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Research article

EFFECTS OF INDIAN PAN MASALA (PLAIN AND BLENDED - PMP & PMT) ON TESTIS AFTER LONG TERM FEEDING (PO) IN MICE

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ABSTRACT: Pan masala plain (PMP) with or without tobacco (PMT) cause testis impairment. Three sets of mice (n = 60), 2% PMP and PMT diet were fed for12 & 13 months. Further six sets of mice in two groups plus control (n=20) received by oral feeding (PO) a commercial brand PM (at doses of 8, 40 and 100 mg/kg b.w. in olive 0il by oral administration as a single dose) and zarda (PMT) too was given same doses and the controls were kept at staple diet only and by oral administration 0.5 ml vehicle for a period of 3 months. The animals were sacrificed (n=10) after 2 weeks and 3 months to study graded dose effects of PM plain and blend on sperm abnormalities. The plain and zarda group showed graded dose related elevated sperm abnormalities compared to control. The damages were high with 100 mg zarda group after 3 months period. The plain and Zarda group when compared, dose-duration wise, at each dose level, and in between two test groups, showed significant structural and functional changes of sperm head and mutilations. The sperm head anomalies were maximum in 12 and 13 months PMP and zarda fed groups in terms of morphology and other abnormalities. The damage is more in 13 months fed mice ($p<0.01$) and 12 months fed mice ($p<0.05$) when compared to the control group. Both the groups of PM, showed different types of sperm head abnormalities i.e., banana, beak, hammer shaped, amorphous etc. Testis of 12 and 13 months PMP and PMT groups were processed for histopathology. It was found that PMP and PMT inducts no effects after 2 weeks exposure, whereas caused liver tumor after 56 weeks exposure. PMP and PMT in comparison, and both significantly effect mouse testis showing degenerative changes in seminiferous tubules and interstitial tissue being more prominently effected in PMT group. .

Key words: Pan Masala, Testis, Mice

INTRODUCTION

The chewing of betel quill without tobacco is a habit of orient, wide spread and a great antiquity when Portuguese blended betel with tobacco in 1600 and named as betel quid which was later as pan masala (PM) composed with areca nut, betel leaf, catechu, lime and tobacco. Wide consumption of betel quid was linked with oral cancer (Nigam, SK and Venkatakrishna-Bhatt H, 2004). Since two decades, a powdered chewing mixture, commercialized as *pan masala (PM)* has been in abundant use in India among non-smokers as well as to refrain smoking and tobacco among smokers. Areca nut, a major portion of *PM* is a clastogenic, genotoxic and carcinogenic in animals (Panigrahi GB, Rao AR,1986). Catechu, an ingredient of *PM* causes hepatic irritation, hyperplasia of oral mucosa (Dunham LJ, Herrold KM, 1962), dominant lethal mutation and chromosomal damage in mouse bone marrow cells[4]. Over lakh fresh benign cancer incidence from south-east Asian countries were reported not only due to smoking but also chewing of betel quid and *PM* (I.A.R.C (1985). Betel quid is a mixture with different proportions of catechu, lime, areca nut, tobacco with spices, coconut, sugar and *gulkhand* (rose leaves and sugar).

The different types of betel quid with certain addictive commercial products in handy one dose sachets of familiar, popular brand are sold. *PM* is widely consumed as a brain stimulant to remain anorectic and to compensate extra work-load by young and old, since it is a mood-elevator, a relief in misery and grief. India and neighboring countries are now in focus: Assam hails top in common cancers of lip, buccal and pharyngeal areas due to pan, betel nut and tobacco as betel quid or *PM* (Jussawala, D. J. and V. A. Deshpande, (1971). Practice of reverse smoking in tribal, smokeless tobacco cause precancerous lesions and predominantly at the squeeze and sucking oral sites of the tobacco quid (N. C. R. P Consolidated Report (1987). Different proportions of *PM* and its ingredients is region based. Assamese incubate betel nut with fibrous peri-carp in pits for 4 months and then strip pericarp and use endosperm (*tamul*). Gujarathis make *mava* with dried betel nut and lime with or without tobacco. Raw kennel betel nut, lime and tobacco are known as KWMP at Meghalaya. South Indians use betel leaf, nut, lime and tobacco. Currently north Indians and rest consume *PM*. World over 200 million people chew betel quid (Hoffman D, Hecht SS (1988). Juvenile start would go up to 30-40 chews of betel quid per day in youth focus development of buccal ulcer, stomatitis and pre-cancerous lesion. Many sites such as tongue, alveolus, buccal mucosa, hard palate, tonsils, oropharanx, larynx and esophagus are affected by chewing *PM*, smoking tobacco which may act synergistically causing leukoplakia (Khirme RD et al, 1991), sub-mucous fibrosis and cancer of oral cavity, oropharynx, larynx and esophagus. The habit of chewing *PM* is comparatively new, all over the world but it is more prevalent in Indian sub-continent. The use of *PM* is receiving social acceptance and consumption is also increasing with an increase of variable products of *PM* every day. The habit of *PM* chewing is also slowly getting foothold in western countries. *PM* constituents with genotoxic potential is reflected by an increase in rate of sister chromatid exchange (*SCE*) and chromosomal aberrations of mouse bone marrow cells. Aqueous and ethanol extracts of different brands *PM* showed mutagenic potential[11]. Thus *PM* is focused for carcinogenic, tumourogenic, terratogenic and mutagenic potential in a preliminary report. In continuation we studied the effects of oral *PM* and its blend for long periods i.e. 13 months exposure in respect of histological changes in testicular tissue in mice.

MATERIALS AND METHODS

Adult Swiss Albino mice, 8-10 weeks old weighing 23 ± 3 gm were selected from an inbred colony. All the groups received staple diet. The PMP, PMT fine powder in 100 gm quantity was mixed thoroughly with 4900 gm of feed given to mice routinely besides control animals which received only staple diet. Mice usually eat 5 gm feed per day thus 100 mg of *PM* was consumed by the experimental animals every day. Animals of the same sex were housed in a cage in an air-conditioned animal house (humidity 55.6% constant 12 hr light/dark cycle) and were provided with food prepared in the Institute. They were maintained on standard mice feed and water *ad libitum*. Their diet consisted of wheat 70%, cracked Bengal gram 20%, fish meal 5%, and yeast powder 4% and shark liver oil 1% in the form of dry mesh. Three sets of mice (n = 60), 2% PMP and PMT diet were fed 12 & 13 months. Further six sets of mice in two groups plus control (n=20) received by oral feeding (PO) a commercial brand PM (at doses of 8, 40 and 100 mg/kg b.w. in olive 0il by oral administration as a single dose) and zarda (PMT) too was given same doses and the controls were kept at staple diet only and by oral administration of 0.5 ml vehicle for a period of 3 months. The animals were sacrificed (n=10) after 2 weeks and 3 months to study graded dose effects of PM plain and blend on sperm abnormalities. A minimum of 10 animals each from *PM* treated i.e., Pan Masala Plain (*PMP)* and *PMT* groups were sacrificed after 12 and 13 months feeding. While representative animals 10 from control group were also sacrificed for comparison. The testis and cauda epididymies were cleanly dissected out. The testis were fixed in Bouin's fluid and processed for histopathological study. Five u thick sections of testis were cut and stained with haematoxylin and eosin. The sperm were stained with 0.5 % of aqueous eosin Y solution for about 30 minutes. Sperm in each group in large numbers were scored in order to identify anomalies and qualitative analysis of the sperm head shape changes using the criteria of Wyrobek and bruce (Wyrobek AJ, Bruce WR, 1978).

RESULTS AND DISCUSSION

Histopathological study of testis revealed mild damage to the tubular structure as well as to the spermatogonial cells as compared to the control which showed normal seminiferous tubules and interstitial cells. (Fig.1).

However, the interstitial tissues were gradually over the span of short period showed early degenerative changes in seminiferous tubules and in the interstitial tissue in the *PMT* treated group (Fig.2). The histological changes were much similar in both *PMP* and *PMT* treated groups (Fig.3). The effect is further intensified by extensive degenerative changes in the seminiferous tubule and interstitial cells in PMP treated group as revealed by the presence of sperm in the lumen of the testicular tubule (Fig.4). Interstitial tissue surrounding seminiferous tubules resulting in the proliferation and accumulation of edematous fluid was observed (Fig.5). Thus the testis seminiferous tubules showed hyaline degeneration (Fig.6) since profuse edematous fluid accumulates in the seminiferous tubules (Fig.7). The testicular tumor cells were vacuolated with ground glass cytoplasm and small nuclei. Qualitatively, the sperm head shape indicated that *PM* induced different types of sperm head shape abnormalities such as banana, beak, hammer shaped, amorphous etc. in both *PMP* and *PMT* treated groups in comparison with control group. A significant elevation in sperm head shape abnormalities was noticed in both *PMP* and *PMT* treated group as compared to control.

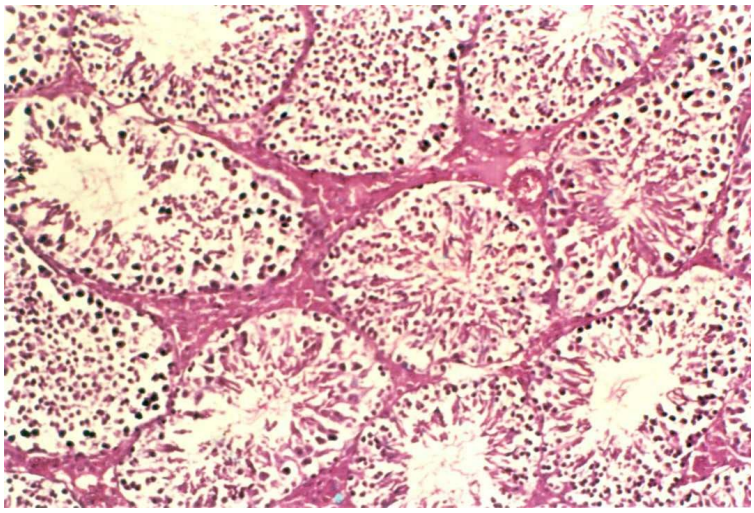

Fig.1. Mouse testis showing normal seminiferous tubules with normal interstitial tissue (H & E 60X).

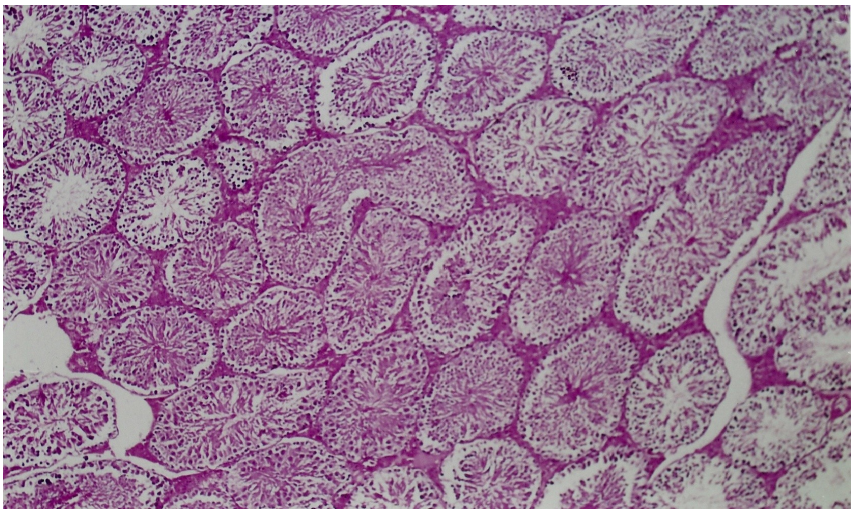

Figure 2. Seminiferous tubules showing changes at various stages. H&E 60 X

Earlier we have shown discrete biochemical changes in testis show decline, (not dose dependent) in the cholesterol and glycogen levels but the protein levels increased at the low doses but reverts to dose dependant decrease at the higher dose levels[13] .In alignment PMT enhanced SGOT, SGOT and alkaline phosphatase levels (Nigam SK, Venkatakrishna-Bhatt H. (2011).

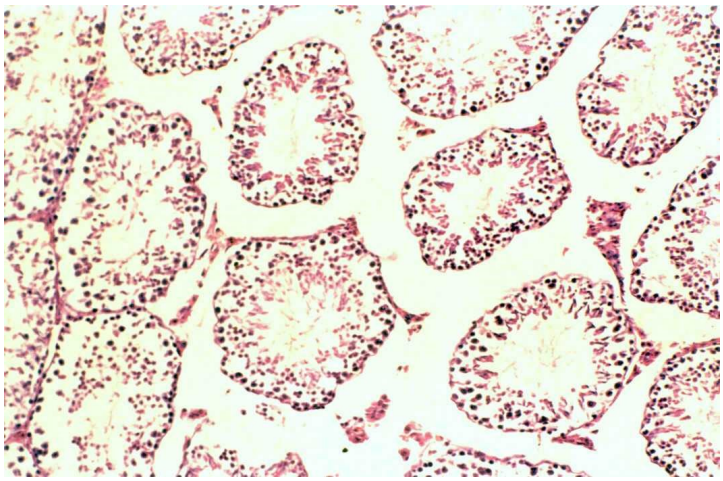

Fig.3. Section of mouse testis with degenerative changes in seminiferous tubules and in interstitial tissue. H & E 6

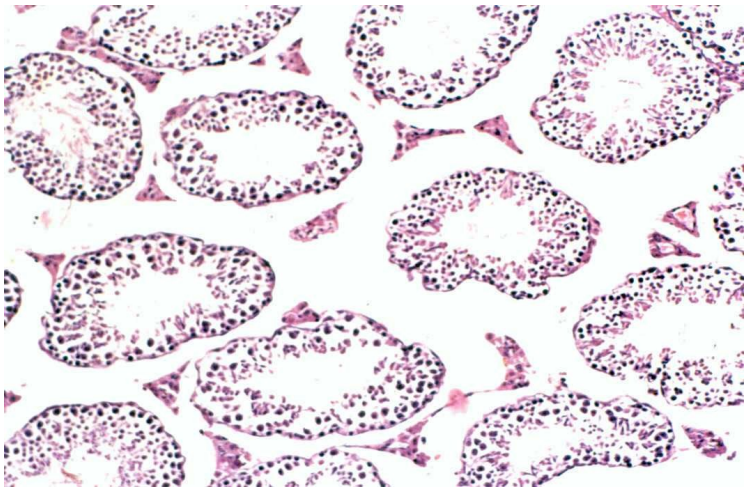

Fig.4. Section of mouse testis showing extensive degenerative changes in tubules and interstitial tissue. H & E 60X.

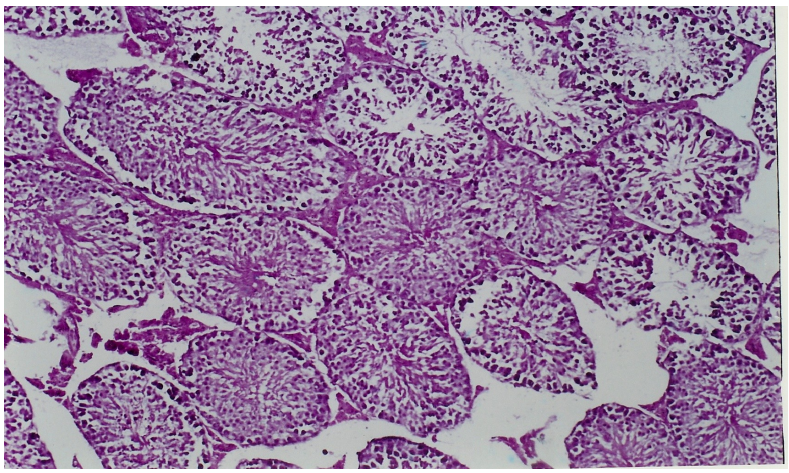

Fig. 5. Interstitial tissue surrounding seminiferous tubules showing proliferation and accumulation of edematous fluid H & E 60X.

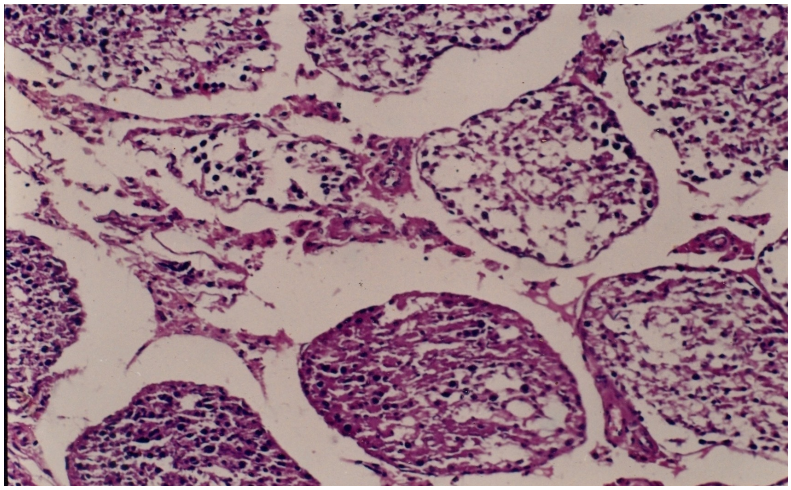

Fig. 6. Mouse testis seminiferous tubules showing hyaline degeneration H & E 60X.

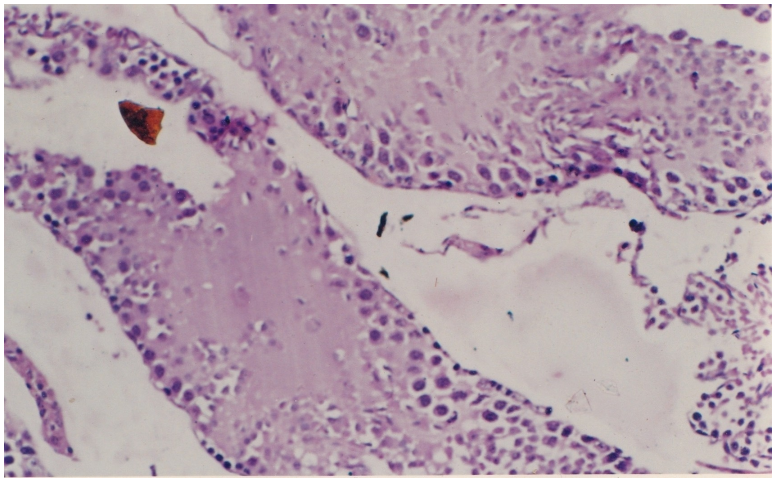

Fig.7. Mouse testis seminiferous tubules showing accumulation of edematous fluid. H & E 60X.

Several reports on the genotoxic potential of *PM* (Panigrahi GB and Rao AR 1986, Giri, et al, 1987, Mukherjee A, Giri AK, 1991, Bagwe et al, 1990) and sperm head abnormalities on exposure to varying periods to *PMP* and *PMT* (Nigam, S.K and Venkatkrishna Bhatt, H 2004) both *in vivo* and *in vitro* experiments prompted us to look into the chronic histopathological changes that occur over a period of 13 months on the testis of mice. *PMP* and *PMT* was found to induct tumor on exposure to 56 weeks and no effect within 16 weeks and therefore the sustained and cumulative effect of *PM* with or without tobacco is obvious on the mouse testis. The *PMP* and *PMT* effects in between though insignificant, yet significantly showed impairment of mouse testis showing degenerative changes in seminiferous tubules and interstitial tissue (Figure 2) being more prominent in the *PMT* treated group (Figure 3) when compared to control group (Figure 1). This study is in alignment with *PM* induced anomalies in sperm shape and structure and the reproductive dysfunction in animals. The sperm head abnormalities were more in 13 months fed mice ($p<0.01$) and 12 months fed mice ($p<0.05$) when compared to the control group which showed variable non-significant changes from the commencement to the termination period (Table.1) There were significant sperm head abnormalities in both groups of mice in comparison with placebos. There was conspicuous dose-duration cumulative significant damage indicating sperm head abnormalities in between (PMP and PMT fed mice) as well as in comparison with mice on staple diet when examined during autopsy periods of 6, 9, 12, 13th months (n=10).

The sperm head structural changes were maximum in the PMT group whereas structural shape distortion commence from 3rd month onwards, abnormalities significantly more from 9th month to 12th month in both test groups being very high in the PMT fed mice ($p<0.01$) after 13 months exposure. However reproductive dysfunction and by deficit sperm count, structural damage in its configuration as well as break down end points caused by chronic oral PMP and PMT were observed in spite of variable consumption in mice though mice of the same age group receiving uniformly composed diet and reared under stable environmental condition's.

Table 1. Effect of oral administration of Manikchand Pan Masala on the sperm after 2 weeks and 3 months exposure.

Group	Dose (mg/kg b wt)	Sperm abnormalities (%)	
		2 weeks	3 months
Control (Distilled water)	0.2 ml	1.42	1.48
Manikchand Plain	8	1.56	1.82
Manikchand Plain	40	1.82	1.96
Manikchand Plain	100	2.26	3.64
Manikchand with Zarda	8	2.18	2.62
Manikchand with Zarda	40	2.62	2.88
Manikchand with Zarda	100	3.82	4.66

10 Animals in each group

In forcebly fed mice the different dose dependent and dose duration impaired effects of sperm head abnormalities were exponential, being very significant ($p<0.01$) between bland and blended zarda groups even when compared to the three dose levels within the same category.. Since *PM* is a mass consumption without a restriction on the quantum, among well to do as well in labor class as a brain stimulant and this type of conditioning, may be to avoid smoking or to overcome exhaustion, besides reflex stimulation and its constituents causing tissue damage. This is especially on the male reproductive organs on long time exposure in adults and developing children. *PM* oral feeding in animals cause not only sperm head abnormalities but also significantly increase frequency of breaks at high doses. Its constituents, betel nut which is the prime component which has terratogenic effects mainly attributed to arecoline. Arecanut alkaloids and tobacco specific N-nitrosamine have cytotoxic and clastogenic potential and deleterious to the testis (Sinha A and Rao AR (1985). This study on the histology of mouse during chronic oral exposure to *PMP* and *PMT* reveals presence of sperm in the lumen of testicular tubule of *PMP* treated group while it was not observed or very few in *PMT* treated group. Thus the histological changes in the present study may be due to adverse effects of various ingredients of *PM* especially arecanut alkaloids and tobacco specific nitrosamine on the cell compartment of reproductive system as revealed by the histological alterations in this spermatogonia cells in the testis (S.K.Nigam and H.Venkatakrishna-Bhatt (2006).

The results of this study endorse further confirmation by studying the reproductive and endocrine system and their markers on different species of mammals using various doses of *PM* with different scheduled and time intervals (S.K.Nigam and H.Venkatakrishna-Bhat (2007). Currently, no reports are available regarding reproductive effects of *PM* in humans since reproductive dysfunction if any due to *PM* may go unnoticed due to lack of appropriate data of PM composition, its consumption both in quantity and duration. Henceforth well knit public health (case control, cohorts) studies are desired.

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