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Teratogenic Impacts of Tobacco Specific Carcinogen 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone (NNK) in Swiss Albino Mice Exposed during Gestational Period

Running Title: Teratogenic Malformations Induced by NNK in Albino Mice

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Abstract Marketed tobacco products, both smoking as well as non-smoking, contain pro-carcinogenic component 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in high quantities. NNK when ingested, gets activated by action of Cytochrome P450 (CYP450) enzyme present inside living system. In presence of CYP450 enzyme, NNK forms active metabolites NNAL and NNAL-glucs. These metabolites are responsible for adduct formation with DNA and proteins, in turn causing mutation and cancer induction. This study aims at teratogenic effects of tobacco carcinogen NNK on offspring of pregnant Swiss albino mice when exposed to it during gestational period. In this study we have injected a single dose of 100mg/Kg B.wt./ml normal saline of NNK in 6 pregnant dams on 14th day of gestation to find out the embryotoxic and teratogenic effects induced by it. Body weight as well as deformities of the litters produced were recorded. The dams delivered stillbirths with various malformations viz. ascites, ring haemorrhage on tail base, cleft palate, exophthalmia etc. The survived pups developed external tumours on different parts of the body. This indicates that NNK can cross the transplacental barrier between mother and the foetus to induce toxic stress in foetal tissue. It disturbs the smooth progression of the period of organogenesis, as a result initiating various organ deformities. Further extensive studies are required to understand how NNK plays a role in producing teratogenic effects in offspring of mothers exposed to it either active or passive way.

Keywords NNK, Carcinogen, Teratogenic Impacts, Transplacental Crossing, Gestational Period

1. Introduction

Human beings are being exposed to a number of xenobiotics in everyday life, which are hazardous in turn slowly paving the path of painful end. These xenobiotic components may enter our body system through different routes such as dermal, ingestion and inhalation. These external unwanted agents such as pesticides, herbicides,

growth hormones, food additives, fruit ripening agents, preservatives, dyes, surfactants, synthetic drugs and polycyclic aromatic hydrocarbons (PAH) may lead to impairment of different physiological functions leading to short as well as long term effects on prolonged exposure. Xenobiotic exposure is one of the main causative factors of serious possible toxic impacts including carcinogenesis, tumorigenesis and mutagenesis. The changing lifestyle habits are also responsible for the increasing health issues to a greater extent. Consumption of alcohol and tobacco is seen in all spheres of the society irrespective of the gender, including both generations; young and old.

Tobacco consumption in smoking or non- smoking ways is hazardous as it is one of the main risk factors for development of mainly, lung, oral and oesophageal cancers. Tobacco specific products such as cigarette, bidi, khaini, zarda, paanmasala etc. contain nicotine, nitrosamines as well as other toxic components like formaldehyde, polycyclic aromatic hydrocarbons (PAH) etc. Nitrosamines are the key components which are carcinogenic. Four major types of nitrosamines so far detected in tobacco products include Nicotine-derived-nitrosamine-ketone (NNK), N-nitrosonornicotine (NNN), 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and N-Nitrosoanabasine (NAB) [1, 2]. Amongst these, NNK and NNN are the most potent carcinogenic agents. Although NNK is a pre-carcinogen, the Cytochrome P450 enzymes in our body react with it forming DNA reactive metabolites viz., NNAL or4-(methylnitrosaminmo)-1-(3-pyridyl)-1-butanol and NNAL-gluc., in turn inducing the methylation, pyridyl-oxo-butylation and hydroxy-butylation of nucleotide bases in DNA [3-6]. Nicotine, another toxic component of tobacco plays an important role in cancer induction and metastasis by activating various cancer signalling pathways [7-13].

Evaluation of contribution of carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone **(**NNK) in causing mutagenic and teratogenic impact in human is difficult as it is present in small quantities in cigarette smoke. Records have been found that in human, NNK is capable of crossing the transplacental barrier and causes adduct formation in placenta from smoking women [14]. The assay also has established that the level of maternal exposure to cigarette smoking is directly related to the birth weight of the offspring. Furthermore, radiolabelled NNK exposure in gestational period in C57BL mice showed NNK metabolites covalently bound to lungs, liver and nose tissue of 18th day old foetuses [15]. Our goal in this study was to find out the possible teratogenic impacts in NNK treated albino mice exposed during gestational period.

2. Materials and Methods

2.1. Experimental Animals

Swiss albino mice of age (8 ±1) weeks, weighing about 20-30gm were selected for the experiment that were healthy and pathogen free. Animals were acclimatized for about 15 days before commencement of the experiment. Animals were provided standard mice feed and water *ad libitum*. Male and female mice were caged together in 1:3 ratio and kept under observation. Before conducting the experiment, approval of IAEC was taken vide letter no. 5/IAEC/CU/05/01/2021.

2.2. Chemicals

4(N-methyl-N-nitrosamino) 1, (3-pyridyl), 1-butanone or NNK was purchased from Sigma-Aldrich Co. and of purity (≥ 98.5 %). The powdered form of NNK was dissolved in normal saline (0.9%) for intravenous application in experimental animals.

2.3. Dose Determination and Experimental Design

Relevant studies performed on NNK were considered for the selection of the dose in this experiment. The LD_{50} for NNK in mice was found to be 150mg/kg and the dose 100mg/kg was responsible for pulmonary and hepatic tumours in litters of NNK treated dams [16]. For this reason we selected the dose 100mg/kg body weight for the experiment.

In the experimental work six female virgin albino mice were used. Three females and one male are caged together for one night. Confirmation of mating is done by checking the vaginal plug in the next morning. This was counted as gestational day 0 (GD-0). Males were removed from the cages after four days. A single dose of NNK (100mg/Kg/ml normal saline) was administered to the pregnant animals intra-peritoneally on 14th day of gestational period. Body weights of the animals were measured every alternate day throughout the experimental period. Around 19th and 20th day of gestational period, five out of six dams delivered pups. One female dam showed signs of pseudopregnancy.

Tumorous outgrowths formed in pups were dissected out from skin, fixed in formalin, processed and sectioned into 4µm thickness. Staining was done following H&E standard method and observed under microscope.

To report the significant changes of NNK treated groups to control groups, the following standard formula was used,

$$\text{Mean} \pm \text{Standard Error}$$

ANOVA (Analysis of Variance, One way) was used to test the significance of sample means between different experimental groups in different days.

3. Results

Four of the NNK treated mice delivered all stillbirth litters. Only 5 pups of the other one survived but their growth was stunted. Different types of abnormalities were recorded in both dead and alive litters as listed in figure 1

and table 1. The dead foetuses showed significantly ($P<0.05$) reduced body weight when compared to the control. Fluid accumulation inside abdominal cavity (ascites), dark coloured ring haemorrhage at the tail base, cleft palate, club foot were noticed in case of stillbirth litters of the carcinogen treated group (Figure 2, 3, 4). The body growth of survived pups of treated group reduced drastically and were less active than the controlled ones. For most of the time, the pups of NNK treated groups were lethargic and showed a sleeping tendency at the cage corner. Three-week-old survived pups had no properly opened eyelids, no forelimbs and showed stunted body growth as shown in figure 5. Two of the survived pups developed external tumours near the neck and the head region (Figure 6). In addition, one of the five survived pups of the treated group died after six weeks of birth.

The histopathological sections of external tumour growths under light microscope showed sheet of cancerous cells with eosinophilic cytoplasm originated from keratinocyte cells of squamous epithelium. Nuclei patterns were bizarre and hyperchromic. High proportions of mitotic figures were detected indicating hyper proliferation of cells. Concentrated keratin centres denoted as "pearl of epithelial cells" were present in the tissue section, as shown in figure 7. Infiltration of inflammatory cells (plasma, lymphocytes) to the area was detected. Granulation as well as fatty degeneration in cytoplasm of cells. With these specifications, the external tumorous growth was identified as carcinoma of squamous cells in skin.

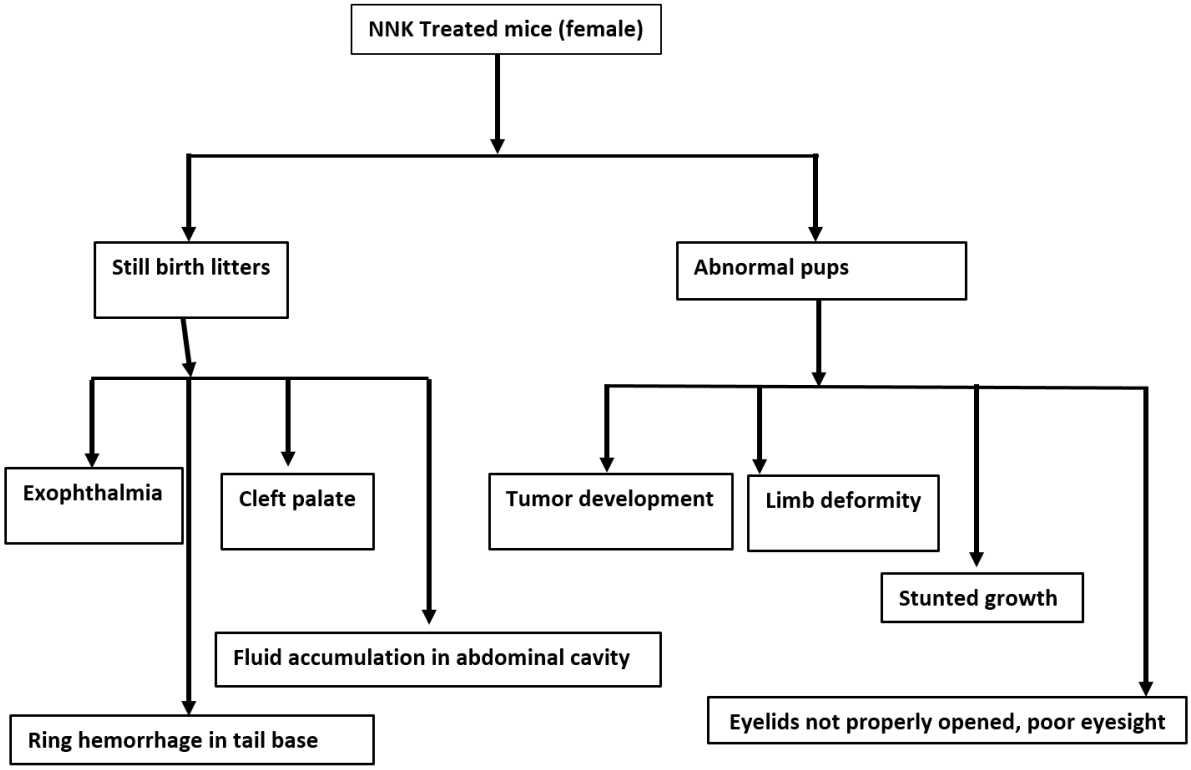


Figure 1. Schematic representation of different deformities in stillbirth as well as survived pups of NNK treated dams

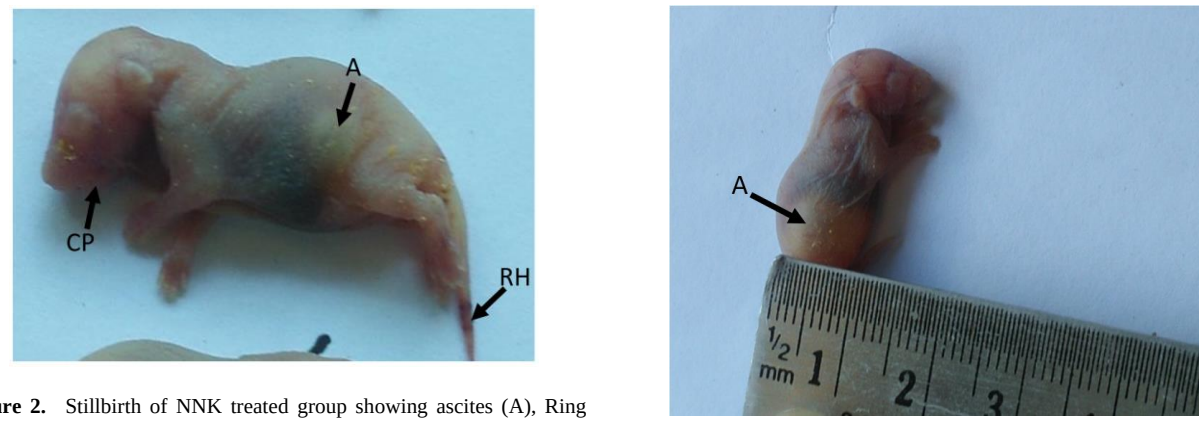


Figure 2. Stillbirth of NNK treated group showing ascites (A), Ring Haemorrhage (RH) at the tail base, Cleft palate (CP).

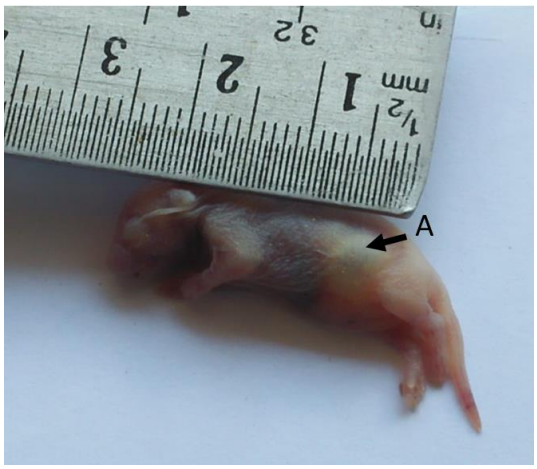
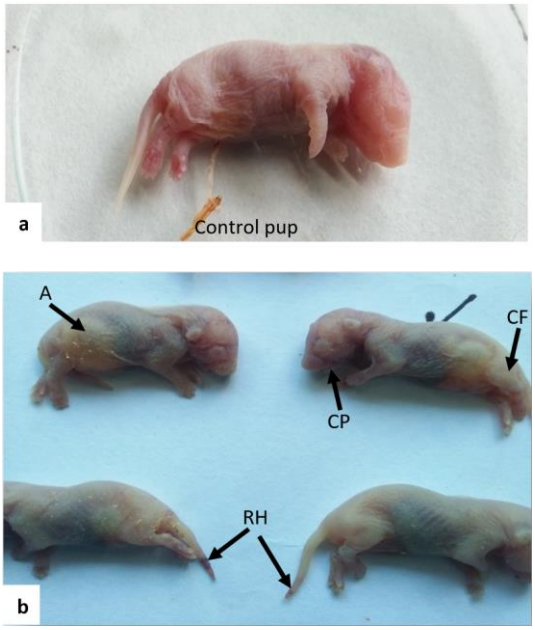
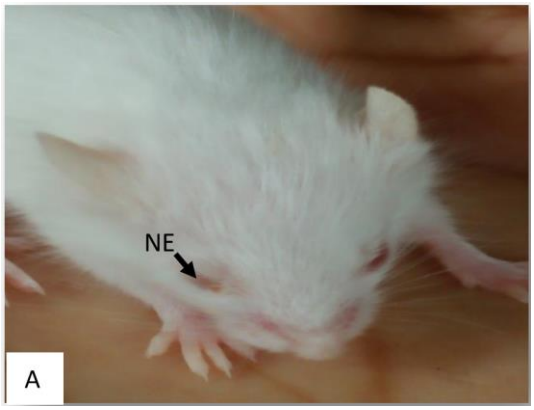


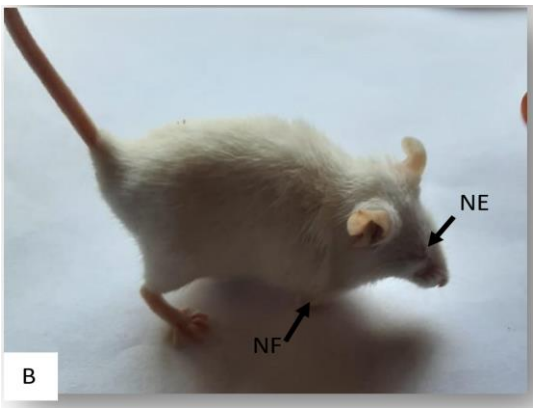
Figure 3. Stillbirth with Ascites (A) (fluid accumulation in the abdominal cavity)



A- Ascites, CP- Cleft palate, CF- Club foot, RH- Ring haemorrhage

Figure 4. (a) Litter from control group with no abnormalities (b) Dead litters from NNK treated groups with different abnormalities.





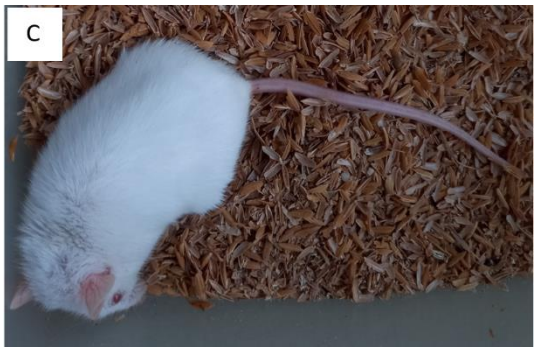
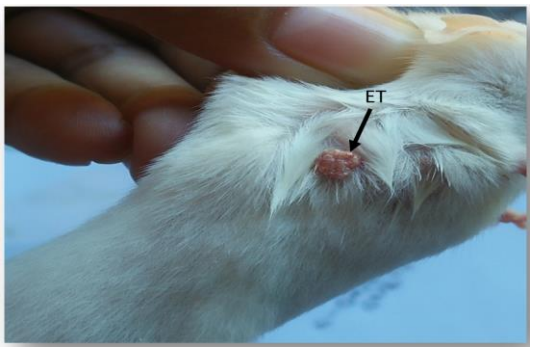


Figure 5. **A**. 3 weeks old pup of NNK treated group with no properly opened eyelid (NE), **B.** Pup with NE and no forelimb (NF) and **C.** 2 months old pup in moribund condition.



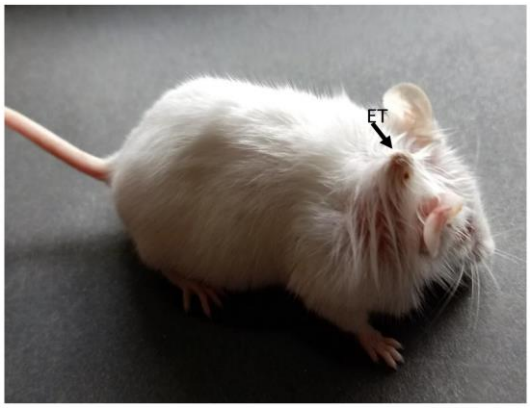
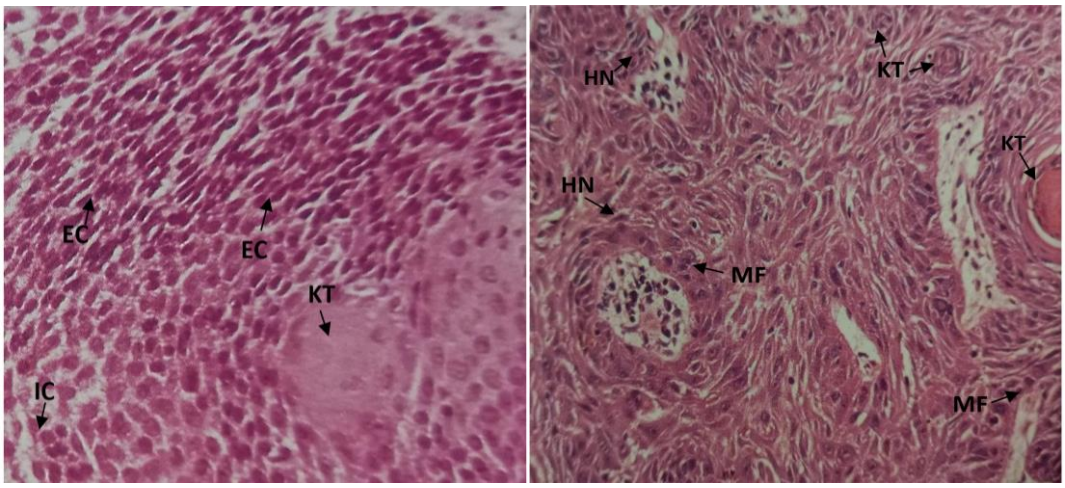


Figure 6. External tumour (ET) growth in 2 months old survived pups of NNK treated group



(A) (B)

Figure 7. Photomicrograph of histological section of dermal squamous carcinoma showing hyperchromic nuclei (HN), keratinization (KT) of squamous cells, eosinophilic cytoplasm (EC), Mitotic figures (MF) and individualisation of cells (IC).

Table 1. Different teratogenic malformations recorded in litters of control and NNK treated groups. Values are calculated as (Mean± S.E.)

Teratogenic impacts		Control	Treated
Total litter production	Survived	10.02 ± 0.82	5
	Stillbirth	-	8.01 ± 2.76
Body weight		11.6 ± 0.45	9.62 ± 0.45
Cleft palate		-	7.19 ± 0.07
Exophthalmia		-	9.57 ± 0.04
Tail ring haemorrhage		-	10.33 ± 0.11
Defected eye	Single eye	-	4.01 ± 0.05
	Both eyes	-	1.12 ± 0.13
Limb deformity		-	3.02 ± 0.02
Stunted growth		-	4
Tumour development		-	10.22 ± 0.23

4. Discussion

NNK is a pre-carcinogen present in both smoking and smokeless tobacco products. This study showed that when injected with NNK intra-peritoneally on 14th day of gestational period, it can penetrate the transplacental barrier and initiate various deformities in foetuses. NNK gets metabolized in mother and foetus tissue in turn converting itself into active carcinogen [17]. Embryotoxic nature of NNK was recorded by Winn et al. [18], in pregnant CD-1 dams.

In this experiment, four of the pregnant dams treated with NNK delivered stillbirth litters. This indicates the extent of embryotoxicity effect of NNK. Organ malformations like cleft palate, deformity in limb formation, eyelid formation in dead foetuses were recorded. This result is concomitant with Winn et al. [18]. Significant reduction of body weight of the litters compared to controlled group is recorded. From the survived pups, external tumour growths were seen on different body parts. This may be due to activation of cancer signalling pathways by NNK metabolites by mother as well as foetal tissue. Accumulation of NNK metabolites was found in foetal nasal tissue, hepatic and pulmonary tissue and in amniotic fluid of the dam, when intra-venous injected radiolabelled [carbonyl- 14C] NNK on 13th day of gestation [15].

The pups of treated group were less likely to move around and with decreased appetite for food and water than the control group. This led to moribund condition and death of all the survived pups within 2-6 weeks after born. Blair et al. [19] has established that in case of human, increased incidences of death of babies due to sudden infant death syndrome is corelated to duration of exposure to maternal as well as household exposure to cigarette smoke before and after birth.

Incidence of NNK exposure responsible for foetal mortality and transplacental genotoxicity was well recorded by Alaoui-Jamali et al. [20], when injected intraperitoneally on 14th day of gestational period in Syrian golden hamster. Micronucleus formation was found in foetal tissue indicating toxic stress caused by activation of NNK and its conversion to NNAL as well as protein binding metabolites, indicating NNK as a potent transplacental carcinogen. This attributes formation of squamous carcinoma on skin of survived pups, weeks after pre-natal NNK exposure.

5. Conclusions

This study shows that NNK initiates various organ malformations and tumours in the foetus of pregnant mice if exposed during gestational period. Long-term exposure to smoking as well as smokeless tobacco can not only pose

threat to the pregnant women but also to the developing foetus. Further extensive study is required to find out whether these embryotoxic effects induced by NNK can retain through generations even though the offspring are no longer exposed to it.

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Conflict of Interest

Authors share no conflict of interest.

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