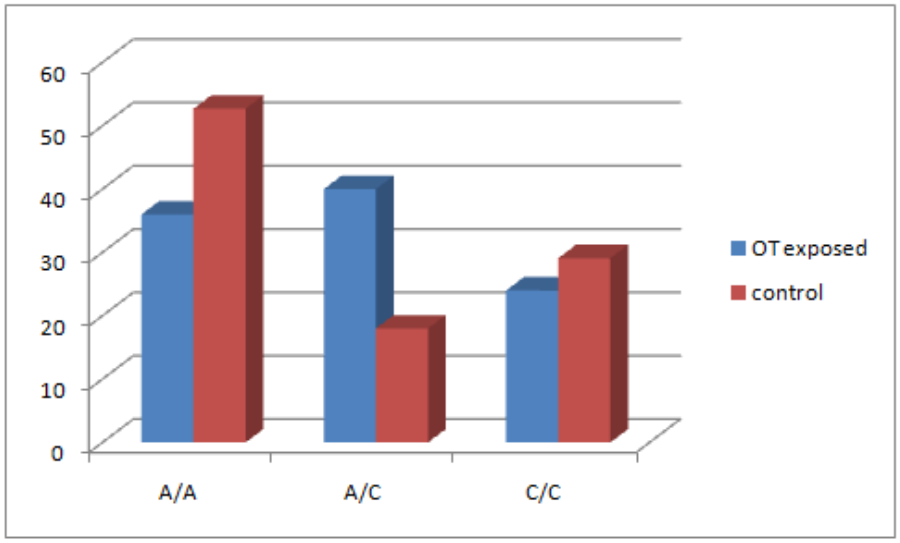

FIG:15,.genotype frequency in males of a1298c MTHFR gene

The frequency of **1298A/A** genotype MTHFR gene in males was36%in patients and 52.7% in controls. The odds ratios (ORs) of the MTHFR **1298A/A** (OR = 1.00); while the frequencyof **1298A/C** genotype in males was 40% in patients and 18%in controls. The odds ratios (ORs) of the MTHFR **1298A/C** (OR = 0.31; 95% confidence interval (CI) = (0.10-0.92). The **P-value** is 0.03.The frequencyof **1298C/C** genotype in males was 24% in patients and 29.1%in controls. The odds ratios (ORs) of the MTHFR **1298C/C** (OR = 0.83; 95% confidence interval (CI) = (0.26-2.65). The **P-value** is 0.75.

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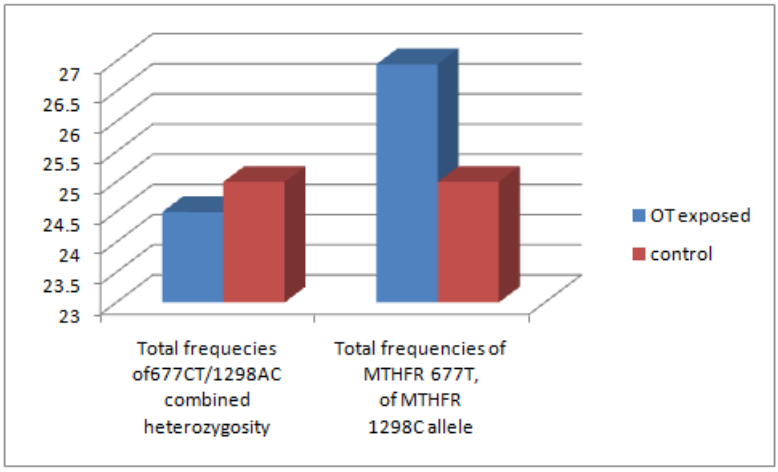

FIG:16,combined genotype&allele frequencies of c677t/a1298CMTHFR gene

The frequency of 1298A/C genotype MTHFR gene was 35.5% in patients and 32.5% in controls. The frequency of homozygote genotype was 19.7% in patients and 23.8% in controls. The frequencies of 1298C allele were 38% in patients and 40% in controls. No statistically significant difference in the frequency of A1298C MTHFR gene mutation was detected between the two groups (P = 0.17). The frequencies of MTHFR 677T and MTHFR 1298C alleles were (11%, 38%) in patients and (10%, 40%) in controls, respectively (Figure 4). The total mutant allele frequencies were 24.5% in women experiencing RSA and 25% in controls. All possibleMTHFR C677T/A1298C genotype combinations were represented in both groups (Table 3). The frequencies of 677CT/1298AC combined heterozygosity in patients were 26.95% and 25% in the controlgroup. Our findings indicated that combined MTHFR C677T/A1298C genotype distribution has no statistically significant differences. The odds ratios (ORs) of the MTHFR 677C/T (OR = 1.04; 95% confidence interval (CI) = (0.48-2.23) and the MTHFR 1298A/C (OR=1.14; 95% confidence interval (CI) =0.58-2.23).

IV. Discussion

Many pieces of literature have discussed the matter that MTHFR gene mutations might be a risk factor for exposure to OT anaesthetic gases; hence, we investigated the prevalence of C677T and A1298C, two common MTHFR gene mutations in OT exposed personnel, to determine whether these mutations related with OT exposure. The genotypes distribution of C677T MTHFR gene mutation was compared in the two studied groups. It is clear from Table 2 and Figure 3 that the heterozygosity in nucleotide 677th of the MTHFR gene& homozygosity for C677T genotype of the MTHFR gene has no statistically significant difference among the two groups

, which were concordant with previous reports . The total frequency of 677T alleles for MTHFR gene 677T was also compared between OT exposed & control groups (11%,10%), respectively. The genotypes distribution of C677T MTHFR gene mutation was also compared in sex wise distribution. ,On the whole, our data has indicated no Statistically significant difference in the prevalence of C677Tmutation between the two groups. These observations are in contrast with a previous report on the literature . This difference may be explained by differences in the populations or by using low numbers of samples.

Furthermore, the frequency of A1298C MTHFR gene mutation was also compared in patients and healthy women. The genotypes distribution of C677T MTHFR gene mutation was compared in the two studied groups. It is clear from Table 3 and Figure 7 that the heterozygosity in nucleotide A1298C of the MTHFR gene& homozygosity for A1298C genotype of the MTHFR gene has no statistically significant difference among the two groups , which were concordant with previous reports . The total frequency of 1298Calleles for MTHFR gene 1298Cwas also compared between OTexposed&control groups (11%,10%), respectively. The genotypes distribution of A1298C MTHFR gene mutation was also compared in sex wise distribution.In OT exposed females **OR (95% CI) 2.42 (1.02-5.73)*** ,it shows increased prevalence of A1298C mutation than control female group. In OT exposed males **OR (95% CI) 0.31 (0.10-0.92)***,it shows decreased prevalence of A1298C mutation than control male group.On the whole, our data has indicated Statistically significant difference in the prevalence of A1298C mutation in sex wise distribution between the two groups. These observations are in accordance with a previous report on the following literature.

Bodin L, Axelsson G, et al: The association of shift work and nitrous oxide exposure in pregnancy with birth weight and gestational age Nitrous oxide exposure was associated with higher ORs for low-birth-weight (OR, 3.4; 95% CI, 0.9 –3.4) and small-for-gestational-age babies (OR, 3.0; 95% CI, 1.2–7.2).[1]

Axelsson et al. done a study on Shift work, nitrous oxide exposure, and spontaneous abortion among Swedish midwives And found an association of spontaneous abortions with night and shift work (OR, 1.63; 95% CI, 0.95–2.81) but not with nitrous oxide exposure (OR, 0.95; 95% CI, 0.62–1.47) involving 1,717 pregnancies in 3,985midwives.[2]

Boivin et al 121 performed Risk of spontaneous abortion in women occupationally exposed to anaesthetic gases: a meta-analysis of 19 studies completed between 1971 and 1995 and found a relative risk of spontaneous abortion with nitrous oxide exposure of 1.48 (95% CI, 1.4 –1.58).[3]

Rowland AS, et al Reduced fertility among women employed as dental assistants exposed to high levels of nitrous oxide. authors concluded that 1 unscavenged nitrous oxide exposure (which they estimated to exceed 1,000 ppm) for greater than 5 h per week was associated with reduced ability to conceive.[4]

Hoerauf KH, et al Genetic damage in operating room personnel exposed to isoflurane and nitrousoxide. In a study of 50 physicians (25 anesthesiologists and 25 unexposed controls), occupational exposure to sevoflurane (8.9 _ 5.6 ppm) and nitrous oxide (119 _ 39 ppm) was associated with increased levels of sister chromatid exchange. [5]

Sharer et al.: Effects of chronic exposure to nitrous oxide on methionine synthase activity study on showed that a 24-h exposure to concentrations of 860 parts per million (ppm) or lower of nitrous oxide did not significantly change methionine synthase function in Sprague-Dawley rats.[6]

Koblin.51 study on Inactivation of methionine synthetase by nitrous oxide in mice. demonstrated that nitrousoxide (70%) inhibited methionine synthase activity in liver biopsies with a 50% reduction in activity predicted after approximately 1.5 h.[7]

Reynoldset al observed neurologic injury has also been noted after a routine nitrous oxide–based anesthetic in patients with cobalamin deficiency, such as those with pernicious anaemia, although the injury did not become apparent for many weeks

selzer et al done a study on adverse effects of nitrousoxide in a child with 5,10-MTHFR deficiency. Myelin degeneration after chronic nitrous oxide exposure has been observed.[8]

Badner NH, et al: initially studied the effects of nitrous oxide administration on plasma homocysteine levels. Nitrous oxide-induced increased homocysteine concentrations are associated with increased postoperative myocardial ischemia in patients undergoing carotid endarterectomy.[9]

Nagele P, et al Influence of mutations in the MTHFR gene on homocysteine levels after nitrous oxideanesthesia. Increased homocysteine levels are an independent risk factor for cardiac morbidity, potentially through causing endothelial dysfunction and procoagulation. [10]

ENIGMA 2 TRIALin patients undergoing anaesthesia for major surgery avoidance of N2O will reduce the cardiac complications ,stroke or death when compared with otherwise identically managed surgical patients receiving N2O as a component of their anesthesia .

Our study was in contrast with following Literature

Heidam L: et al Spontaneous abortions among dental assistants, factory workers,painters, and gardening workers: A follow up study. In the subgroup exposed to nitrous oxide (dental assistants), a 94% response rate was noted, and the OR for spontaneous abortion was 1.0 (CI, 0.8–1.2) and therefore the group exposed to nitrous oxide was not at increased risk.[11]

Ericson and Kallen et al study on Hospitalization for miscarriage and delivery outcome among Swedish nurses working in operating rooms 1973–1978. this study design did not reveal any differences in the incidence in perinatal deaths or malformations in anesthesiology/operating room nurses.[12]

Duncan et al. on Fetal risk of anesthesia and surgery during pregnancy. found no teratogenic effect and no difference in the incidence of spontaneous abortion with controls. [13]

Koblin et al.64study on Effect of nitrous oxide on folate and vitamin B12 metabolism in patients did not find any changes in two markers of methionine synthase function (urinary formic acidand formimino glutamic acid urinary excretion) after 3 h of nitrous oxide exposure during hip replacement in elderly patients.[14]

Deleu D, et al study on Long-term effects of nitrous oxide anaesthesia on laboratory and clinical parameters in elderly patients: A randomized double-blind study. stress that nitrous oxide is unlikely to precipitate neurologic injury secondary to demyelination in the majority of cases; this is exemplified both by the prevalence of nitrous oxide use and by the relative scarcity of the case reports reporting this form of injury.[15]

Mazze and Kallen: et al studied on Reproductive outcome after anesthesia and operation during pregnancy addressed this issue in a large registry study of 5,405 patients and found no association between different methods of anesthesia and adverseoutcomes.[16]

V. Conclusion

C677: in intron 2 was genotyped using Hinf1 restriction enzyme. No significant difference was observed in the distribution of genotypes,allele&sex wise frequencies between the two groups **(Table2b,2c,and 2d).**

A1298C: MTHFR was genotyped using MboII **r**estriction enzyme . The distribution of genotypic&allele frequencies(table 3band 3c) revealed no significant association. In sex wise distribution genotypic frequencies (table 3d) revealed significant association.In OT exposed females **OR (95% CI) 2.42 (1.02-5.73)*** ,it shows increased prevalence of A1298C mutation than control female group. In OT exposed males **OR (95% CI) 0.31 (0.10-0.92)***,it shows decreased prevalence of A1298C mutation than control male group.On the whole, our data has indicated Statistically significant difference in the prevalence of A1298C mutation in sex wise distribution in OT exposed personnel.

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