Indian Journal of Biological Sciences, 25 : 5-15, 2019 : ISSN No. 0972-8503

DETERMINATION OF LD_{50} AND ED_{50} VALUE OF NICOTINE DOSES IN RELATION TO BODY AND ORGANS WEIGHT ALONG WITH HAEMATOLOGICAL PARAMETER: A DAY-DEPENDENT EXPERIMENTAL STUDY ON MALE ALBINO RATS.

[1]Monalisa Paul, [1]Chitali Kundu, [1]Merina Yasmin,[2]Dr. Suman Kar and [1]Sandip Kumar Sinha*

[1]Department of Human Physiology, Vidyasagar University, Midnapore: 721102, West Bengal, India, Pin-721102

[2]Department of Conservative Dentistry and Endodontics, Awadh Dental College and Hospital, Jamshedpur, Jharkhand, India

ABSTRACT ■ As we know that nicotine toxicity is accountable for several damages at cellular level. Cellular damage directly or indirectly causes unhealthy impact on body and organ weight. Still now a little research work has been conducted regarding the nicotine induced changes in body and organ weight (Heart, Liver etc)along with haematological (RBC, WBC, Platelets and hemoglobin) parameters in male albino rat. In this study we tried to find out the effects of $LD_{50}$and ED_{50} of nicotine on above parameters. We appliedLD$_{50}$ and ED$_{50}$ doses of nicotine on day-dependent study (10, 15 and 20 days) and observed its effect on rat model. In this experimental study the doses of LD_{50} and ED_{50} of nicotine were 51mg/kg body weight and 3.5mg/kg body weight respectively. After 10 days of treatment, no significant changes were observed. But during 15 days of nicotine treatment, it produced toxic effects on the studied parameters. But all those parameters were more aggravated during 20 days of nicotine application. All the results were highly significant at $P<0.05$ level in compared to that of control group. In conclusion, it is suggested that the effective dose of nicotine of 3.5mg/kg body weight caused toxic effects in this day-dependent study.

Key words: *Nicotine, LD50, ED50, Hb, Blood cells, Body weight, Hemoglobin*

1. INTRODUCTION

Tobacco consumption is one of the major risk factors for hepatic and heart diseases. Nicotine is a risky addictive alkaloid of tobacco products. Acetylcholine, dopamine, noradrenaline, serotonin, GABA, glutamate and opioid peptides like neurotransmitters are secreted in the brain due to nicotine action. Basically, nicotine acts through the cholinergic receptors.A toxic substance necessarily worried for its harmful effects which are recognized by clinical analysis. Thedosage-response and day extended study relationship is the most fundamental single principle in toxicological study. For the determination ofacute toxicity ofa chemical, LD_{50}andED_{50} values are very important for common practice of toxicity study in experimental animals.In a rat model, Benwell and his group (1995) applied three dosages like 0.25, 1 and 4 mg/kg body weight per day of nicotine to determine the effective dose that commonly increased plasma nicotine level up to 25 mg /ml and enhanced

* **Corresponding author** : e-mail: sandipkrsinhavu@gmail.com

mesolimbic dopamine responses. Dey et al. (2016) suggested that after application of nicotine (2.0mg/kg body weight) for 28 days, hepatic and renal toxicity level significantly increased than that of the control level. But earlier Mondal and his group(2004) studied nicotine toxicity on female albino rats with normal and protein restricted diet and established LD_{50}(93 mg/kg body weight) andED_{50} (3.5 mg/kg body weight)regarding the changes of body and organ weight (Ovary and Uterus). Similarly, Chattopadhyay and Chattopadhyay (2008) in their animal experiment revealed that after nicotine (3.5 mg/kg body weight) treatment on adult female albino rats (Wister strain) for 15 days with special protein restricted diet supplements, the ovary and plasma showed toxicity and lipid profile level became changed. There is a lack of investigation regarding the nicotine toxicity related to ED_{50} value and day dependent study on male albino rat's especially on body and organ weight along with hematological parameters. With this background in this investigation we tried to observe the toxicological effects of nicotine on male albino rats. Therefore, it is very much necessary to assess the dose dependent effects of nicotine in relation to mortality rate of the animals for the determination of actual effective concentration of nicotine. This is applicable as an acute or chronic dose suitable for the animal studies. The dosages were determined as follows: the minimal dosage will produce minimal intended effect and the largest dose will produce the maximal effect in the majority of the animals. At the same time, we applied some effective dosage on animals for day-dependent studies and observed the changes in body and organ weights as well as in hematological parameters.

2. METHODS AND MATERIAL

2.1Animal Care, grouping and mode of treatment:

Experiments were carried on with male Albino rats (100-120 gm body weight) which were obtained from a standard animal supplier (Regn. No. 1828 / PO / BT / S / 15 / CPCSEA)and those were kept in CPCSEA approved animal house of the institution (Registration No. 2013/ GO/Re/S/18/CPCSEA/ 2018). An ethical clearance was taken from the animal ethical committee of the Institution (Ref. no. VU/ IAEC/2/4,Vidyasagar University) before conducting the study. The animals were housed in a clean polypropylene cage in a standard temperature (20±2ºC) with relative humidity (45–60%) under 12-h light and dark cycles during the whole study period. Then animals were acclimatized in the animal house for 15 days before starting the experiment. Then animals were divided into 5 groups on the basis of nicotine dose of 20,40,60,80 and 100 mg/kg body weight respectively and each group had 10 animals. For the nicotine treatment, we used nicotine tartrate (Sigma Aldrichmade). The applied nicotine doses were started from higher to lower concentrations and the percentage of motility rates were noted after 24 hours. The motility rates percentage (Probit value) were imputing in a graph against logarithm of the dosage and the LD_{50} (Lethal Dose-50) was determined from this graph (Finney 1952). The dose which makes a quantal consequence in 50% of the population was called effective dose (ED_{50}), that referring to the 50% population base. Generally, ED_{50} is used for the checking of drug toxicity level during animal experiments. When ED_{50} would be significantly less than LD_{50} for a drug then it would be therapeutically relevant (Finney, 1952 and Gaddum ,1933).

For determining an effective dose (ED) the

animals were divided in to nine (9) groups on the basis of applied doses of nicotine of 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 mg/kg body weight respectively for a study of 15 days. Each groupcontained5 animals. Effective dose was determined from graphical presentation and the effective dose was calculated on the basis of serum phosphates activity (Bowers et al., 1966). The Phosphates activity is a very important biomarker for cardiac and hepatic disorders and it has clinical significance in intermediary metabolism. After that we applied the ED_{50} of nicotine for day duration study. In this phase of study the animal were divided into three groups for day-dependent study with durations of 10, 15 and 20days respectively. In each group contained 10 animals and these three groups were again divided into two subgroups (Control and Nicotine treated group) with five animals in each subgroup. Throughout the experiments the animals were provided normal standard balanced diet with water ad-libitum. For all cases, before nicotine treatment, the body weight of each rat was measured regularly. After the end of the treatment, the rats were kept overnight with fasting condition and in the next day the animal were sacrificed in the morning between 9 am to 11 am by cervical dislocation. Then heart and liver tissues were collected after the removal of tissue fluid and blood. Tissue weights are recorded and expressed as relative weight (mg/gm body weight).

2.2Protein Estimation and Alkaline phosphatase activity (ALP): This experiment was conducted for the determination of ED_{50}.In ALP enzyme study, 50µl tissue homogenate was added to 0.5ml of alkali buffer substrate (0.05M glycine buffer containing 5.5mM PNPP, pH10.5) and incubated for 30 minutes at 37^0C. Then 5 ml of NaOH (0.02N NaOH) was added to the mixture and then OD was recorded at 400nm, after 5 minutes. ALP activity was determined as ìmol/mg protein (Bowers et al., 1966).The protein was estimated using BSA (bovine serum albumin) as standard according to Lowry et al., 1951.

2.4Hematological study:

Total count of RBC and WBC: During hematological studies, the total count of RBC and WBC was determined by a hemocytometer and the blood samples were diluted to 1:200 and 1:20 with RBC and WBC diluting fluid respectively and cells were counted under high power (40X) objective by using a Neubauer's counting chamber. The numbers of cells were calculated as the number of cells / cu.mm of whole blood (Gottfried. 1979).

- **Platelet count:** The platelet diluting fluid was used for counting the platelets. This fluid contains 0.2 ml of neutral formaldehyde and 0.1 gm of brilliant cresyl blue in 100 ml of deionized water. The RBC counting chamber of hemocytometer was used for total platelets counting. Then anticoagulant mixed blood samples were diluted at the ratio of1:200 and the platelet count was determined by the number of cells / cu.mm of whole blood (Rees and Ecker, 1923).
- **Hemoglobin estimation:** For hemoglobin estimation, Cyanmethemoglobin method was used with Drabkin's solution according to Dacie and Lewis (1968).

2.5 Statistical analysis:

The percentage of mortality rate was converted to probit value and a graph was plotted against logarithm of the applied dosage of nicotine (Finney, 1952 and Gaddum, 1933). All the data were expressed

as Mean ± Standard Error of Mean (SEM) and One-Way ANOVA was used for statistical analysis of data by Origin 6.0 professional software.

3. RESULT:

3.1 LD_{50}, ED_{50} values of nicotine:

We used the logarithmic conversion of doses for LD_{50} and ED_{50} calculation (Gaddum.1933). The toxic effects of nicotine on living organs required for the measurement of therapeutic index. The LD_{50} and ED_{50} values were used for this index. Both $LD_{50}$and ED_{50}values indicated the lethal doses and effective dose of the drug respectively. In this study, LD_{50} dose was 51mg/kg body weight (Figure-1 and Table-1) and ED_{50} dose was 3.5mg/kg body weight as shown in Figure-2 and Table-2.

3.2 Effect of nicotine on total body weight and organ weight (liver and heart) of rats on day duration study:

In this study we represented the growth rate of control and nicotine treated groups in Figures 3 (a, b, and c). There was no such significant alteration of body weight after 10 days of nicotine treatment (123.02±1.8gm) compared to control group (124.44±1.7gm) as shown in Figure 3a.The cellular growth rate of the nicotine treated animals were gradually slowed down during 15 and 20 days of day duration study compared to that of control animals. It was noted that up to 3

Table1.Determination the LD_{50} value after application of nicotine tartrate subcutaneously on the basis of death rate of male rat.

Dose (mg/kg bw)	log dose	% of Death	Number of death cases	Probit value
20	1.3	0	0	3.04
40	1.6	20	2	4.2
60	1.8	50	5	5
80	1.9	70	7	5.52
100	2	100	10	6.96

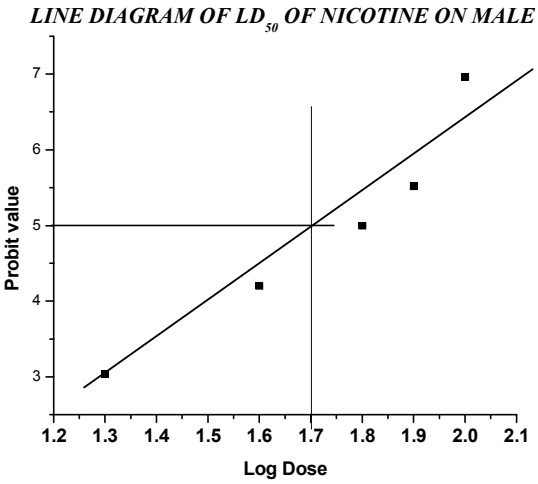

Fig. 1: Probit values against logarithm of doses of nicotine tartrate applied subcutaneously on male rat for determination of LD_{50}

Table 2: Determination the ED_{50} value after application of nicotine tartrate subcutaneously on the basis of serum phosphatase (ALP) activity study in male rat

Dose (mg/kg body weight)	Percentage of ALP	log dose	Probit value
1	43.92	0	4.82
1.5	44.73	0.17	4.85
2	46.16	0.3	4.87
2.5	47.56	0.39	4.92
3	49.11	0.48	4.97
3.5	50.06	0.54	5
4	51.68	0.6	5.03
4.5	55	0.65	5.08
5	55.1	0.69	5.08

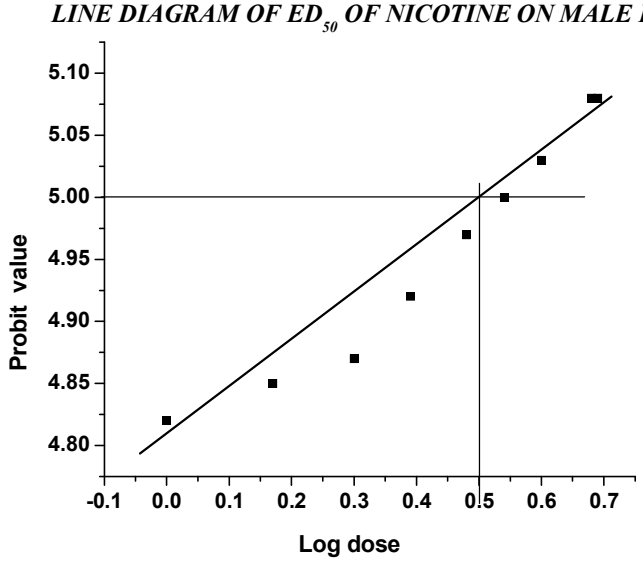

Fig. 2: Probit values against logarithm of doses of nicotine tartrate applied subcutaneously on male rat for determination of ED_{50}

days of treatment, the growth rate of body weight of the animals of two groups (control and nicotine) was not significantly differed with each other. But it was found that after 15 and 20 days of nicotine treatment the body weights were significantly ($P<0.05$) reduced compared to control group (Figure 3b and 3c).

Heart and liver tissues were collected after the removal of tissue fluid and blood. The tissue weight was recorded after sacrificing the animal and expressed as relative weight of gm/100gm of body weight. After 10 days of nicotine treatment, the organ (liver and heart) weight were didn't show any significant change when compared to that of with control group. However, after 15 and 20 days of nicotine treatment, the heart weight (0.49 ± 0.02gm and 0.42 ± 0.02gm / 100 gm body weight respectively) and liver weight

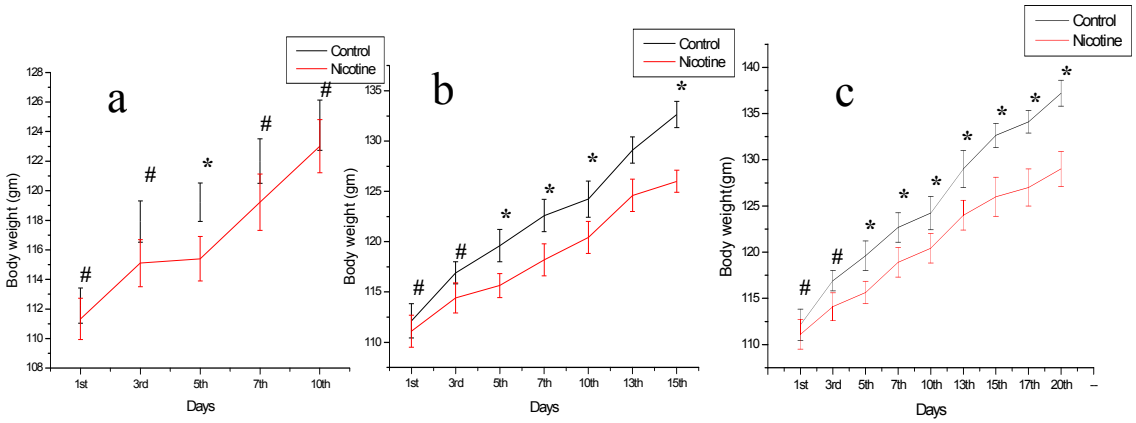

Fig. 3: Changes body weight (gm) due toapplication of nicotine (3.5 mg/Kg body weight) subcutaneously according to different day durations (10, 15, and 20 days). Here "a", "b" and "c" represent 10, 15 and 20 days durations of nicotine treatment respectively. [* P <0.05 with respect to control; # no significant change between control and nicotine treated groups]

(3.74±0.1gm and 3.00±0.1gm / 100 gm body weight respectively) were significantly (P<0.05) decreased in comparison to that of the control group (Figure 4). It was also noted that after 15 and 20 days the weights of heart of the control group was 0.54±0.01gm, 0.55±0.01 gm / 100 gm body weight respectively and the weights of the liver of control group was4.61±0.1gm, 4.67±0.01 gm / 100 gm body weight respectively .So the day-dependent study showed that the effective dose of nicotine (3.5mg/kg)had a notable influence on the body and organ weight in case of male albino rat.

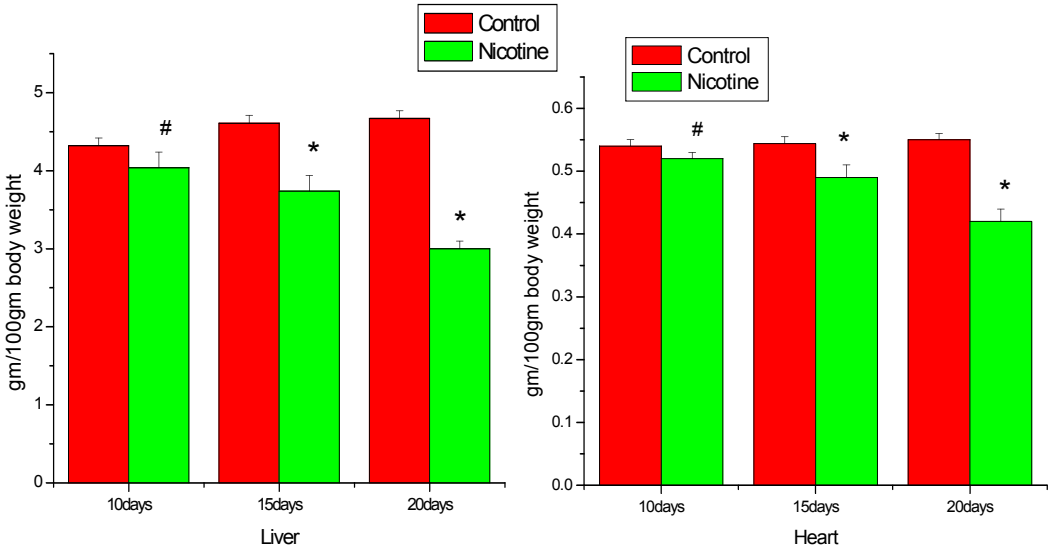

Fig. 4: Changes of organ weight (liver and heart) of Control andNicotine treated groups[*P < 0.05with respect tocontrol group; # no significant change between control and nicotine treated groups]

3.3 Effect of nicotine on hematological parameters in day-dependent study:

The results of the above study have been presented in Figure 5. The data analysis of this study by ANOVA showed that after 10, 15 and 20 days of nicotine treatment, the WBC count (3.6±0.22, 4.0±0.2 & 5.0±0.2 $10^3/mm^3$ respectively) was significantly ($P<0.05$) increased as compared to the control group (2.18±0.2,2±0.2 and 2.18±0.2 $10^3/mm^3$ respectively). However, the statistical analysis showed a significant ($p<0.05$) decrease in RBCs count (6.0±0.32, 4.07±0.35 &2.5±0.45 $10^6/mm^3$ respectively) in respect to control group animals (7.13 $10^6/mm^3$). The reduction of RBC concentration directly affects the haemoglobin concentration. So, the haemoglobin concentration, after 10, 15 and 20 days of nicotine treatment, was significantly ($p<0.05$) decreased to 9.66±0.46gm/dl, 7.16±0.69gm/dland 5.96±0.89 gm/dl respectively in comparison to haemoglobin concentration of 10.92 gm/dlin control animals. Platelets are other important blood cell components for maintaining the biological hemostasis. After 15 and 20 days of nicotine treatment the platelet count (1011±10and 1027±11 $10^3/mm^3$ respectively) was significantly ($P<0.05$) increased from that of control group (816±11

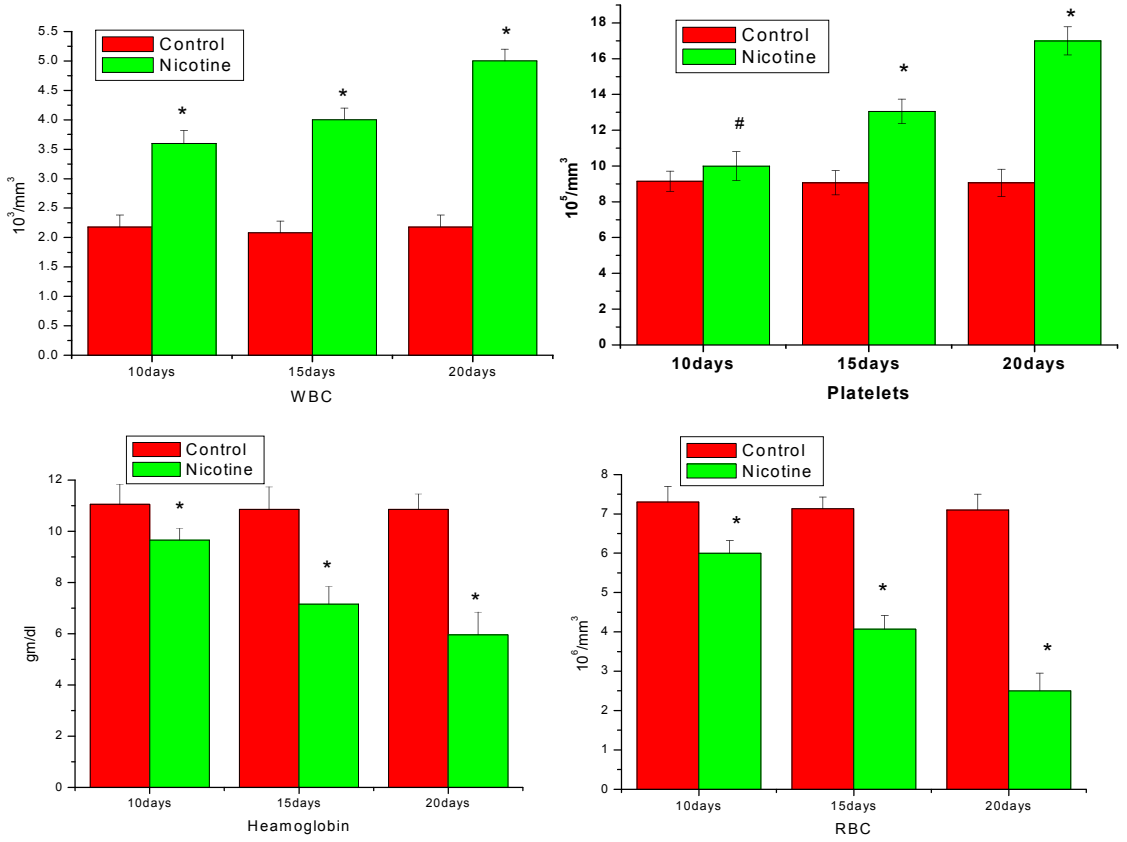

Figure 5: Changes of haematological parameters (WBC, RBC, Platelets and haemoglobin) in Control and Nicotine treated groups. [*P < 0.05with respect tocontrol group; # no significant change between control and nicotine treated groups]

and816±10 $10^3/mm^3$ respectively). Here during 10 days of nicotine treatment no significant change in the platelet counts was observed. From the above results it is suggested that nicotine toxicity adversely affect the haematological parameters which might cause the blood coagulation imbalance.

4. DISCUSSION:

The LD_{50} measurement is an important part of biological standardization for drugs development study. According to Dutch Expert Committee on Occupational Standards (DECOS, 2004), the LD_{50}values of nicotine through dermal, oral, inhalation, intraperitoneal injection were 19.3, 50 to70, 140, 30 mg/kg body weight respectively. Mandal and his group (2004) established the LD_{50} value of 93 mg/kg body weight in female albino rat fed with both normal and high protein diet. They observed body and organ (ovary and uterus) weights in their study. However, in the present study the LD_{50} of nicotine in case of male albino rats was 51mg/kg body weight (Figure-1 and Table-1). Another important indicator like ED_{50} (median effective dose) is the dose of a medication that produces a specific effect on 50% of the population. The ED_{50}value also depend son the subject's weight, height, fat content, pharmacokinetics, and pharmacodynamics. In clinical studies the recommended dose of any drug is derivedon the basis of ED_{50}. The phosphatase activity is a very important biomarker for cardiac and hepatic disorders and it has clinical significance in intermediary metabolism. So, we measured alkaline phosphatase level (ALP) for determining ED_{50}value.In our study the ED_{50} of nicotine was 3.5mg/kg body weight (Figure-2 and Table-2). Then we applied this effective dose on male albino rats for the physiological changes , body, heart and liver weights along with haematological parameters during conducting day dependent (10, 15 and 20 days) studies.

A study by Bellinger et al., (2003) reported that after nicotine (2 or4 mg/kg body weight) administration (i.p) on male and female rodents for 14 days, the body weight gained of rodents was more slowly compared to that of the control group. Martha and his associates (2004) also observed that the use of tobacco product (smoking) induced a slow gain of body weight in rat or weighed significantly less ($p<.0.001$) than control group's animals. Similarly, in another study, after nicotine (0.5 mg/kg body weight) (s.c) application on female rats for 30 and 60 days, the weights of different organs like ovary, kidney, pituitary, liver, heart and uterus were significantly ($P<0.05$) diminished compared to that of the control group (Iranloye and Bolarinwa, 2009). In our study we observed that 3.5 mg/kg body weight nicotine dose showed a significant ($P<0.05$) change after 15 days of treatment. After 15 days, the growth rate of body weight was significantly ($P<0.05$) decreased in respect to that of the control group. At the same time after 20 days of nicotine treatment body weight also showed a greater decrease than that of 15 days treatment and it was statistically significant ($P<0.05$).On the other hand the weight of liver and heart was also significantly ($P<0.05$) diminished after 15 days of treatment when compared to that of control group (Figure 3 and 4). It is well known that nicotine acts through nicotinic Ach receptor. Similarly, it also acts on various neurotransmitters, which augment catecholamine, dopamine, no repinephrine, Ach, ã-amino butyric acid, glutamate and serotonin release. Among them catecholamine, dopamine and serotonin, which are involved in regulating energy

homeostasis through changing the feeding behavior, suppress appetite leading to reduced food intake and lower BMI (Chiolero et al., 2008 and Dey et al., 2016). As we know that nicotine induced oxidative stress causes production of ROS elements. Then it increases lipid peroxide which is responsible for cell apoptosis. Similarly nicotine is also responsible for adipose tissue lysis and release of free fatty acids (Jutkiewicz et al., 2011). So, from these above mentioned cellular effects of nicotine it may be suggested that this dose (3.5mg/kg body weight) of nicotine caused a reduction of body and organ weights. This diminished weight ultimately reflected on variety of physiological responses including rejection / avoiding of food or water because of reduced palatability, induced anorexia or systemic toxicity, which in turn reflected on organs weight.

In case of haematological study, we estimated RBC, WBC, platelets and hemoglobin level of blood. After tobacco intake, nicotine is normally carried out by the circulating system. For that reason, the blood cells are vigorously affected by nicotine toxicity. High WBC count is a dangerous issue for atherosclerotic vascular disease and ischemic stroke (Chong et al., 2001). Tobacco smoking agent like nicotine is responsible for increasing numbers of WBC including neutrophils, lymphocytes, monocytes, eosinophils, and basophils. Primarily C-reactive protein (CRP) is produced from the liver hepatocytes but also synthesized by smooth muscle cells, macrophages, endothelial cells, lymphocytes, and adipocytes (Sproston and Ashworth, 2018).Here it was noted that the WBC count was significantly ($P<0.05$) increased within 10 days of nicotine treatment (3.5mg/kg body weight) compared to that of the control group. It is evident that elevation of catecholamine synthesis due to nicotine causes increased numbers of leukocyte in the blood. In other way nicotine also induces oxidative stress, inflammation and cellular toxicity which are responsible for increased leukocyte numbers (Iyer et al., 2014). Several reports suggested that high concentration of nicotine and cotinine could increase RBC hemolysis (Asgary et al., 2005 and Robert et al., 1996). Lipid peroxidation, vascular pro-inflammations (moderate and strong) and plug formation inside the vascular lumen could cause hemolysis (Jean- Michel and Martin-Ventura, 2020). Nicotine promotes the production of ROS, which stimulates the activation of caspases system as well as cell death (Khademi et al., 2019).On the other hand, in our study the RBC count and hemoglobin level were significantly ($P<0.05$) diminished during 10 daysof nicotine treatment (3.5mg/kg body weight) compared to that of the control group. Nicotine induced oxidative stress causes rupture of the RBC membrane. The cotinine (metabolized product of nicotine) causes 13.8% RBC membrane peroxidation which is higher than nicotine. Due to destruction of RBC, huge amount of haemoglobin become released from the cell and haeme molecule is metabolized by bilirubin and biliverdin path way and consequently less haemoglobin level was found in blood. So due to nicotine toxicity bilirubin level is increased (Asgary et al., 2005). But on the other hand, insignificant changes($P<0.05$) werefound in case of platelets count after the 10 days of nicotine (3.5mg/kg body weight) treatment when compared tothe control group. The platelets count was significantly ($P<0.05$) increased as a result of 15 days of nicotine (3.5mg/kg body weight) treatment in comparison to that of the control animal. It is already established that smoking influences platelets production

and its activity along with increased circulating thrombomodulin protein level. Simultaneously nicotine also causes for endogenous epinephrine release and increase platelet aggregation under flow conditions. These are the conditions which could cause of damage of cardiovascular health and stroke might occur (Lupia et al., 2010).

5. CONCLUSION:

In the present investigation we tried to standardize the ED_{50} and LD_{50} of nicotine. The ultimate effective dose of nicotine was 3.5mg/kg body weight and lethal dose was 51mg/kg body weights which were applied in male albino rats for day–dependent study. This particular effective dose (3.5mg/kg body weight) of nicotine was very much responsible for changing of body and organ (heart and liver) weights. So in conclusion it may be stated that any form of tobacco (nicotine) consumption is directly or indirectly responsible for changing the body and organ weights along with haematological parameters. In this research study we were unable to measure the serum nicotine and cotinine levels, which were the indicators of the rate of nicotine toxicity on body.

6. ACKNOWLEDGEMENT:

This study was supported by Vidyasagar University, Midnapore for providing the financial support through personal research grant (PRG) and the UCIC of the University for providing instrumentation facilities.

7. REFERENCES:

Asgary S., NaderiGH.,Ghannady A. (2005). Effects of cigarette smoke, nicotine and cotinine on red blood cell hemolysis and their -SH capacity. ExpClin Cardiol.10(2): 116–119.

Bellinger L., Cepeda B A., & Wellman P J. (2003). Meal patterns in male rats during and after intermittent nicotine administration. PharmacolBiochemBehav, 74(2), 495-504.

BenowitzNL., Jacob P. (1984). Daily intake of nicotine during cigarette smoking. ClinPharmacolTher. 35: 499–504.

Benwell MEM., Balfour DJK.,Birrell CE. (1995). Desensitization of the nicotine-induced mesolimbic dopamine responses during constant infusion with nicotine. Br J Pharmacol. 114:454–560.

Bowers DG and Seashore SE., (1966). Predicting Organizational Effectiveness with a Four-Factor Theory of Leadership. Administrative Science Quarterly. 199:24-38.

Chandrupatla DM. Molthoff CF., Ritsema WI.,Vos R. (2018). Prophylactic and therapeutic activity of alkaline phosphatase in arthritic rats: single agent activity and in combination with methotrexate.Translational Research.77(2):25-30.

Chattopadhyay K.,ChattopadhyayBD. (2008). Effect of nicotine on lipid profile, peroxidation & antioxidant enzymes in female rats with restricted dietary protein, Indian J Med Res. 127: 571-576.

Chiolero A., Faeh D., Paccaud F., Cornuz J. (2008). Consequences of smoking for body weight, body fat distribution, and insulin resistance. The American journal of clinical nutrition (review). 87(4):801-809.

Chong D L, Aaron R F, Nieto F J, Lloyd E C, Shahar E and Wolfe AD. (2001) White Blood Cell Count and Incidence of Coronary Heart Disease and Ischemic Stroke, and Mortality from Cardiovascular Disease in African-American and White Men and Women: The Atherosclerosis Risk in Communities Study. Circulation. 103:1357–1358.

Dacie JV, Lewis SM. (1968). Practical Haematology, Churchill Livingstone, London, UK, pp. 566-568.

DECOS (Dutch Expert Committee on Occupational Standards) .(2004). Nicotine. Committee on Updating of Occupational Exposure Limits, Health Council of the Netherlands, The Hague. http://www.gezondheidsraad.nl/sites/default/files/00@15105.pdf

Dey KS, Dolai PD, MandalD, Das B, DashKS, RoyD , Roy S. (2016). Protective effect of

AndrographispaniculataNees and Vitamin-C in nicotine-induced oxidative stress in liver and kidney.World J Pharm Sci. 4(9): 345-352.

Finney DJ. (1952). Probit Analysis. Cambridge, England, Cambridge University Press.

GaddumJH&Sc D. (1933). Section of Experimental Medicine and Therapeutics. Proceedings of the Royal Society of Medicine. 47 (7):11-20.

Gottfried EL. (1997). Erythrocyte indexes with the electronic counter. N Engl J Med. 300:1277.

Iranloye OB, BolarinwaAF. (2009). Effect of Nicotine Administration On Weight And Histology Of Some Vital Visceral Organs In Female Albino Rats. Nigerian journal of physiological sciences. 24(1):7-12.

Iyer RA., Joshi AR &Esmaeil H. (2014). Effect of cigarette smoking on leukocytes count in human adult males. *Int J Phys*; 2:107-111.

Jain A., Agrawal S & Flora JSS. (2015). Arsenic and nicotine co-exposure lead to some synergistic effects on oxidative stress and apoptotic markers in young rat blood, liver, kidneys and brain. Toxicology Reports. 266(2): 1334–1346.

Jean- Michel B and Martin-Ventura J, (2020) Red Blood Cells and Hemoglobin in Human Atherosclerosis and Related Arterial Diseases. Int. J. Mol. Sci.; 21: 6756.

Jutkiewicz EM., Brooks EA.,Kynaston AD., Rice KC& Woods JH. (2011). Patterns of nicotinic receptor antagonism: nicotine discrimination studies. J PharmacolExpTher. 339:194–202.

Khademi F , Totonchi H , Mohammadi N , Zare R , and Zal F(2019) Nicotine-Induced Oxidative Stress in Human Primary Endometrial Cells International Journal of Toxicology 38(3): 202-208.

Lowry O H., Rosebrough J N., Farr A L., and Randall R J. 1951. Protein measurement with the folinphenol reagent. *J.Biol.Chem*. 193: 265.

Lupia E, Bosco O &Montrucchio G. (2010). Thrombopoietin contributes to enhanced platelet activation in cigarette smokers. Atherosclerosis. 210:314–319.

Mandal K., Dasgupta S &Chattopadhyay BD. (2004). Nicotine induced alterations on reproductive organs of female rats under protein deficient condition. Indian J Exp Biol. 42: 330-2.

Martha JGN., Elizabeth V T., Mary U S., Carol A C., and Laurel C P. (2004). A Rat Model to Determine the Biomedical Consequences of Concurrent Ethanol Ingestion and Cigarette Smoke Exposure. AlcoholClinExp Res. 28(7): 1120–1128.

Mohamed SG and Amer SA. (2014). Protective Effects of Vitamin C against Nicotine-Induced Oxidative Damage of Rat Liver and Kidney. Josr-jestft. 12(8):50-63.

Rees HM and Ecker EE. (1923). An improved method for counting blood platelets. JAMA Network. 192(80):621.

Robert J B, Mario RF, and Manfred S(1996) Catecholamine-Induced Leukocytosis: Early Observations, Current Research, and Future Directions Brain, Behavior, and Immunity. 1996;10: 77–91.

Sproston RN and AshworthJJ. (2018) Role of C - reactive protein at Sites of Inflammation and Infection.FrontImmunol.; 9: 754.