



(RESEARCH ARTICLE)



Genotoxic activity of betel nut on germinal cell in Sarcoma 180 ascites tumour bearing male mice

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Abstract

The genotoxicity of the ethanolic extract of betel nut was evaluated using sarcoma 180 tumour bearing mouse considering sperm motility, sperm viability, biochemical estimation of fructose in seminal fluid and sperm head morphology assays. Sperm head morphology was studied by H-E staining and Toluidine blue staining method. But Toluidine blue staining method is a reliable method to evaluate the DNA damage of sperms. Ethanolic BNE (betel nut extract) can suppress the percentage of sperm motility, sperm viability and seminal fructose level. In addition, it can also enhance the percentage of DNA damaged sperms. Moreover, histological sections of testes have been studied in control and BNE treated sarcoma 180 tumour bearing mice to highlight the potential toxic effect of BNE. The significant decreasing rate of seminal fructose concentration, sperm motility as well as viability and increasing rate of sperm head abnormality in different doses of treated series may be as a result of different toxic alkaloid ingredients present in BNE. Therefore, the results showed the potential of the BNE to induce different types of germ cell abnormalities in tumour bearing male mice.

Keywords: Betel nut extract; Sarcoma 180; Seminal fructose; Seminiferous tubule; Sperm abnormality; Viable cell

1. Introduction

The analysis of semen and sperm chromatin abnormalities has been studied extensively for predicting male fertility [1]. Different types of biochemical components have been found in seminal fluid [2]. Fructose – the most important carbohydrate is found among different biochemical substances that acts as a donor of energy to the spermatozoa [3,4]. Low level of seminal fructose is positively correlated with low seminal fluid volume and low sperm motility [5]. So, fructose is essential for normal growth of sperm head morphology, sperm viability and motility. It is well known that exposure to some plant products cause different physiological as well as cytological abnormalities in experimental animals [6]. Areca nut or betel nut is used to treat different types of disorders such as leprosy, cold, worm infection and leukoderma in ancient period [7]. Now a day, betel nut is very popular as it is consumed by people of India and other Asian countries. The genotoxicity and carcinogenicity of betel nut extract was reported by earlier researchers [8]. Research since last four decades has generated enough evidence to involve betel nut, as a carcinogen in human [9,10,11]. Moreover, the high incidence of oral, oesophagus, liver, stomach and pancreas cancer has been associated with the habit of betel nut chewing [11,12,13,14]. Several studies have also suggested that betel nut extract can enhance the chromosomal aberration rate of mice in vivo system [15,16]. Betel nut treated experimental male mice showed low percentage of sperm viability and motility [17]. Our earlier studies have demonstrated that betel nut significantly enhanced the sperm head abnormality with steady decrease of sperm motility, haemoglobin percentage, and total count of RBC and survival rate of normal Swiss albino mice [17]. But detailed study about the effect of betel nut extracts in sarcoma 180 tumour bearing mouse is insufficient with respect to semen and germinal cell abnormality. The present study has therefore, been oriented to evaluate the dose dependent effect of BNEs on seminal fluid and germinal cell of

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in vivo sarcoma 180 tumour bearing mouse considering fructose concentration of seminal fluid, sperm motility, sperm viability, sperm head abnormality test by H E (haematoxylin and eosin) staining and toluidine blue staining methods.

Moreover, BNE treated histomorphology of testis in Sarcoma 180 tumour bearing mice were evaluated as betel nut acts as potential deleterious toxic component and its interaction with histological parameters in such tumour model has not been thoroughly evaluated.

2. Material and methods

2.1. Collection, Identification and Extraction of betel nut

Fresh betel was purchased from local market of Kolkata, India and authenticated by the Botanical survey of India, Botanical Garden, Howrah, West Bengal, India. BNE was prepared by slight modifications of the technique as described by earlier researchers [6,15,18,19]. 100gm betel nut seeds were cut into small pieces and kept in 90% ethanol for overnight. Then ethanol-soaked betel nut pieces were placed in thimble of Soxhlet apparatus for extraction. After 48 hours of extraction, ethanolic betel nut solution was collected and filtered. Then this solution was placed in the incubator at 50°C to dry. Sticky betel nut extract was kept for future experiment.

Different concentrations of aqueous solutions of BNE (1 gm /100 ml, 2 gm /100 ml and 3 gm /100 ml) were prepared for intraperitoneal injection to tumour bearing male mice [15]. A parallel group for positive control was done using sterilized distilled water (because BNE solution was prepared in distilled water). S-180 tumour bearing mouse was used as negative control group during the experiment.

2.2. Experimental animals

Male Swiss albino mice (*Mus musculus*) were purchased from registered animal supplier in Kolkata. All the male mice (with average 20 gm weight) were kept in laboratory for acclimatization for seven days. The animals were provided dry animal pellet and water ad libitum. The animals were also maintained hygienic condition with suitable temperature in animal house for acclimatization.

2.3. Selection of animal tumour model and tumour transplantation

Murine specific S - 180 ascitic cell line was selected for the present experiments. The ascitic Sarcoma – 180 cells were inoculated (1×10^6 cells / animal) by intraperitoneal injection and maintained *in vivo*. The transplantation process was done aseptically. The average time for appearance of ascitic tumour was 6 - 7 days (log phase) as standardized by the earlier researchers [20,21,22,23].

All experiments were strictly regulated by the rules and guidelines of IAEC (Institutional Animal Ethics Committee) of Rammohan College, Kolkata, and Animal House Registration No. - 1795/PO/ERe/S/14 CPCSEA, for maintenance and care of laboratory animals.

2.4. Standardization of dose and acute toxicity study

All experiments of Male Swiss albino mice were divided into 5 groups, each contains 6 healthy mice. Three different concentrations of BNE (i.e., 100 mg/kg, 200 mg/kg and 300 mg/kg of body weight) were prepared. The different doses of BNE and sterilized distilled water (vehicle or positive control group) were administered intraperitoneally for the determination of acute toxicity of experimental mice.

3. Seminal fluid and sperm chromatin analysis

3.1. Seminal fructose analysis

Fructose concentration in seminal fluid of control, vehicle and BNE treated mice was determined by the Resorcinol method [3,24]. Diluted seminal fluid concentrations were analysed on the basis of the optical density by PC based double beam spectrophotometer (Systronics 2202) on 546 nm wavelength and seminal fructose value was measured on the basis of different fructose standards [25].

3.2. Sperm motility analysis

Sperms were separated from cauda of epididymis of different control and treated mice, diluted with 0.3 ml 0.9% normal saline and kept for 15 minutes at 37° C. Then 0.02 ml of diluted sperm containing seminal fluid was charged to Neubauer chamber of Haemocytometer for counting. The motile and immotile sperms were manually counted in each square of WBC counting chamber and total 16 squares counted by 10 x 40 magnifications under binocular research microscope [17,26,27].

3.3. Sperm viability analysis

Sperms were collected from caudal part of epididymis of control and treated mice, mixed with 0.3 ml of 0.9% normal saline and kept for 15 minutes at 37° C to allow them to become immotile. Number of living and dead sperms were analysed in haemocytometer by conventional Eosin Nigrosin staining method [26,27].

3.4. Sperm head abnormality test by Haematoxylin and Eosin stain

Sperms head morphology analysis by using Hematoxylin and Eosin staining is an important parameter to analyze the male germinal cell toxicity. Sperms were collected from caudal part of the epididymis of control and treated mice, kept in 0.9% normal saline for 30 min. The fluid containing sperms was smeared, dried and stained by Hematoxylin and Eosin. The slides were then washed and studied under binocular research microscope at 10 x 100 magnifications. Different types of sperms i. e. normal, big head, pin head, amorphous head, banana shaped etc. were observed. Total 1500 number of sperm heads of each treatment was analyzed [17, 28,29].

3.5. Sperm DNA damage analysis by toluidine blue

Toluidine blue (TB) is a basic nuclear dye and is used as a sensitive structural probe for DNA structure and packaging. This staining method is an established conventional method to analyse the DNA packaging of spermatozoa [1]. This method also indicates poor sperm integrity with severe DNA damage and morphological assessment of sperm cells. Sperms of control and treated mice were collected from cauda of epididymis and kept in 0.9% normal saline for 15 minutes. The solution containing sperms was smeared, dried and dipped in pre-chilled 95% ethanol for 5 minutes for fixation. Then fixed slides were transferred to 0.1N HCl for 5 minutes at 4°C for hydrolysis. The slides were then washed in distilled water twice and stained in 1% TB solution (pH 4) for 12 minutes. Finally, the TB-stained slides were gently washed in distilled water and observed under binocular research microscope at 10 x 100 magnifications [30].

3.6. Testicular Histology analysis

Testis of sacrificed mice in controlled and treated groups was collected and testicular tissue was fixed in Bouin' s fixative for 1 hour at 4°C. Then Bouin' s fixed small pieces of tissues were kept in the same fixative for 24 hours. The tissues were dehydrated in graded alcohols, cleared in xylene and embedded in paraffin wax. The paraffin block was sectioned into 0.5 mm thickness and stained by Haematoxylin and Eosin by the following the method of earlier researchers [31,32,33]. The stained testis tissue slides were examined under binocular research microscope in different magnifications.

Seminiferous tubules of control and treated series were analysed by software - open CASA plugin of image J [33] from the haematoxylin and eosin-stained tissue sections of testis.

4. Results

4.1. Seminal fructose

The mean seminal fructose concentration in BNE treated mice was lower in comparison with control as well as vehicle. It is interesting to note that the mean seminal fructose concentration gradually decreased with increasing concentrations of BNE treatment which were highly significant ($p^{**}>0.001$) in both 200mg BNE /kg and 300mg BNE /kg body weight treated mice but in 100mg BNE /kg body weight treated tumour bearing mice it was slightly lower than control and vehicle series. (Table: 1 and Figure 1A).

4.2. Sperm motility

The percentage of motile sperms gradually decreased with increasing concentrations of BNE which was highly significant ($p^{**}>0.001$) in both 200mg BNE /kg and 300mg BNE /kg body weight treated mice and significant ($p^{*}>0.05$) in 100mg BNE /kg body weight treated tumour bearing mice (Table:1 and Figure 1B) in comparison to control and vehicle series.

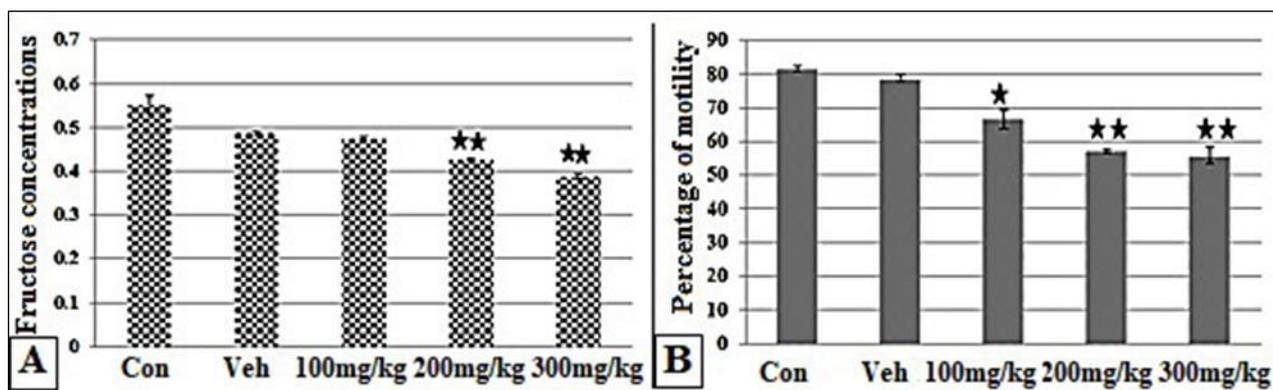


Figure 1 A. Graphical representation of seminal fructose concentration (mg/l) analysis of control, vehicle and treated series. Fructose concentration in both 200mg BNE /kg and 300mg BNE /kg body weight treated mice was lower in comparison to control, vehicle and other treated group which was highly significant ($p^{**}>0.001$). Values are expressed as mean \pm SE (n=3). **B.** Graphical representation of sperm motility analysis in control, vehicle and treated mice. Percentage of motile sperm was lower in BNE treated tumour bearing mice which was significant ($p^{*}>0.05$) in 100mg BNE /kg body weight treated mice and highly significant ($p^{**}>0.001$) in both 200mg BNE /kg and 300mg BNE /kg body weight treated mice. Values are expressed as mean \pm SE (n=3)

4.3. Sperm viability analysis

The percentage of viable sperms gradually decreased and non-viable sperms increased with increasing concentrations of BNE which were significant ($p^{*}>0.05$) in both 200mg BNE /kg and 300mg BNE /kg body weight treated mice (Table: 1 and Figure 2) in comparison to control, vehicle and 100mg BNE /kg body weight treated series.

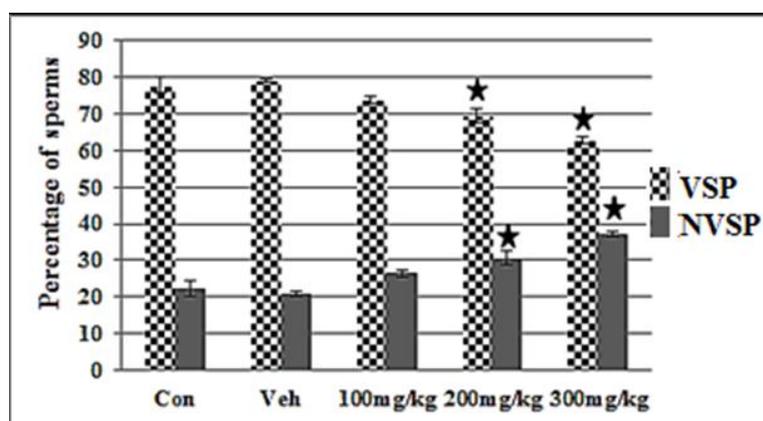


Figure 2 Graphical representation of percentage of viable and non-viable sperms in control, vehicle and BNE treated series. Viable sperms were significantly ($p^{*}>0.05$) less but non-viable sperms were significantly more in 200mg BNE /kg and 300mg BNE /kg body weight treated series. VSP=Viable Sperms, NVSP=Non-viable Sperms. Values are expressed as mean \pm SE (n=3)

Table 1 Analysis of seminal fructose concentration, sperm motility, sperm viability in control, vehicle and treated groups of tumour bearing mice

Treatment	Seminal fructose concentration (mg/l)	Sperm motility (%)	Viable sperms (%)	Non-viable sperms (%)
Control	0.551±0.022	81.67±0.87	77.78±2.25	22.22±2.25
Vehicle	0.486±0.004	78.68±0.88	79.14±0.58	20.86±0.58
100mg BNE/kg	0.475±0.005	66.66±2.74*	73.59±0.18	26.41±0.97
200mg BNE/kg	0.426±0.004**	57.1±0.52**	69.39±0.12*	30.61±1.94*
300mg BNE/kg	0.388±0.005**	55.95±2.45**	62.73±0.40*	37.27±0.81*

* = Significant ($p^{*}>0.05$) and ** = Highly significant ($p^{**}>0.001$)

4.4. Sperm head abnormality analysis by Haematoxylin and Eosin stain

Different types of sperm head abnormalities such as amorphous head, big head etc. were observed from the haematoxylin-eosin-stained slides in control, vehicle and BNE treated tumour bearing mice (Figure 3 A, B). The percentage of abnormal sperms in BNE treated tumour bearing mice was more and highly significant ($p^{**}>0.001$) in comparison to control and vehicle series (Table 2 and Figure 3C).

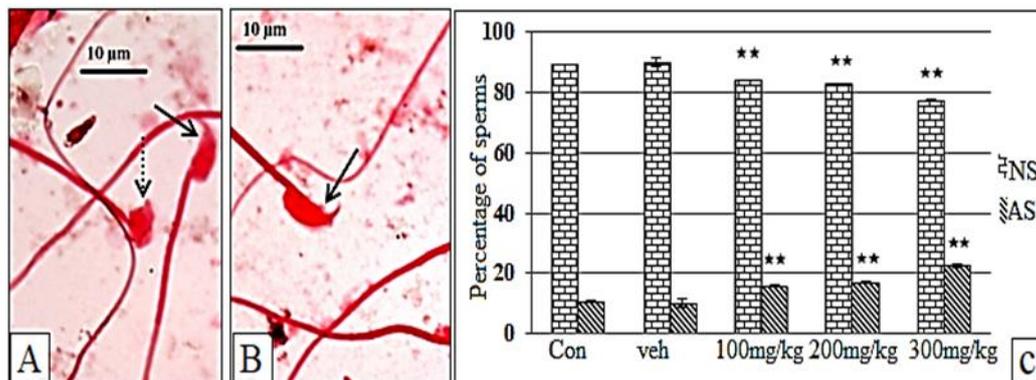


Figure 3 A: Sperm head morphology in treated mice with normal (straight arrow) and amorphous sperm (dotted arrow) 10 x 100 magnifications. **B:** Sperm head morphology with normal sperm (straight arrow) in control 10 x 100 magnifications. **C:** Graphical representation of normal and abnormal sperms in control, vehicle and treated groups. In treated groups (100mg BNE/kg, 200mg BNE/kg and 300 mg BNE/kg body weight) the percentage of abnormal sperms was significantly high ($p^{**}>0.001$) in comparison to control and vehicle series. NS=Normal sperms, AS =Abnormal sperms. Values are expressed as mean \pm SE (n=3)

4.5. Sperm DNA damage analysis by toluidine blue staining

Damaged or distorted sperm head abnormalities were also observed by toluidine blue staining methodology in control, vehicle and BNE treated tumour bearing mice (Figure 4 A, B, C). The abnormal features of sperm head were high in BNE treated tumour bearing mice which were significant ($p^{*}>0.05$) in 100 mg BNE / kg body weight treated mice and highly significant ($p^{**}>0.001$) in both 200 mg BNE /kg and 300mg BNE / kg body weight treated mice (Table 2 and Figure 4 D).

Table 2 Analysis of normal and abnormal sperm heads by H E stain and Toluidine blue stain in control, vehicle and treated groups of tumour bearing mice

Treatment	Normal Sperm head morphology by H E stain (%)	Abnormal Sperm head morphology by H E stain (%)	Normal Sperm head morphology by Toluidine blue stain (%)	Abnormal Sperm head morphology by Toluidine blue stain (%)
Control	89.13 \pm 0.15	10.87 \pm 0.15	86.97 \pm 0.81	13.03 \pm 0.81
Vehicle	90.00 \pm 1.53	10.00 \pm 1.53	85.60 \pm 0.21	14.40 \pm 0.21
100mg BNE/kg	84.07 \pm 0.18**	15.93 \pm 0.18**	81.03 \pm 1.27*	18.97 \pm 1.27*
200mg BNE /kg	82.80 \pm 0.12**	17.20 \pm 0.12**	78.67 \pm 0.47**	21.33 \pm 0.47**
300mg BNE /kg	77.40 \pm 0.40**	22.60 \pm 0.40**	74.60 \pm 0.59**	25.40 \pm 0.59**

* = Significant ($p^{*}>0.05$) and ** = Highly significant ($p^{**}>0.001$)

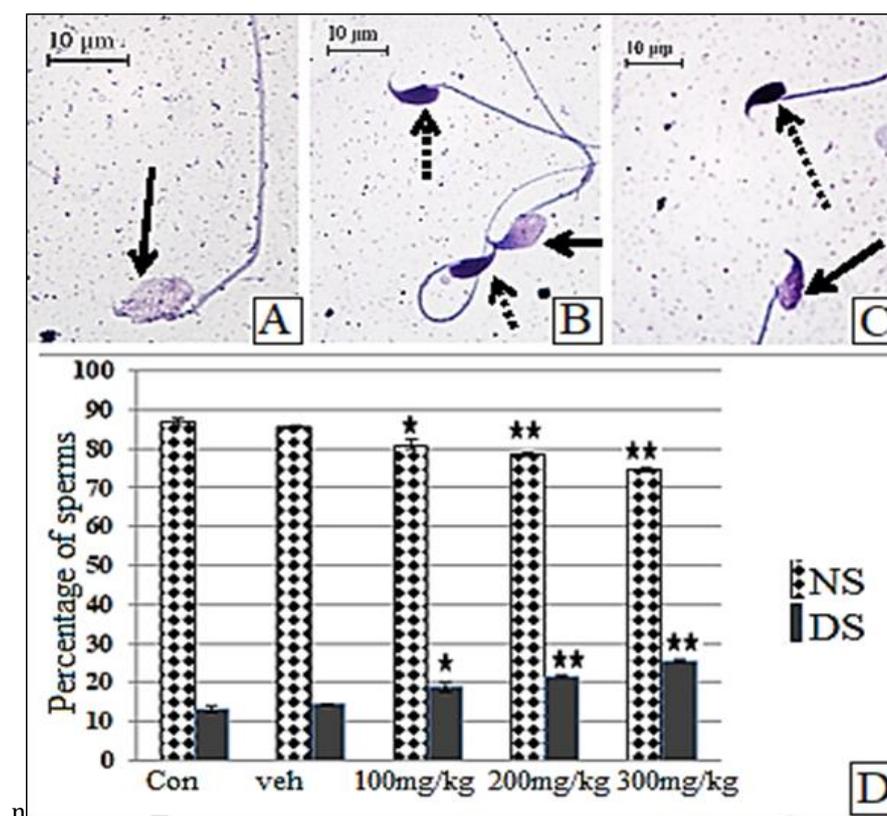


Figure 4 Toluidine blue stained sperm head morphology. **A:** Abnormal shaped or damaged sperm head (straight arrow) 10 x 100 magnifications. **B** and **C:** Normal Sperm head (dotted arrow) and abnormal sperm head (straight arrow) 10 x 100 magnifications. **D:** Graphical representation of percentage of normal and abnormal sperm in control, vehicle and treated groups. In 100 mg BNE /kg body weight treated group, the percentage of abnormal sperm was significantly high ($p^* > 0.05$) but in 200 mg BNE /kg and 300 mg BNE /kg body weight treated groups, the percentage of abnormal sperm was high that was highly significant ($p^{**} > 0.001$) in comparison to control and vehicle series. NS=Normal sperm, DS = Damaged or Abnormal sperm. Con.=Control, Veh. =Vehicle. Values are expressed as mean \pm SE (n=3)

4.6. Testicular Histology analysis

It was observed that the average number of spermatozoa of seminiferous tubules are more in control and vehicle series than in different treated groups (Table: 3). In addition, the percentages of damaged or abnormal seminiferous tubules (i.e., separation of germinal epithelium, vacuolization, presence of detached cellular material in the tubule lumen, atrophied tubules etc) were gradually increased with increasing concentrations of BNE which was highly significant ($p^{**} > 0.001$) in 300mg BNE /kg body weight treated mice (Table: 3 and Figure 5 A, B, C, D, E, F) in comparison to control, vehicle and other treated groups.

Table 3 Analysis of Haematoxylin and Eosin-stained testicular tissue of control and treated series

Types of treatment	Average concentration of spermatozoa	Percentage of abnormal seminiferous tubules
Control	41.33 \pm 0.88	12.33 \pm 0.88
Vehicle	40 \pm 0.58	12.67 \pm 0.33
100mg BNE /kg	31.33 \pm 0.88*	16 \pm 1.15
200mg BNE /kg	25.33 \pm 0.88**	20.67 \pm 0.89*
300mg BNE /kg	22 \pm 1.15**	29.33 \pm 0.34**

* = Significant ($p^* > 0.05$) and ** = Highly significant ($p^{**} > 0.001$)

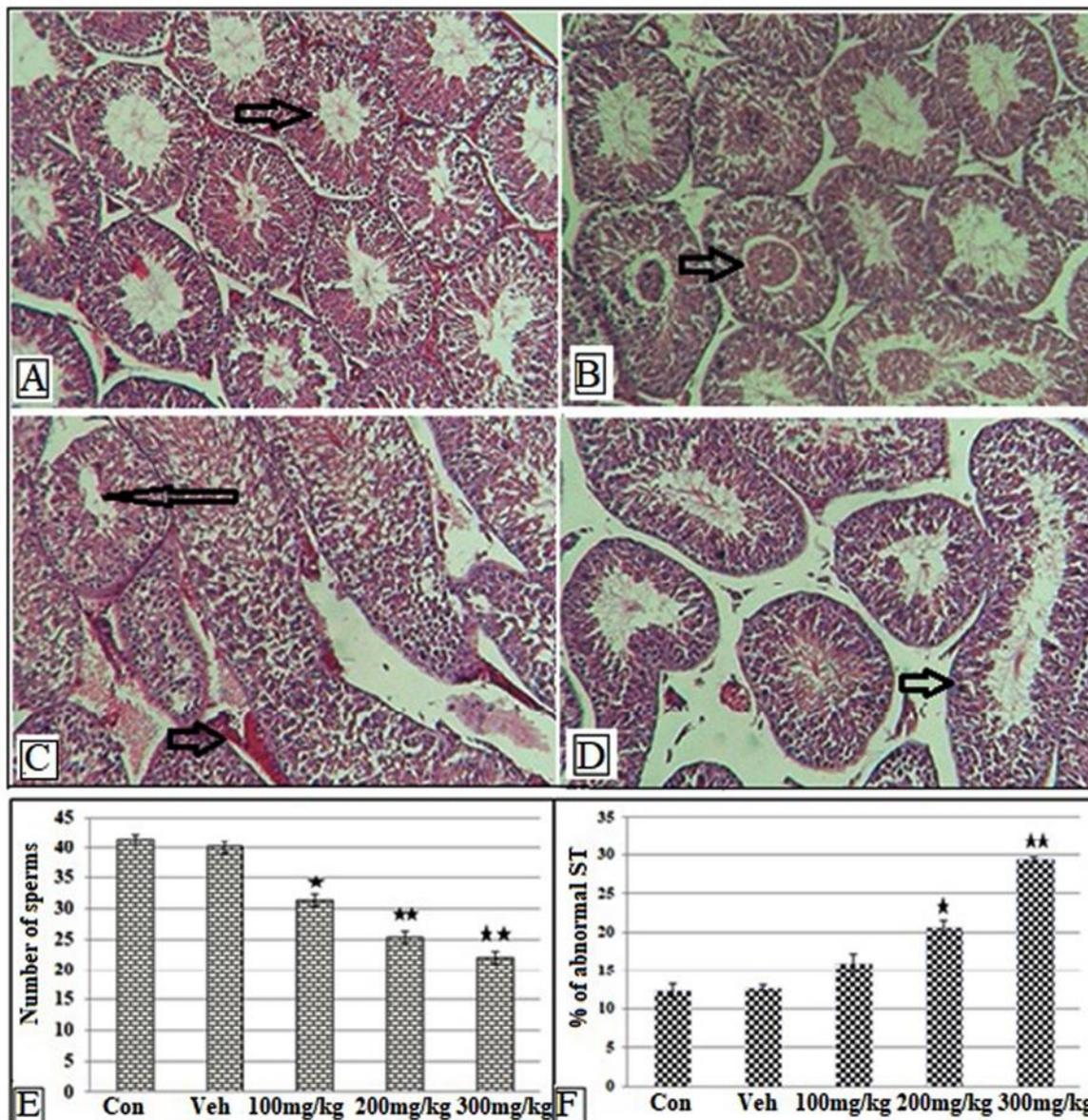


Figure 5 H & E-stained testicular tissue. **A:** Control mouse showing maximum number of normal seminiferous tubules (arrowed). **B:** Treated mouse showing detached cellular material in the tubule lumen (arrowed). **C:** Treated mouse showing abnormal seminiferous tubule (ST) with small atrophied tubule lumen (long arrow) and mild interstitial edema (small arrow). **D:** Treated mouse showing irregular shaped seminiferous tubule (arrowed). **E:** Graphical representation of average sperm concentrations of each seminiferous tubule of control and different treated groups. Sperm concentrations are low in different treated series which is significant ($p^* > 0.05$) in 100mg BNE /kg body weight treated mice but the values are highly significant ($p^{**} > 0.001$) in both 200mg BNE /kg and 300mg BNE /kg body weight treated groups. **F:** Graphical representation showing percentage of abnormal ST (seminiferous tubules) in control and different treated groups. The abnormal seminiferous tubules are maximum in treated groups which is significant ($p^* > 0.05$) in 200mg BNE /kg and highly significant ($p^{**} > 0.001$) in 300mg BNE /kg body weight treated series. Values are expressed as mean \pm SE (n=3).

5. Discussion

Different genetical, physiological, environmental and lifestyle factors are responsible for cancer initiation and progression [34]. However, exposure of some plant species and their bioactive components are also responsible for initiation and progression of cancer. Betel nut chewing not only causes different types of cancers such as oral, oesophagus, liver, pancreas but also induces cardiovascular, neurological and psychoactive disorders [35]. Our earlier studies showed that, ethanolic betel nut extract on in vivo sarcoma – 180 tumour bearing mice induced the rate of cell

proliferation with steady increase of chromosomal aberrations and hematological toxicities [15]. Stich (1991) demonstrated that presence of high phenolic components in diet can cause chromosomal aberrations as well as DNA breaks in animals [36]. According to our earlier observations [15], BNE contains large amounts of phenolic components that can induce toxic effects which support the concept of Stich [36]. Seminal fructose concentration not only helps in the assessment of seminal vesicle dysfunction but also could give a useful indication of male reproductive function [25]. The Sarcoma 180 cell line is a good murine specific cell line to study the effects of carcinogenic and toxicological potentiality of different compounds [37].

Biochemical, cytogenetical and histopathological studies of germinal cells in BNE treated sarcoma 180 tumour bearing male mice are important to evaluate reproductive cytotoxicity. So, in the present study, the relationship between sperm motility and sperm head abnormality with seminal fructose level was evaluated by cytogenetical and biochemical tests and the clinical usefulness of these tests were evaluated. It was observed that the BNE affected germinal cells of tumour bearing mice to a significant extent and cytotoxicity was manifested in the form of different types of sperm head abnormalities. In our results, the percentage of abnormal sperm head abnormality or sperm chromatin damage was significantly higher in case of Toluidine blue staining method in comparison to H E staining method. So, it is suggested that Toluidine blue staining method is a valuable, reliable parameter. It was also observed that both sperm motility and viability were equally sensitive to the cytotoxic action of BNE. Furthermore, BNE exposure induces some undesirable toxic effects in respect to quality of seminal fructose concentration and abnormal, damaged seminiferous tubules in tumour bearing mice.

6. Conclusion

Present study focused to analyse the biochemical, cytogenetical and histopathological abnormalities considering the male germinal cell parameters of betel nut extract treated Sarcoma 180 ascitic tumour bearing mouse. On the basis of above results and discussion the present authors may conclude that higher doses such as 200 mg BNE / kg and 300mg BNE /kg body weight treatment cause more adverse effects than 100mg BNE /kg body weight and control groups on seminal fructose concentrations, sperm motility, viability, sperm head morphology, sperm DNA organization and seminiferous tubule morphology.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Statement of ethical approval

This study was approved by the Institutional Animal Ethics Committee of Rammohan College, Kolkata (Animal House Registration No. - 1795/PO/ERe/S/2014 CPCSEA dated 20 January 2016).

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