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CYTOTOXIC EFFECT OF BETEL NUT ON SEMINAL FLUID FRUCTOSE CONCENTRATION AND SPERM MOTILITY OF NORMAL MALE MICE

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ABSTRACT: Different types of substances (i.e. citric acid, fructose, glucose, potassium, zinc etc) have been found in seminal fluid. These substances are important for viability and motility of sperm. Fructose, the major carbohydrate, is essential for sperm head morphology, viability and motility, which serves as an energy source for spermatozoa. Till now, little work has been done about the impact of seminal fructose concentration on sperm head morphology or sperm characteristics. Moreover, knowledge about the effect of betel nut extracts on seminal fructose concentration and sperm head abnormality in mammals is insufficient and inconsistent. Therefore, the present study was aimed to investigate the dose dependent effect of betel nut extracts (BNEs) on seminal fluid of *in vivo* Swiss male albino mouse model considering sperm head abnormality test, sperm motility, fructose concentration of seminal fluid as parameters. Additionally, haematological parameters i.e. percentage of haemoglobin, total number of RBC and WBC, differential counts and the life span of both control and BNE treated mice were determined to evaluate the effect of toxicity. The present findings indicate that BNE being a potent, cytotoxic plant extract induces a variety of deleterious effects on the seminal fluid fructose level, sperm head abnormality and sperm motility of the mice. Moreover, analysis of haematological parameters showed a toxic effect in BNE treated groups. It is interesting to note that the different concentrations of BNE significantly reduced the mean survival time in mice in compare to control and vehicle groups.

KEYWORDS: Betel nut extract, seminal fructose concentration, sperm motility, big head sperm, normal sperm head.

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1.INTRODUCTION

Biochemical and cytogenetical analysis of seminal fluid are useful in establishing clinical diagnosis of male reproductive tract disorders [1]. Different substances i.e. citric acid, fructose, glucose, flavins, potassium, zinc etc. have been found in the seminal fluid. These substances provide nutritional support, viability and motility to the spermatozoa. Fructose is produced by the seminal vesicles and is the major carbohydrate found in seminal fluid. It is essential for sperm viability and motility which serves as an energy source for spermatozoa [2]. Fructose is therefore, a suitable marker for the secretary function of the accessory reproductive gland. Sometimes, the seminal fructose can be used for the diagnosis of different reproductive duct related diseases [1]. Number of normal sperm cells is also important for successful fertilization. So, the development of sperm head abnormality has been used as a reliable short-term biological indicator for the evaluation of chemical cytotoxicity or genotoxicity [3], [4]. It is known that exposure to different environmental agents cause major pathological symptoms in the experimental animals and also in human male reproductive system [5], [6]. Research works have been done about the impact of seminal fructose concentration on sperm head morphology or sperm characteristics. Betel nut or areca nut is widely chewed in India and South East Asia. It is a well-known fact that an excessive and continued consumption of betel nut led the people of India to suffer from the disease – oral cancer. Betel nut extract and its arecoline content have been reported to be carcinogenic in some short term assays and animal experiments [7], [8]. Areca nut extract has been reported to increase the frequency of chromosomal aberrations (CAs) in bone marrow cells of mice *in vivo* [7], [9]. Ghosh and Ghosh [10] also reported that, the extent of sister chromatid exchange (SCE) increase induced by betel chewing, was found to be much more pronounced in pregnant women and women using oral contraceptives, compared with normal women. Saha et al 1995 [11] studied the clastogenic efficacy of two different varieties of betel nut from two different North-Eastern states of India – the raw and fresh betel nuts of Tripura and the processed and widely used ‘Tambul’ of Assam. But, little work has been done about the effect of betel nut extracts on seminal fructose concentration, sperm head abnormality and sperm motility in mammals. It is also known that some plant extracts cause significantly decreased sperm motility and decreased activity of antioxidant enzymes which may cause infertility [12]. Similarly haematological parameters such as percentage of haemoglobin, total RBC and WBC counts and differential count are also useful to evaluate the effect of toxicity of the treatment [13]. Therefore, the present study was aimed to investigate the dose dependent effect of betel nut extracts (BNEs) *in vivo* on Swiss male albino mouse model considering sperm head abnormality, sperm motility, fructose concentration of seminal fluid, percentage of haemoglobin, total count of RBC and WBC, differential counts. Additionally, the mouse survival or the life span of both control and BNE treated mice was determined.

2. MATERIALS AND METHODS

2.1 Test Animals (mice)

Swiss albino mice (*Mus musculus*) of 9- 10 weeks old and weighing about 20 gm. were selected and maintained in the animal house at room temperature. The animals were provided with standard food and purified water. The experiment was performed in accordance with the guidelines formulated by the Institutional Animal Ethics Committee of Rammohan College, Kolkata (Registration Number with date: 1795/PO/ERe/S/14 CPCSEA- 31/12/2014) for the care and use of laboratory animals.

2.2 Preparation of betel nut extracts (BNE)

Betel nut extract (BNE) was prepared by slight modifications of the technique originally described by Chakrabarti et al [14], Bhide et al [15] and Chowdhury et al [16]. Betel nuts were cut into small pieces and kept in 70% alcohol overnight. Then the betel nut sample was placed in the thimble of soxlet and the sample was extracted. After 48 hours extraction, ethanolic betel nut solution was collected and filtered. Then the sample was kept in the incubator (at 50^0C) for two days. A sticky betel nut extract was collected and kept in air tight plastic vial for future use. Sticky BNE was dissolved in sterilized distilled water to prepare different concentration of BN solutions (1 gm. /100ml, 2 gm. /100ml and 3 gm. /100ml) for injection to normal healthy mice.

2.3 Treatment

Thirty (30) male mice of same age group were taken for the present experiment. Six (6) specimens were taken for each set of treatment. In the present experiment, normal mice were intraperitoneally (i.p.) injected with BNE at concentration of 100mg/kg, 200mg/kg and 300mg/kg of body weight respectively for five consecutive days. Normal mice group without any doses was treated as negative control. A parallel positive control group was made to analyse the effect of the solvent (distilled water) as betel nut extract was dissolved in distilled water, considered as vehicle group (Table: 1).

Table 1: Treatment schedule (BNE treatment continued for 5 consecutive days)

Treatment	Route	Dose	Average mice weight
Negative control	-	-	20gm
Positive control/vehicle (distilled water as BNE is dissolved in it)	IP	1ml/100gm body weight	20gm
BNE low dose (100mg/Kg body weight)	IP	1ml/100gm body weight	20gm
BNE medium dose (200mg/Kg body weight)	IP	1ml/100gm body weight	20gm
BNE high dose (300mg/Kg body weight)	IP	1ml/100gm body weight	20gm

2.4 Sperm head abnormality Test

Sperm head morphology was studied in experimental series to observe germ cell toxicity. Sperm cells of control and BNE treated mice were collected from the caudal part of epididymis and minced, kept in 0.5ml normal saline for 30 minutes. One drop of solution (containing sperm) was taken on a clean slide to draw the smear. Then the smear was dried and stained with haematoxylin and Eosin. Sperm cells were observed by binocular research microscope at 10x100 magnifications. Different types of sperms were observed such as normal, big head, pin or small head, amorphous, banana shaped sperms etc. [17], [18], [19] and [20].

2.5 Sperm motility

Sperms were isolated from caudal part of epididymis of mice and kept in 0.3ml normal saline for 15 minutes at 37ºc. Then 0.02ml diluted seminal fluid, containing sperm, was transferred to Neubauer chamber of Haemocytometer for counting. The motile and immotile spermatozoa were observed and studied from 16 cells of WBC counting chamber at 10x40 magnifications in binocular research microscope [19], [21].

2.6 Quantitative Resorcinol method for estimation of Fructose

Fructose concentration in seminal fluid was determined by the resorcinol method. After single dose of treatment for five consecutive days all mice were sacrificed and seminal fluid was collected to determine the fructose level by resorcinol method [22], [23].

2.7 Haematological Parameters

2.7.1 Estimation of haemoglobin: 0.5 ml blood was collected from the heart of the control and treated mice and mixed with EDTA. Then the percentage of Haemoglobin was estimated by following Sahli's method [24].

2.7.2 Total RBC and WBC count: Total RBC and WBC count of peripheral blood of control, vehicle and treated mice were performed by following the method of Sood [24] and Math [25].

2.7.3 Differential count: Differential count of WBC (i.e. neutrophil, lymphocyte etc.) was studied from control and treated series after slight modification of the original technique of Sood [24]. Percentage of different leukocytes was evaluated from 100 cells in each series of treatment.

2.8 Mouse survivability: Mouse survival or the life span of tumour bearing mouse in both non-treated or control and BNE treated groups was studied according to the specific protocol [17], [26].

% ILS = [(Mean survival time of treated group/Mean survival time of control group)-1] X 100

2.9 Statistical analysis: All data were expressed as a mean ± SE (n=3), were statistically analysed by Student's *t* test [27]. Significance was indicated by an asterisk.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Sperm head abnormality

Different types of sperm head abnormalities (i.e. pin head, big head, banana shaped head, amorphous head etc.) were noted after the treatment of different concentrations of betel nut extracts (100mg/kg, 200mg/kg and 300mg/kg).A steady increase of sperm head abnormalities was noted after the administration of different concentrations of BNE (Fig.1).The frequency of abnormal sperm induced by BNE was highly significant(**p>0.001) at the higher concentration (300mg/kg body wt.) when compared with the control series.

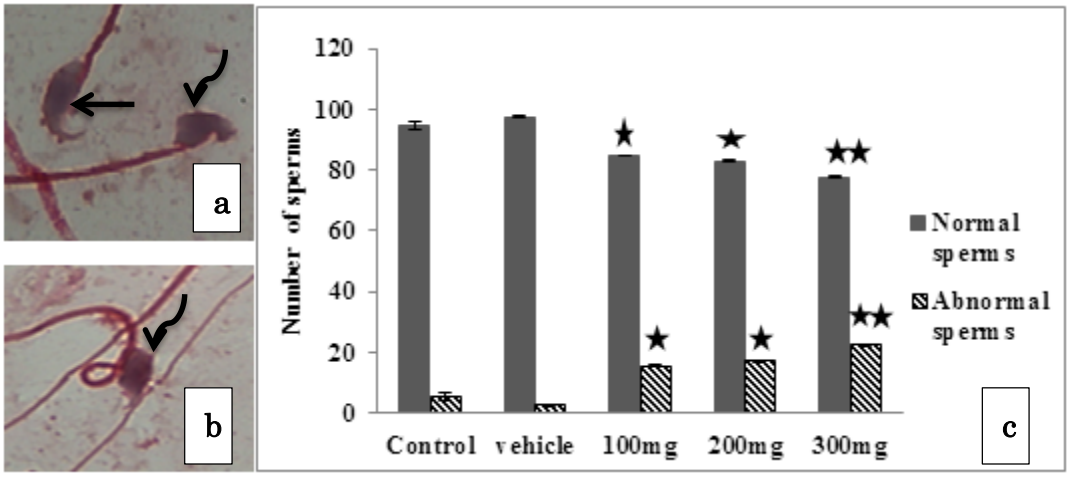

Figure 1: Sperm head morphology analysis of control and BNE treated mouse. Normal head sperms were less whereas affected sperms were more in BNE treated series in comparison to control and vehicle groups. a: Normal head sperm (straight arrowed); b: Amorphous head sperm (curved arrowed) c: Graphical representation of normal and abnormal head sperm in control, vehicle and other treated series. Values are expressed as mean ±SE (n=3).

3.1.2 Sperm motility

The mean sperm motility of control and vehicle mice groups showed less number of immotile spermatozoa than treated series. Number of immotile spermatozoa was increased significantly (**p>0.001) with gradual increasing concentration of BNE treatment (Fig.2). Interestingly, higher concentration of BNE (300mg/kg body wt.) showed maximum percentage of immotile spermatozoa (21.57±0.87) in comparison to control, vehicle and other treated groups.

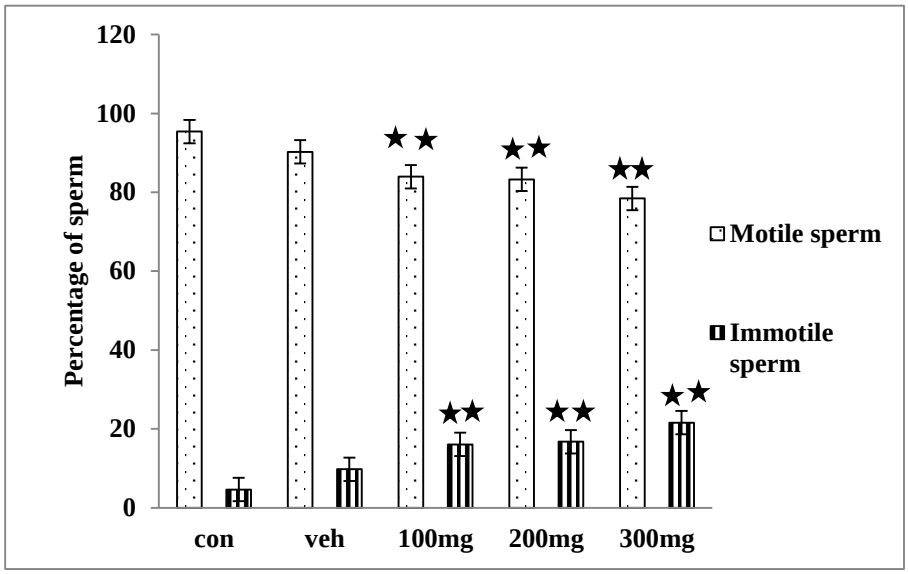

Figure 2: Graphical representation of motile and immotile spermatozoa in control, vehicle and BNE treated series. Values are expressed as mean ±SE (n=3).

3.1.3 Estimation of Fructose

The mean fructose concentration in control as well as vehicle was higher in comparison with different treatment series. It is interesting to note that the mean fructose concentration in 300mg/kg body wt. BNE treated mice was significantly lower (*P > 0.05) compared to control and vehicle series. But the mean fructose concentration in 100mg/kg body wt. and 200mg/kg BNE treated series was slightly lower than control series (Fig.3).

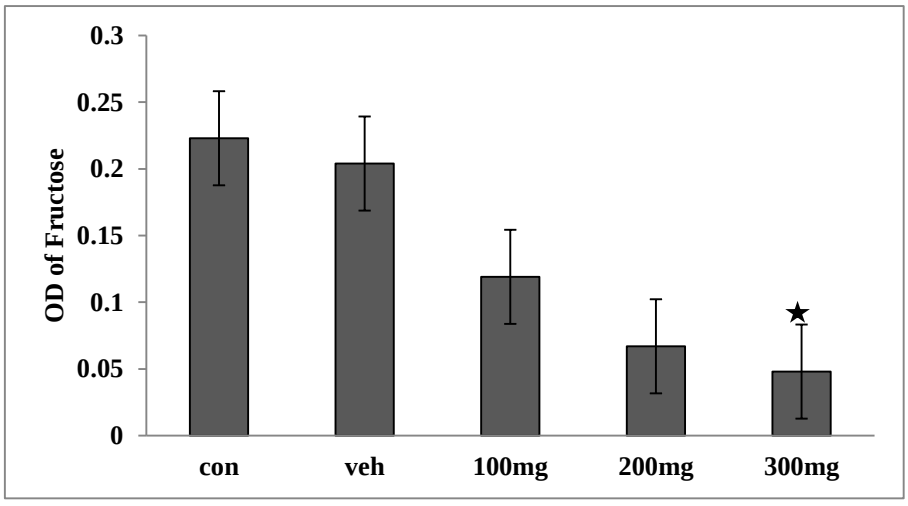

Figure 3: Graphical representation of fructose concentration (mg/l) in control, vehicle and BNE treated series. Values are expressed as mean ±SE (n=3).

3.1.4 Effect of BNE on Mouse survival

Treatment with100mg/kg, 200mg/kg and 300mg/kg body wt. BNE showed considerable decrease of life span of mouse when compared with control group. The survival time was 78± 0.577 (days, mean ± standard error, n=3) in the control group (Fig. 4). Life span of BNE treated series was gradually

decreased with higher dose concentration. In all treated series (i.e. 100mg, 200mg, 300mg/kg BNE) it was highly significant (**p>0.001).

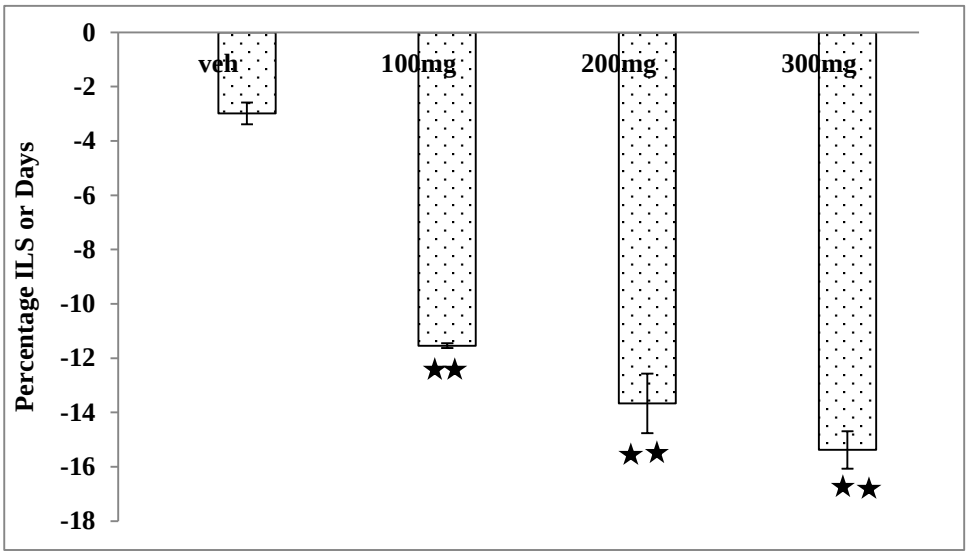

Figure 4: Graphical representation of percentage increase in life span (%ILS) of vehicle (veh) and different BNE treated series. Values are expressed as mean ±SE (n=3).

3.1.5 Haematological parameters

3.1.5.1 Haemoglobin percentage

The haemoglobin content in control (18.13±0.82) and vehicle (18.20±0.35) group was restored, whereas, it was significantly low in different treated series as it was shown in Fig 5. Interestingly, in higher concentration of BNE, the haemoglobin was found to be reduced to 11.80±0.46 as compared to control group.

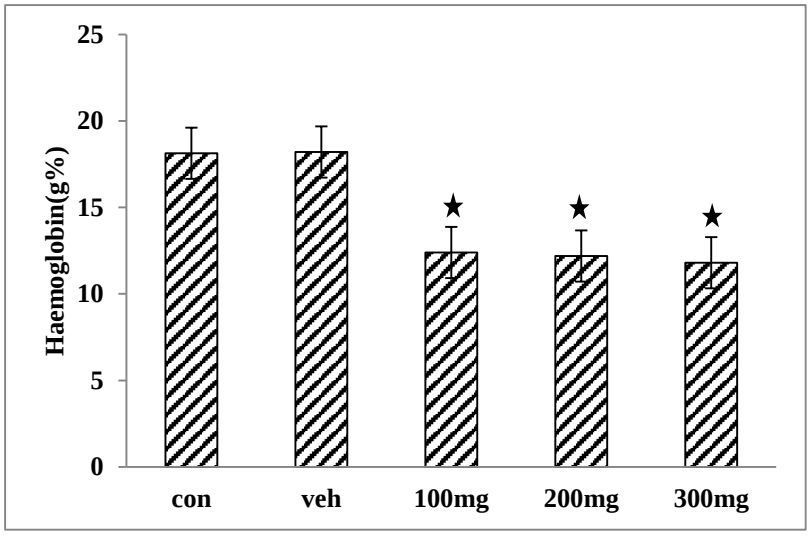

Figure 5: The histographical representation of haemoglobin content in control (con), vehicle(veh) and BNE treated groups revealed that percentage of haemoglobin was significantly (*p>0.05) decreased in 100mg, 200mg, 300mg BNE treatment group. Values are expressed as mean ±SE (n=3).

3.1.5.2 Total RBC count

Control and vehicle groups showed maximum number of RBC in comparison to treatment groups. Average number of RBC count was gradually decreased with increasing concentrations of BNE (Fig.6).

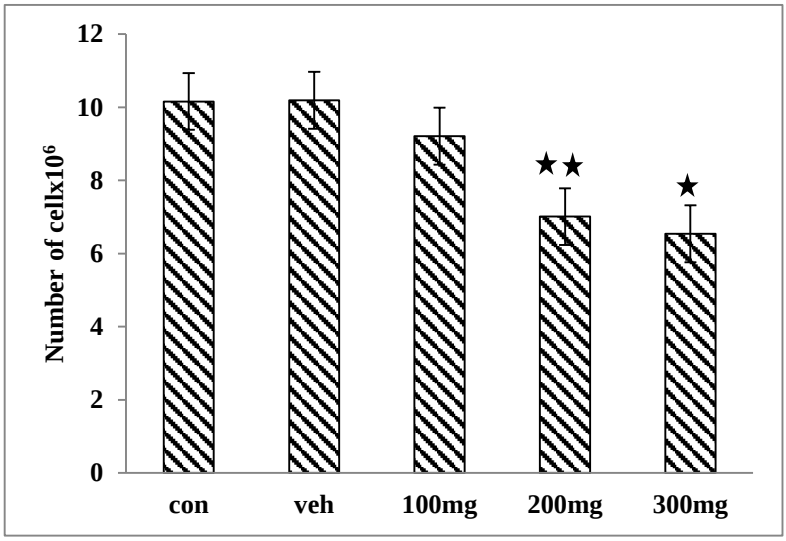

Figure 6: Histographical representation of total count of RBC in control (con), vehicle (veh) and different BNE treated series. Total count of RBC was lower in BNE treated series which was highly significant (**p>0.001) in 200 mg and significant (*p>0.05) in 300 mg BNE / kg body wt. dose. Values are expressed as mean ±SE (n=3).

3.1.5.3 Total WBC count

The Total number of WBC count of treated mice showed results that was opposite of RBC count (Fig.7). Average number of WBC count was gradually increased with increasing concentrations of BNE. Total count of WBC was higher (*p>0.05) in 100 mg and 300 mg BNE / kg body wt. dose.

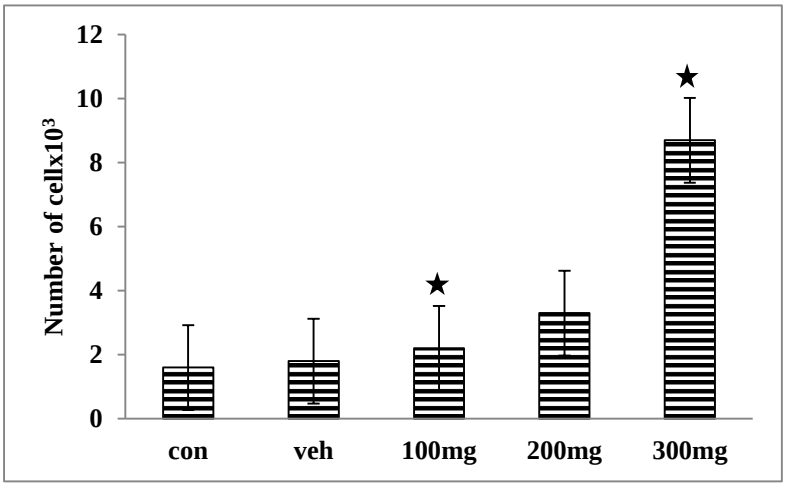

Figure 7: Histographical representation of total count of WBC in control (con), vehicle (veh) and different BNE treated series. Values are expressed as mean ±SE (n=3).

3.1.5.4 Differential count

Differential count of WBC was noticed by a shift from the frequency of neutrophils to the frequency of lymphocytes in control series. In both negative and positive control (vehicle), differential count of leukocytes revealed a less number of neutrophil and large numbers of lymphocytes in comparison to different BNE treated series (Fig. 8). Interestingly, it should be mentioned that in 300mg/kg BNE treated series the lymphocyte population was decreased (28±1.53) and the neutrophil population was increased (72±1.53) in comparison to control and vehicle which were highly significant (**p>0.001).

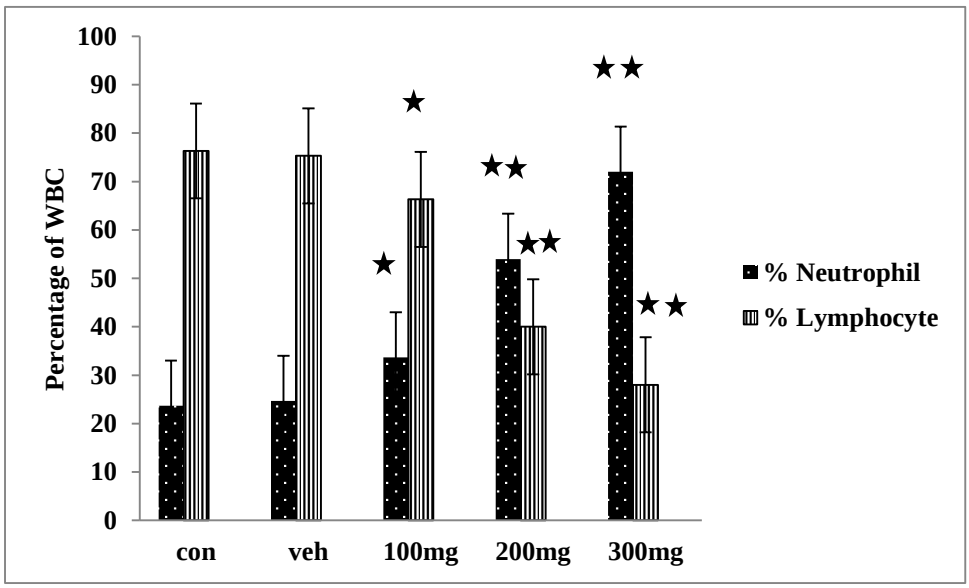

Figure 8: Histographical representation of differential count of Neutrophil and Lymphocyte in control (con), vehicle (veh) and different BNE treated series. Values are expressed as mean ±SE (n=3).

3.2 DISCUSSION

Fructose concentration in seminal fluid is one of the important suitable markers of seminal vesicular function [28]. It is also an energy source for spermatozoa when these are staying in semen [2]. In the present study, in the BNE treated mice the sperm head abnormality and immotile spermatozoa were significantly increased while the fructose concentration was decreased in comparison to control and vehicle groups. A significant relationship was observed between increased sperm head abnormalities and immotile spermatozoa with reduced fructose concentration. So fructose levels in the seminal fluid may be an appropriate indicator of male reproductive function. Haematological parameters of BNE treated mice were found to be significantly altered as compared to normal (negative control) and vehicle group. In the BNE treated series, the total WBC count was increased with a reduction in the RBC count and haemoglobin percentage. Moreover, differential count of leukocytes from peripheral blood revealed a dramatic depletion in lymphocyte count and elevation of neutrophil population in different BNE treated series. Reduction in the RBC count and hemoglobin percentage with increased number of WBC count indicated a sign of toxicity in the BNE treated mice. These haematological abnormalities indicated a toxic effect that resulted in cell death

as well as reduction of life span of treated mice. The survival data revealed that 300mg/kg BNE dose has exerted toxic effect as most of the mice were expired within 3rdor 4thday of the treatment. But the survival time was also affected in case of 100mg/kg and 200mg/kg BNE treated groups where as it was prolonged in control group. The occurrence of good number of different forms of sperm head abnormalities and reduced fructose level in seminal fluid in different concentration of BNE treatment in a dose dependent manner points out that the components present in the BNE are somehow responsible for the alteration of physiological and genetic mechanisms which in turn affects the development of sperm as well as fructose concentration. Male reproductive system is much sensitive to exposure of genotoxic and carcinogenic components like betel nut extract. Seminal fructose plays an important role for nourishment of spermatozoa. Number of normal sperm cells is also important for successful fertilization. So seminal fluid fructose level, sperm head abnormality and sperm motility tests are important parameters for the study of reproductive abnormality.

4. CONCLUSION

The present findings indicate that BNE being a potent, genotoxic plant extract induces a variety of deleterious effects on the seminal fluid fructose level, sperm head abnormality and sperm motility of the mice. Moreover, analysis of haematological parameters showed a toxic effect in BNE treated groups. It was noted that different concentrations of BNE significantly reduced the mean survival time in mice. Additionally, seminal fluid fructose level, sperm head abnormality and sperm motility tests are important, reliable short-term biological indicators for the analysis of reproductive abnormality.

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CONFLICT OF INTEREST

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

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