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## **Effect of Alcohol and Nicotine on Fertility of Male Albino Mice**

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### **Abstract**

Abuse of recreational drugs such as alcohol and nicotine may be genotoxic and may have detrimental effect on the male fertility which was aimed to be investigated through this present study using mice model systems. A total of 60 mice were divided into three groups, viz.; control group (n=20), and two groups of alcohol treated (n=20) and nicotine treated mice groups (n=20), administered in recommended dose amount for 90 days. Representative numbers of mice were sacrificed on 10th, 30th and 90th day from each group and the testes were collected. DNA and RNA was extracted from the collected tissue following standard protocol; followed by RAPD-PCR and Real time PCR based analysis of extracted DNA and cDNA, prepared from isolated RNA, for Rbmy1 gene expression analysis, respectively.  $\beta$ -actin was used as internal normalization control for Real time PCR analysis. Remarkable changes were observed in the RAPD profiles, based on difference in band pattern profile as observed in alcohol treated and nicotine induced cases compared to controls; thereby clearly indicating the effect of recreational drug on the genome of the host in comparison to untreated controls. The differential expression profile of Rbmy1 mRNA in the nicotine model, showed significant down regulation compared to control ( $p=0.045$ ), and non-significant when compared to alcoholic model cohorts ( $p=0.307$ ). The observation also showed differences of Rbmy1 mRNA expression in alcoholic cases compared to controls ( $p=0.782$ ).

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The present study indicates that consumption of recreation drugs such as nicotine may play an important role in disturbing the genome integrity and expression of key gene associated with male fertility, thereby disposing to male infertility.

**Keywords:** Male infertility; Alcohol and Nicotine; Rbm

## 1. Introduction

Male infertility accounts for about 40-50% of infertility cases in humans, a cause of major concern worldwide. Pre-testicular causes including consumption of drugs and alcohol; tobacco smoking, DNA damage, testicular factors including Y chromosome microdeletions; and post-testicular causes has been attributed to be clinically associated with male infertility. Gamete and embryo development are affected by life style factors like insufficient exercise, smoking, alcohol consumption, poor nutrition and environmental factors like substance abuse etc., leading to reduced sperm quality and infertility [31]. The role of recreational use of illicit drugs draws attention while studying the aetiology of male infertility [7]. According to [18] the oxidative stress within the testicle could be due to significant reduction in plasma testosterone in alcohol abusers, which in turn increase the serum lipid peroxidation by-products and a drop in antioxidants. Incidence of poor semen quality has been associated with higher level of alcohol consumption; however, the data was statistically insignificant [21]. Nicotine induces certain biochemical changes in the testes which have toxic effects on gonadal functions [2]. S. Purkayastha and R. Mahanta in 2014 reported that the sperm count in alcohol and nicotine treated group of albino mice showed a decreasing trend [33]. Reference [1] reported that 1.5mg/kg of body weight per day of nicotine in rodents is approximately equal to the accumulation of nicotine by consuming 50 cigarettes a day.

It has been reported that about 10% men are affected by *de novo* deletions of Y chromosome. Again the content of testicular damage or the stage in which the germ cell gets arrested are variable from person to person even though they might have the same deletion [29]. The Y chromosome is divided into seven intervals and the deletions generally occur in the interval 5 and 6 and the cluster has been defined as *Azoospermic factor* (AZF) [17]. From the proximal to the distal end of the long arm of the Y chromosome there exist three regions known as AZFa, AZFb and AZFc region and the deletions occur in this region leading to spermatogenic arrest [26].

For the production of normal spermatozoa Spermatogenesis requires the regulated expression of several genes [23]. Proteins which play an important role in spermatogenesis are encoded by the AZF regions. Genes like DDX3y (DEAD-box helicase Y) in AZFa region, RBMY1 (RNA binding motif on the Y) in the AZFb region and DAZ (deleted in Azoospermia) are considered to be the candidate genes of the AZF as they are exclusively expressed in the testis and it has also been shown that their homologues play an important role in spermatogenesis in other species. Though the Y chromosome microdeletion has been termed as the most important genetic cause of spermatogenic arrest, majority of men with spermatogenic failure do not exhibit the deletion of Y chromosome [20]. One of the most important gene in the AZFb region is the RBMY1 and it has been shown that there are six functional copies of the genes in the region [11]. RBMY1 had been established to have prognostic and diagnostic utility to study the Y chromosome microdeletions and its association with infertility in humans [5, 27]. In our earlier study we found deletion of RBMY1 in 11 infertile males, but though

the testicular histology showed abnormalities in sperm parameters many patients did not show any deletion [30]. This shows that the differential expression level of the gene might be the cause of spermatogenic failure.

The difference in genomic integrity and stability due to toxic components including those which are routinely used as recreational drug like Alcohol and Nicotine has been shown to be associated with multiple health disorder including sporadic reports on infertility, but the available data is still inconclusive and requires addressing. Alcohol affects male reproductive function though the degree of impact and dose-dependent relationship is not yet established [32]. Chronic Alcohol abuse can cause erectile dysfunction, reduction in the libido, and gynaecomastia. Due to alcohol consumption and hepatic cirrhosis the hypothalamic-pituitary gonadal (HPG) axis is altered, finally resulting in testicular dysfunction [13]. There are more than 3000 different chemical compounds in cigarette smoke which can enter the blood circulation of the testes and cause DNA damage, thereby exhibit cytotoxic effect on spermatozoa [35]. Apart from the gaseous phase of the smoke the main particulate component is nicotine [16]. Various cellular processes like gene expression [36], secretion of hormones [3], and enzymatic activity modulation [9] are affected by nicotine. Nicotine induces certain biochemical changes in the testes which have toxic effects on gonadal functions [2].

Due to the complexity it becomes quite difficult for in vitro study of spermatogenesis and thus mouse models are used as an alternative [12]. The analysis of individual molecules involved in gametogenesis as well the interaction of germ cells and sertoli cells have been facilitated by mouse models [37].

In this study an attempt has been made to study the effect of alcohol and nicotine on Male infertility using mice models. With the help of RAPD-PCR and differential expression of the *Rbm1* gene, which is a candidate gene of infertility and present in the Y chromosome. As the primers used in RAPD are of Short length and has relatively short length it can detect high level of DNA polymorphism [14]. This helps in the study of genetic variation among population.

## 2. Materials and Methods

### *Preparation of animal experimental models:*

A total of 60 male mice of about 8-10 weeks old were collected from Department of Zoology, Gauhati University for the study. The mice were kept in animal house of Gauhati University under suitable prescribed conditions. The study was permitted by the institutional ethical committee. The animals were divided into three groups (n=20 each) as mentioned below; of which one of which served as control and the other three as treated

### *Animal grouping and treatment:*

- **Group I:** This group was on normal diet and did not receive any treatment. This group served as controls:
- **Group II:** received intraperitoneal injection of 10-20% ethanol in saline (v/v 14ml/kg) resulting in 1-2.4g/kg of ethanol [4].
- **Group III:** received intraperitoneal injection of 0.5mg/kg per bodyweight of nicotine in saline which

was subsequently increased to 1.5mg/kg per day till the 30<sup>th</sup> day and then with an escalation of 0.5 mg/kg every three days, the dose was increased to 4.5mg/kg per day [19].

### ***Animal Sacrifice and sample collection***

At the end of the treatment days (10<sup>th</sup>, 30<sup>th</sup> and 90<sup>th</sup> days) representative mice from each group were anaesthetized by using an anesthetic mask of di-ethyl ether, and then sacrificed. A “T incision was made from pubic symphysis to the upper thorax to expose the abdominal region. The testis were collected immediately and kept in RNA later, for RNA extraction, and phosphate buffer solution, (PBS) for DNA extraction.

### ***DNA Extraction***

DNA from the mouse testis was extracted with the help of Geneipure Mammalian Genomic DNA purification Kit (Merck Bioscience-Genei, India) using the manufacturers protocol. Lysis of the tissue was done by incubating the sample with Sodium Dodecyl Sulphate (SDS) and Proteinase K solution at 55°C. Chaotrophic salts and ethanol was added to facilitate the binding of the DNA to the membrane in the column provided. The impurities *viz* salts, metabolites and other macromolecules were removed by two subsequent washes with wash buffers and finally the pure DNA was eluted with slightly alkaline buffer.

### ***RAPD-PCR of DNA extracted from Mice Testis***

The DNA extracted from the controls and treated mice testis were used for RAPD analysis. RAPD –PCR was performed using Primers OP-BO3, OP-A14, and OP-C01 as per reported literatures with slight modifications [25] The mastermix contained Genomic DNA approximately about 20ng, 1.5ul of dNTP mix of 2.5mM of each dNTP, 100ng of Random Primer, 1X concentration of 10X Taq DNA polymerase Assay Buffer, 1.5U of Taq DNA polymerase and Nuclease free water to make the volume to 25ul. The amplification conditions were initial denaturation at 94 °C followed by 40 cycles of denaturation at 94 °C for 45 seconds, annealing at 37-38 °C for 1 min and extension at 72 °C for 2 minutes, finally extension at 72 °C for 10 min.

**Table 1:** Nucleotide sequence of the primers.

Name of Primers	Nucleotide Sequence
OP-B03	CATCCCCCTG
OP-A14	TCTGTGCTGG
OP-C01	TTCGAGCCAG

Following PCR the amplicons were mixed with gel loading dye and run in 1.8% Agarose gel containing 0.1ul of Ethidium bromide (10mg/ml).The presence, absence and addition of bands were noted. The RAPD profile was analysed using Genalex analysis Software [28] which gave the Banding patterns based on frequency of alleles. The Principal Coordinate analysis was done to see the genetic variation among the populations.

**Extraction of RNA from Testis tissue.**

RNA was extracted from the Testis sample stored in RNA later using TRIzol purchased from Merck (Genei) using the manufacturer's protocol

**Preparation of cDNA:**

The RNA extracted from mice testis was used to prepare cDNA using MuLV (Moloney Murine Leukemia Virus) Reverse transcriptase enzyme provided in the M-MuLV RT-PCR Kit (Merck-Genei) using the manufacturer's protocol.

**Real Time quantification of relative m RNA expression**

Real Time PCR with SYBR Green dye was followed for relation quantification of m RNA expression of host genome. Expression of *Rbmy-1* was analysed with sequence specific primers normalised with housekeeping gene  $\beta$ -actin. The primer sets used for Real Time Quantification of relative m RNA expression in the patient and control sample are as shown in Table 2.

**Table 2:** Primers for Real Time PCR analysis

Gene	Primer sequence	Length	Tm
Mouse <i>Rbm-1</i>	F: AAGAGACCACCATCCTTTTC	20 bp	58°C
	R: CCCAGAAGAACTCACATTGA	20 bp	58 °C
Mouse $\beta$ -actin	F: 5'TACAGCTTCACCACCACAGC3'	20 bp	56°C
	R: 5'AAGGAAGGCTGGAAAAGACC3'	20 bp	58°C

**Relative quantification on m RNA expression using  $2^{-\Delta\Delta C_t}$  method:**

The expression on m RNA was calculated on the basis of difference of threshold cycle difference between Gene of Interest (GoI) and House-keeping gene (HKG) among cases and controls. The relative change in expression i.e. fold change in gene expression of GoI was calculated as follows:

$$\Delta C_{t(\text{Case})} = C_{t(\text{GoI})} - C_{t(\text{HKG})}$$

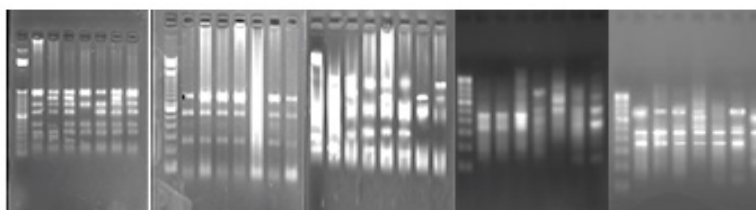
$$\Delta C_{t(\text{Control})} = C_{t(\text{GoI})} - C_{t(\text{HKG})}$$

$$\Delta\Delta C_t = \Delta C_{t(\text{Case})} - \Delta C_{t(\text{Control})}$$

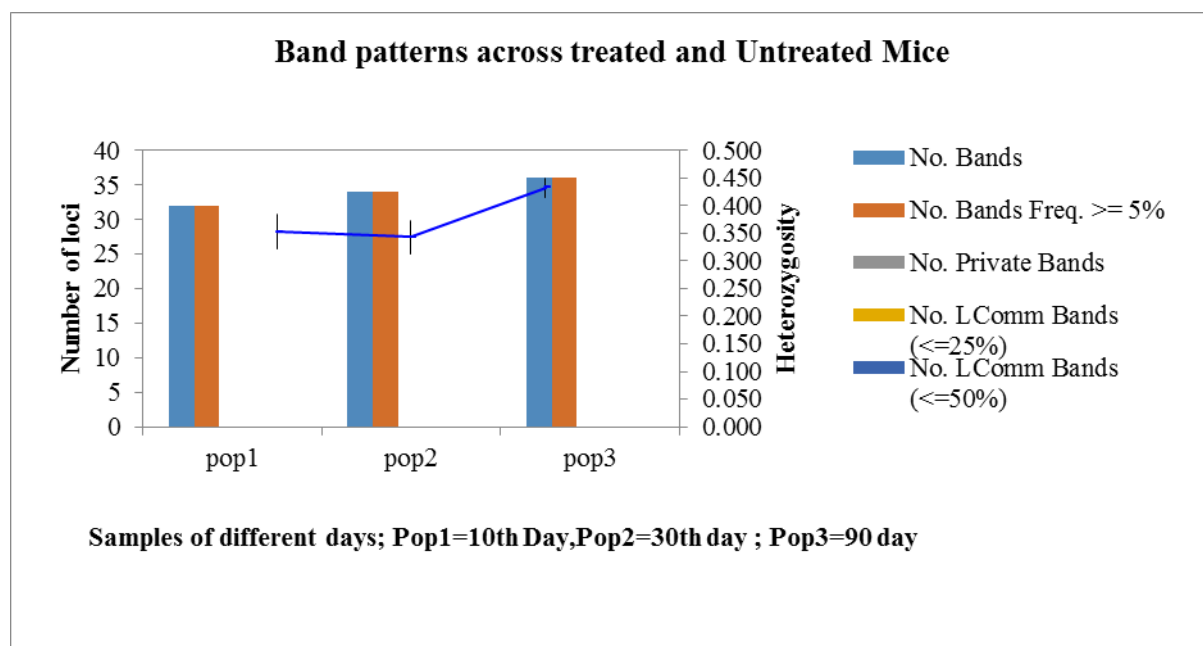
Fold change is calculated as:  $2^{-\Delta\Delta C_t}$

### 3. Results

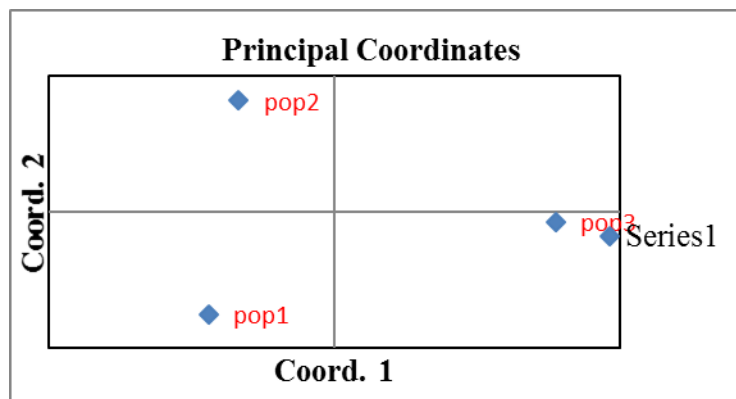
The RAPD PCR showed the presence of 36 loci among the three populations (Pop1 = 10<sup>th</sup> day, Pop2 = 30<sup>th</sup> day and Pop3 = 90<sup>th</sup> day). The bands ranged from 150 bp to 2500 bp. The number of polymorphic bands increased on the 90<sup>th</sup> day of treatment, showing the increase in Mean heterozygosity. The presence and absence of bands was scored as 1 and 0 respectively and the banding pattern was analysed using Genalex software. A total of 102 bands were obtained in the three populations. The Mean heterozygosity ranged from 0.361 in population 1, to 0.343 in population 2 and 0.433 in population 3. The percentage of polymorphic loci ranged from 83.33% in population 1 to 97.22% in population 3. Shanon Indices ranged from 0.517 in population 1, 0.498 in population 2 and 0.022 in population 3 (figure 2 and 3). When Principal coordinate analysis was done the population were divide into three clusters with percentage of variation in the first axis being 56.45 and in the second axis 43.55 (figure 3)



**Figure 1:** A few representative Agarose Gel Photographs of RAPD-PCR with Primers OP-BO3, OP-A14, and OP-C01



**Figure 2:** Banding Pattern as obtained using Genalex data analysis software

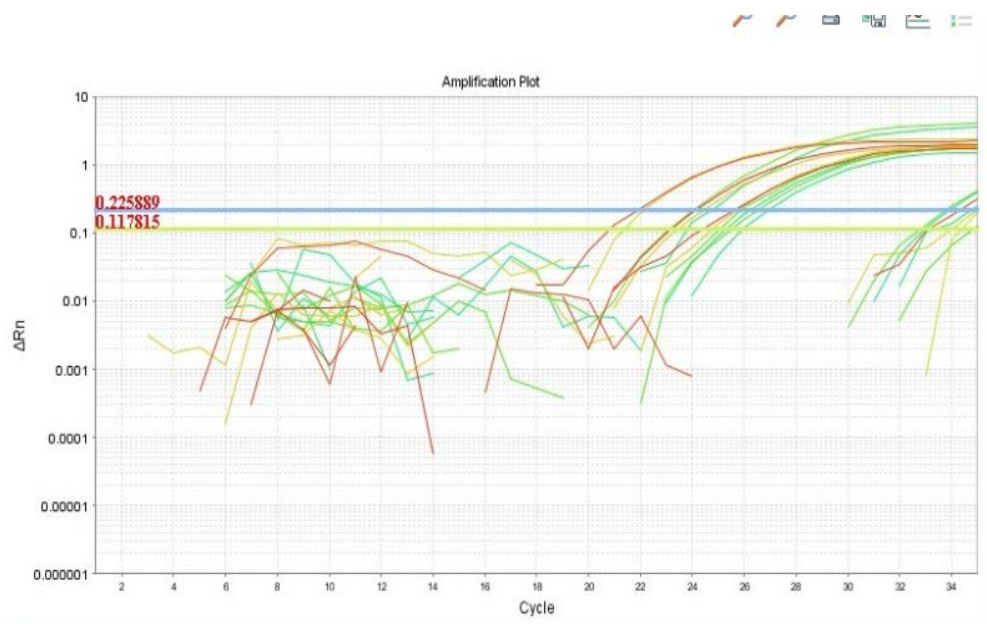


**Figure 3:** Principal Coordinate Analysis

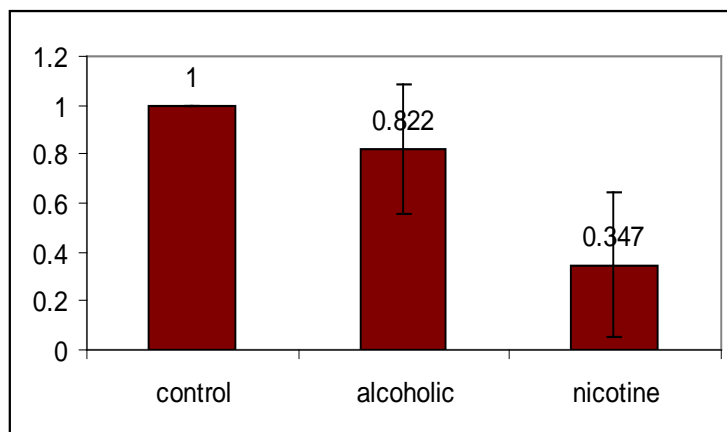
The RAPD results showed considerable variation in the RAPD profile generated by three primers among the population, which reflects that alcohol and nicotine affects the genetic integrity of the mice. Based on this assumption we performed quantitative PCR (qPCR) to study the effect on the treated as well as untreated mice.

#### ***Differential expression of mice Rbmy1 gene***

Differential expression of mice Rbmy1 gene specific for Y-chromosome region associated with infertility, was visualized by Real Time PCR using  $\beta$ -actin as internal control (Figure 5). The differential expression profile of mRNA in the nicotine model showed significant down regulation compared to control ( $p=0.045$ ), and non significantly compared to alcoholic model cohorts ( $p=0.307$ ) (Fig 2). The observation also showed differences of Rbmy1 mRNA expression in alcoholic cases compared to controls ( $p=0.782$ ).



**Figure 5:** Representative Real time amplification plot for *Rbmy-1* and internal normalization control gene  $\beta$ -actin gene in the mice models



**Figure 6:** Fold change in mRNA expression of *Rbmy-1* gene after normalizing with the expression of  $\beta$ -actin expression in the experimental cases, especially in the nicotine induced mice models

#### 4. Discussion

The present study was undertaken to evaluate the role of recreational drugs in inducing male infertility. Ramlau–Hansen et al., 2007 had shown that smoking affects sperm density, motility as well as sperm morphology [8] El-Alfy, *et al.*, 2014, in their study of the effect of mytomyacin C on male albino mice, reported that the RAPD profile clearly showed variation between treated and untreated mice [25]. In our study too we found that the heterozygosity among the population showed increasing trend. The PCoA showed that the percentage of variation of the control and treated group ranged from 56.45 in the first axis and in the second axis 43.55 among the population. This shows that alcohol and nicotine both has an impact on the genetic variation among the population in varying degrees and it is dose dependent.

Hansen, 2012 reported that poor semen quality is associated with higher level of alcohol consumption; however the data was statistically insignificant [22]. It has been reported that alcohol causes a decrease in the number of spermatozoa with normal morphology and increase in irreversible tail defects [10]. As spermatogenesis is a process which involves the regulation of a number of genes the aberration of the process can be due to the differential expression of genes. Nicotine on the other hand affects various cellular processes like gene expression [37] secretion of hormones [3] and enzymatic activity modulation [9]. Nicotine has been shown to cause genetic damage in human and rodents [15,24] but the effects are limited and contradictory. DNA strand breaks in human spermatozoa has been shown to be induced by nicotine [21]. Sen and Sharma in 1991 reported that low doses of nicotine *in vivo* increased the frequencies of sister chromatid exchange and chromosome aberrations in mouse bone marrow [34]. Thus we can assume that there must be certain changes in the expression of genes which caused the polymorphism and genetic variation in our RAPD result. Reference [26] showed that patient with AZFc deletion had reduced expression of RBMY1. To substantiate our RAPD results we performed the study of differential expression of *Rbmy1* gene in the controls and treated mice. We found downregulation of the expression of the *Rbmy1* gene which clearly shows the impact on the fertility of the mice.



The downregulation of the nicotine treated group was significant ( $p=0.45$ ) when compared with the controls and non-significant ( $p=0.782$ ) in case of alcohol.

## 5. Conclusion

From the study we can conclude that the Alcohol and Nicotine has deleterious effect on the genome of an individual. Alcohol and Nicotine also affects male infertility as is seen by the down regulation on expression of the testis specific Y chromosome gene (*Rbmy1*) and thus affects male infertility. The effects are also dose dependent which leads to contradictory results.

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