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Effcets of Synthetic Food Colourants on Spermatogenesis and Alteration in Cauda Epididymal Sperm Characteristics of Swiss Albino Mice

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Abstract: The present study examined effects of Azorubine (E122) and Amaranth (123) on male reproductive system of Swiss albino mice. Control (Group I, n=20) was treated with vehicle. Two treatment groups based on azorubine (Group II) and amaranth (Group III) treatment were designed with sub-groups for doses $^{1/2}LD_{50}$ and $^{1/4}LD_{50}$ (Group IIa, Group IIb, Group IIIa and Group IIIb) consisting of 20 animals each. Five animals from each group and sub-group were sacrificed at 7th, 14th, 21st, and 28th day of administration. Body and testis weight were measured, along with cauda sperm characteristics and testicular histology. Level of reproductive hormones were also examined. Results showed significant deterioration of sperm characteristics after treatment with both dyes. Major types of sperm deformities noticed in azorubine and amaranth treated animals were broken tail, bend tail, cytoplasmic droplet, head-tail separation, coiled tail, and headless sperms. More than 30% decline in motility and abnormality was noted in animals treated with amaranth. Histological observation indicated cytotoxic damages in amaranth treated mice, while azorubine treated animals exhibited large scale disorganization of germ cells and interference in spermatogenesis. Conclusively, azorubine and amaranth at the doses used in this study were detrimental to spermatogenesis. Deformities found in the cauda epididymal sperms indicated consequential impact on the rate of fertility.

Keywords: Azorubine, Amaranth, Spermatogenesis, Sperm characteristics, Germ cells, Reproductive hormones

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Introduction

Food colourants are the most organoleptic compounds that has maximum impact on the consumer's appeal. It provides visual cue relating to quality, taste, and emotion of consumer. Following 1950s, chemical food colourants were introduced in the food industries mainly containing petroleum-based ingredients for its cheap and wide-range of colours. Use of food colourants is global, involving large number of food products including ice creams, ice lollies, candies, desserts, cakes, snacks, soft drinks, pastries, and confectioneries (Lidon and Silvestre, 2007, 2010; Commission Regulation, 2011). There is a huge demand of these food products in children which increases the risks of side-effects

as body weight to dose ratio decline sharply with respect to older consumer (Asif *et al.*, 2021). In addition, food colourants can have both short-term, and long-term side-effects such as; allergic reactions and behavioural alterations (Silva *et al*., 2022).

There are stringent measures and regulation for use of food colourants based on doses and types of colourants list of acceptable dyes varies substantially between nations. Some of the most used food colourants are Riboflavin (E 101), Tartrazine (E 102), Carminic acid (E 120), Erythrosine (E 127), Indigotine (E 132), and Brilliant blue FCF (E 133) (Merinas-Amo *et al*., 2019). Despite acceptability of food colours and prescribed daily doses much of the above dyes have been found related to adverse effects such as-- DNA damage (Sasaki *et al.*, 2002), hyperactivity (McCann *et al*., 2007), alteration in central nervous system (Novembre *et al*., 1992), allergic reaction (Wüthrich *et al.*, 1997; Inomata *et al.*, 2006), altered cognition and behaviour (Ganesan *et al.*, 2011) and testicular toxicity (Dixit and Goyal, 2013) etc.

Although there are not much scientific evidences that relate food colourants to human fertility, however, recent study by Wopara *et al*. (2021) reported that synthetic food dyes (Tartrazine and Erythrosine) could distort general tissues in testis and caused shrinkage of seminiferous tubules. Another study by Gray *et al*. (1992) reported that maternal exposure of azo dye could reduce size of testes in male offspring and induced sub-fertile ovaries in female offspring. Spermatogenesis is a complex process that involves multiple cell division (mitotic and meiotic), morphological transition into mature spermatozoa, and synchronization of hypothalamic-pituitary-gonadal axis regulated hormones. Thus, slight modification in the cellular milieu can variably impact spermatogenesis at various stages. In the present investigation, two azo dyes namely; Azorubine (E 122) and Amaranth (E 123) were evaluated for its possible effects on male reproductive system. Both dyes are allowed to use in certain food and food products under stringent dose conditions. The Codex Alimentarius has not listed any provision for Azorubine (FAO/WHO, 2019), while Amaranth has been associated with cancers (Jusufranic *et al.*, 2014) and banned in many countries. It was hypothesized that both azo dyes have distinct effects on spermatogenesis and cauda epididymal sperms.

Materials and Methods

Test food dyes:

Azorubine ($C_{20}H_{12}N_2Na_2O_7S_2$) (CAS No.: 3567-69-9) and Amaranth ($C_{20}H_{11}N_2Na_3O_{10}S_3$) (FD and C Red Dye No. 2; CAS: 915-67-3) were purchased commercially from Sigma Aldrich (MO, USA). Chemical structures of both dyes are shown in Figure 1.

Animal model and ethical approval:

In the present study, male Swiss albino mice (*Mus musculus*) of age 6-8 weeks were used. Randomly bred male animals of proven fertility were selected for the study. These animals were maintained in polypropylene cages of size 43 x 27 x 15 cm. Animals were kept in departmental facility, provided equal hours of light and dark ratio. Drinking water was provided *ad libitum*. Animals were maintained under supervision of veterinary expert. All experiments were carried out according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, 2010). Ethical approval for parameters and protocols were obtained from Institutional Animals Ethics Committee (IAEC). In addition, guidelines of Indian National Science Academy, New Delhi for Care and Use of Animals were strictly followed.

Experimental design:

Based on median lethal dose evaluation (LD_{50}), two doses each of azorubine and amaranth were examined. These two doses were ½ and ¼ of the LD_{50}. Calculated doses for azorubine were 1500 and 3000 mg/kg body weight, whereas, for amaranth were 1200 and 2400 mg/kg body

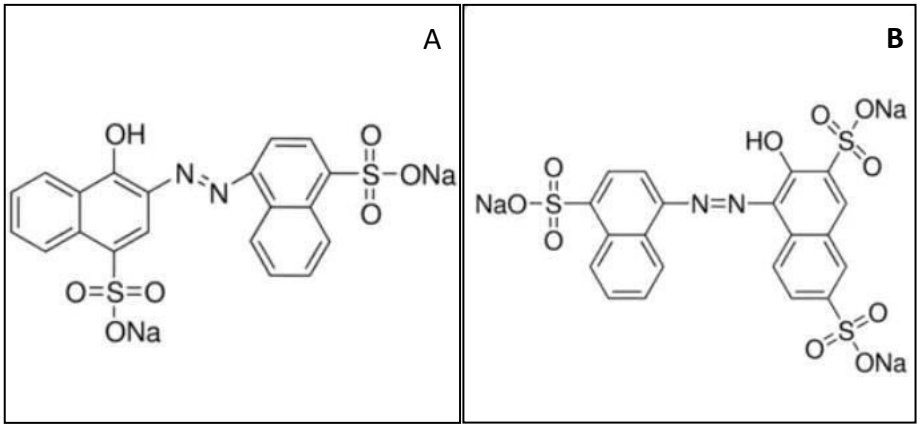


Fig. 1: Chemical structure of Azorubine (A) and Amaranth (B).

Table 1: Experimental groups and specifications

Groups	Specifications*
Group I	Control (vehicle administered)
Group IIa	Administered daily with $^{1/4}LD_{50}$ dose (1500 mg/kg b wt) of azorubine
Group IIb	Administered daily with $^{1/2}LD_{50}$ dose (3000 mg/kg b wt) of azorubine
Group IIIa	Administered daily with $^{1/4}LD_{50}$ dose (1200 mg/kg b wt) of amaranth
Group IIIb	Administered daily with $^{1/2}LD_{50}$ dose (2400 mg/kg b wt) of amaranth

*Five animals from each group were terminated from treatment and sacrificed at 7th, 14th, 21st, and 28th day of administration.

weight. Experimental parameters were carried out at four periodic intervals i.e. 7th day, 14th day, 21st day, and 28th day of exposure. Five groups were designed consisting of 20 animals each. From each group 5 animals were terminated at each periodic interval. Doses of azorubine and amaranth were dissolved in ddH_2O and administered through oral gavage. Control animals were treated with vehicle only (i.e. ddH_2O). Table 1 illustrates groups and their respective specifications.

Body and organ weight:

Body weight was recorded at the commencing and termination day of experiment. Following schedules sacrifice, testes were dissected out and weighed, an average of both right and left testes were recorded.

Cauda sperm characteristics and morphology:

Sperm samples were collected by chipping off cauda epididymis in 1 ml normal saline. A drop of the sample was placed on the slide and count, motility and abnormality were recorded according to the guidelines explained in WHO method manual (WHO, 1999). This sample was further washed thoroughly and examined morphologically by Papanicolaou staining (WHO, 1999).

Testicular histology:

A portion of testis dissected and fixed in 10% formalin, dehydrated in ethanol, cleared in xylene and embedded in paraffin wax. 5 µm thick sections were cut and fixed onto a glass slide. Tissue samples were then stained with haematoxylin and eosin for light microscopic observation.

Hormonal analysis:

Level of testosterone, follicle stimulating hormone and luteinizing hormone were estimated by commercially available ELISA kits.

Statistical analysis:

Numeric values of parameters were represented as Mean±SE. One way ANOVA in combination with

Tukey's multiple comparison were applied to estimate deviation in variance (MINITAB, Pennsylvania, USA). Box plot was made to observe change in body weight during period of investigation (EXCEL, Microsoft, USA).

Results

Body and organ weight:

Body weight of animals administered with azorubine and amaranth indicated significant decline in weight gain. Animals treated with azorubine showed slightly nominal decline in weight gain by 28th day comparing those administered with amaranth. On 28th day mice treated with azorubine ¼LD_{50} and $^{1/2}LD_{50}$ showed body weight of 21.30±0.19 and 20.32±0.38 g, respectively against control which was 25.07±0.51 g. Thus, at least 15-20% decline in weight gain was recorded in mice treated with azorubine. In contrast, body weight of animals administered with amaranth on 28th day was measured as 21.66±0.22 and 19.87±0.13, respectively, amounting to nearly 18-25% decline when compared with control (Fig. 2A). The box plot also indicated black negative boxes for higher doses of both food dyes, indicating gross toxicity (Fig. 2B).

Since, body weight gain in animals administered with both investigated dyes were depreciated, it was expected to see decline in testis weight. Expected results were observed for higher dose of azorubine and both doses of amaranth. Testis weight in both groups showed substantial decrease indicating impact of daily doses, however, direct influence was not confirmed. Maximum decline in testicular weight was observed following 28 days of continuous exposure of amaranth. Similar results were observed for azorubine too, however, this effect was only limited to higher dose (Table 2).

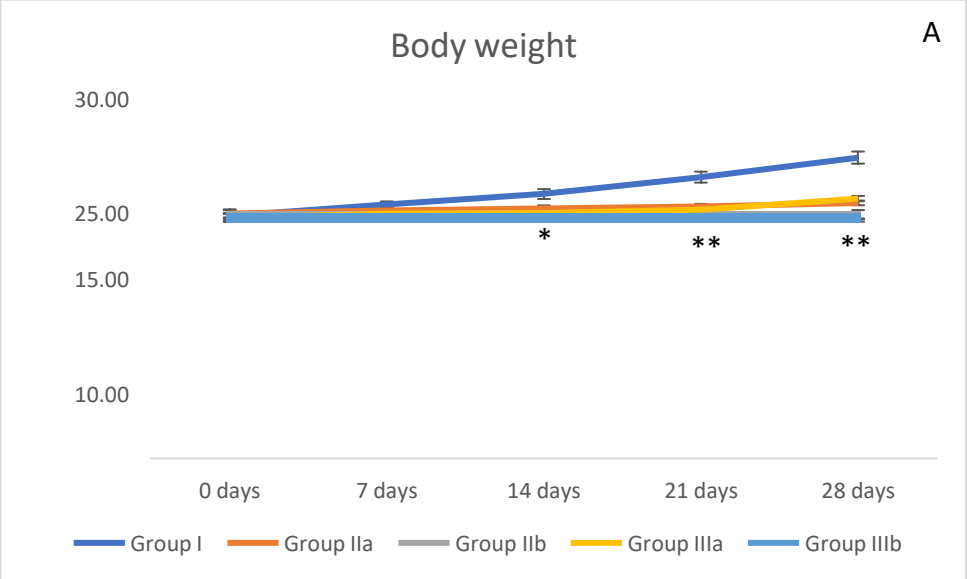
Sperm characteristics and morphology:

Observation of density, motility and abnormality in animals exposed to azorubine and amaranth clearly showed impact of varying degree. Where cauda epididymal sperm density was least affected by both food dyes, indicating low interference in sperm storage and production. Significant reduction in sperm density was evident in animals administered with amaranth, mostly following 21 and 28 days of daily doses (Fig. 3A). Unlike density, motility and abnormality in cauda epididymal sperm was mostly invariably present at all intervals after exposure to both dyes. However, highly significant decline (>30%) in motility was observed only in animals administered with amaranth (Fig. 3B). Likewise, most damages in cauda epididymal sperms were evident at maximum doses of amaranth. Although percentage abnormality in cauda epididymal sperm was significantly higher in all dose groups of azorubine treated animals, but the increase in abnormality was extremely high in animals treated with amaranth (20-30% higher following 21-28 days of administration) (Fig. 3C).

Types of abnormalities observed by Papanicolaou staining in cauda epididymal sperms was broken tail, irregular plasma membrane around sperm tail, bend tail, cytoplasmic droplet, head tail separation, coiled tail, and tails without head (Fig. 4). Although all types of abnormalities were present in all groups nonetheless, appearances of some abnormalities were predominantly present in amaranth treated animals, such as-- head less sperm, coiled tail and broken tail (from mid-section) (Fig. 4).

Histological observations:

Typical damages were witnessed in histological architecture of testicular tissue. A progressive disorganization in germ cells order was apparent in animals treated with azorubine. Lumen of seminiferous tubules contained low sperm as compared to control. In most cases basal lamina was either thin or disintegrated. Germ cells appeared to have fallen into the lumen, indicating severe interference in spermatogenesis (Fig. 5). Leydig cells and Sertoli cells were mostly unaltered indicating possibilities of resumption following withdrawal of treatment. On the other hand, disoriented spermatogonia and number of



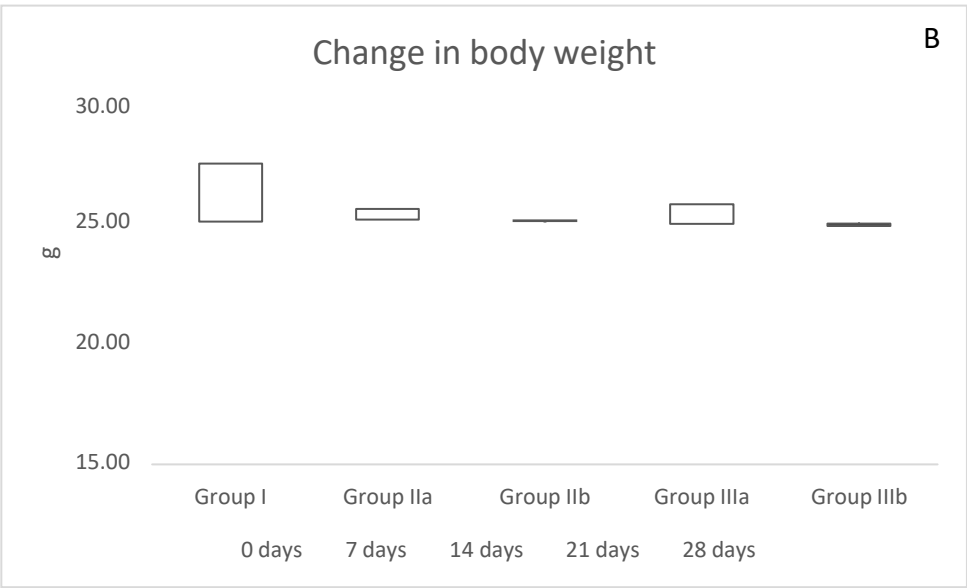


Fig. 2: (A) Body weights (g) were measured for each group at various time intervals. (B) Box plot was drawn to observe increase in weight with respect to initial body weight (white box increase positive growth and black box indicate negative growth). All comparisons were made against Group I (Control). *P<0.05, **P<0.01 against Group I.

Table 2: Testis weight of animals exposed to Azorubine and Amaranth at periodic intervals.

	7 days	14 days	21 days	28 days
Group I	472.36±27.48	465.09±29.61	473.29±33.56	469.71±21.58
Group IIa	468.39±23.49	458.92±33.09	462.78±35.01	446.61±44.61*
Group IIb	459.71±35.09**	445.66±31.44*	440.29±30.39**	438.77±44.78**
Group IIIa	463.76±30.29*	454.09±35.39*	439.21±28.61**	435.78±28.01**
Group IIIb	455.81±36.29**	453.74±30.61*	432.61±28.49***	429.41±25.8***

Values represented average of right and left testes weight and shown as Mean±SE. *P<0.05, **P<0.01, ***P<0.001 against Group I.

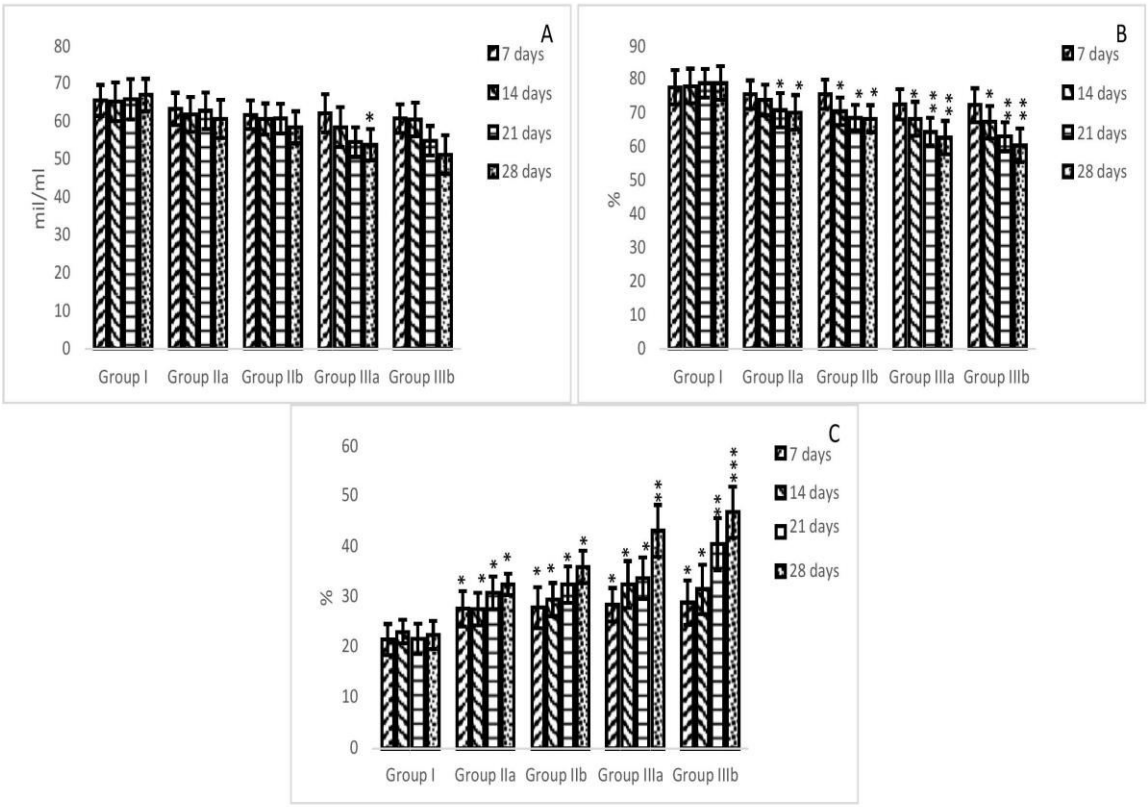


Fig. 3: Sperm characteristics (A: Density, B: Motility, C: Abnormality) of animals treated with Azorubine and Amaranth following administration of variable doses. *P<0.05, **P<0.01, ***P<0.001 against Group I.

amaranth. Appearance of vacuoles in the seminiferous tubules and loss of Leydig cells and basal lamina was common in animals treated with amaranth for longer duration. Unlike azorubine exposure, damages appeared in testicular histology of amaranth treated animals were more permanent and resumption highly unlikely (Fig. 5).

Hormonal analysis:

Interestingly level of FSH in animals treated with azorubine and amaranth were similar. At low dose ($^{1/4}LD_{50}$) although decline in level of FSH was recorded but it was not significant when compared with control. However, at higher dose ($^{1/2}LD_{50}$) highly significant decline in FSH was apparent (Fig. 6A). Level of LH indicated dose dependent decline in animals treated with both azorubine and amaranth. Nonetheless, extent of decline in level of LH was maximum in animals treated with amaranth. Extremely significant decline in LH was recorded in animals treated with both doses of amaranth (Fig. 6B). In contrast, animals exposed to azorubine only showed extreme decline in animals administered with $^{1/2}LD_{50}$ that is too following maximum days of exposure. There was not much deviation between both doses of amaranth, indicative of low variability based on dose (Fig. 6B). Level of testosterone clearly affected by both azo dyes, among which amaranth was more effective in reducing levels by almost 50% when compared with control. Higher dose of azorubine ($^{1/2}LD_{50}$) indicated extremely significant decline in the level of testosterone (Fig. 6C). In all dose groups for both dyes a progressive decline in level of testosterone was apparent indicating dose dependent response. However, at low dose exposure of azorubine animals indicated nominal change in level of testosterone following 7 days of

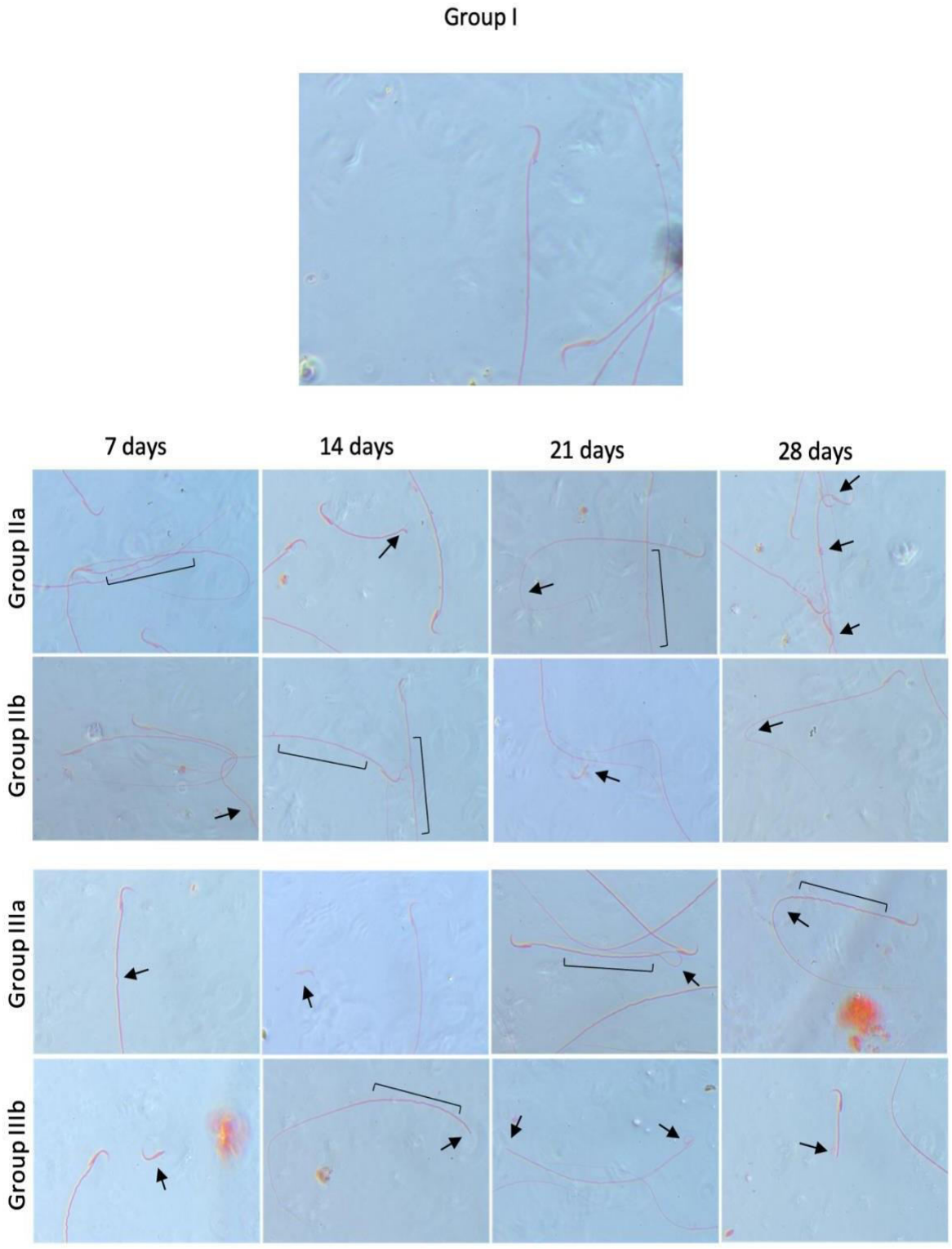


Fig. 4: Cauda epididymal sperm morphology following treatment with azorubine andamaranth, at respective days of exposure. Control was used only for representation as no significant days wise alterations was present. Pointed arrows are showing types of damages predominantly present in the group.

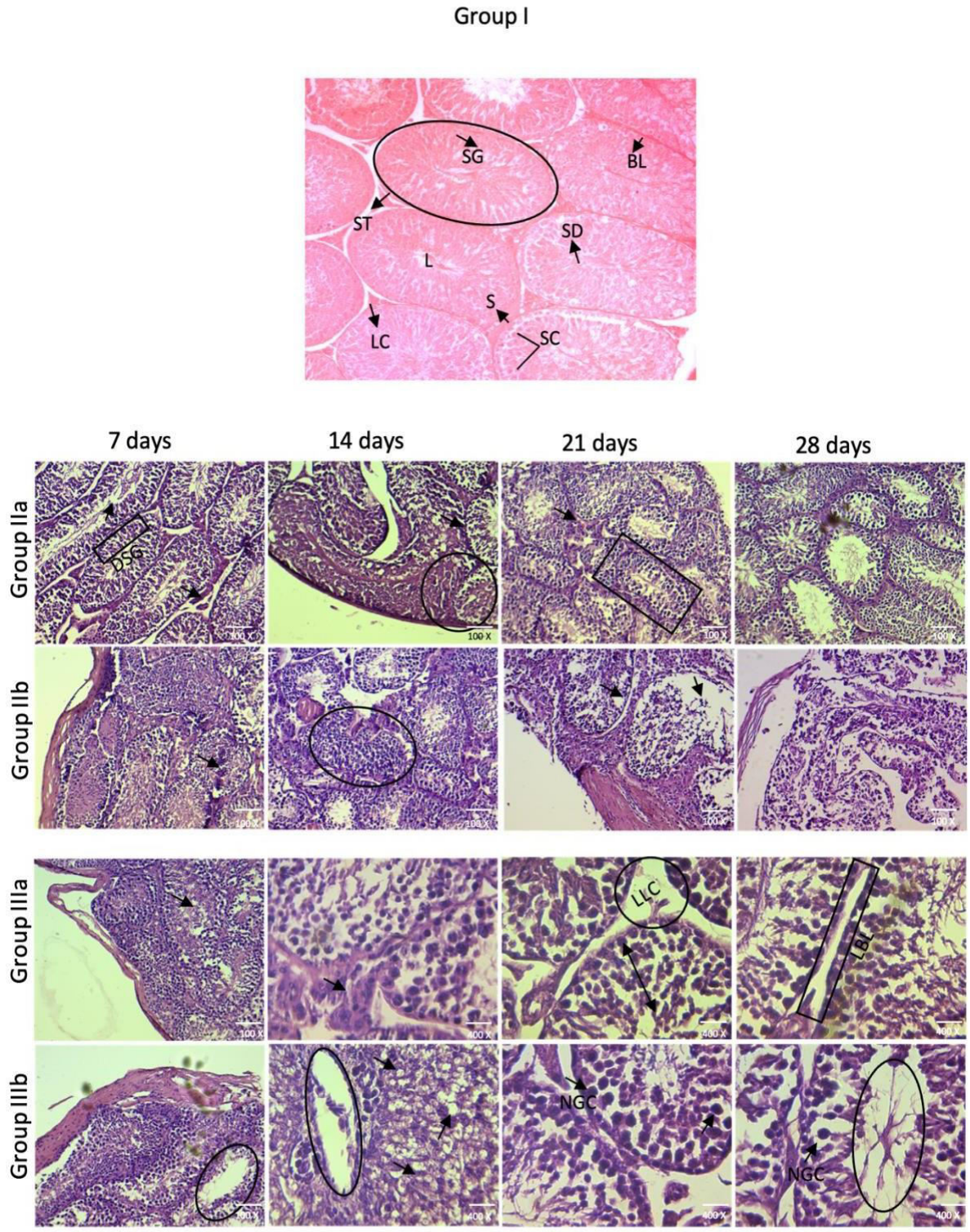


Fig. 5: Histological architecture of testis of animals administered with Azorubine and Amaranth for 7, 14, 21 and 28 consecutive days at various dose levels. Due to similar attributesone representative image is shown for control testis. SG: Spermatogonia; BL: Basal Lamina; L: Lumen; SC: Spermatocytes; LC: Leydig cells; S: Sertoli cell; ST: Seminiferous tubules; DSG: Disoriented spermatogonia; LLC: Loss of Leydig cells; LBL: Loss of Basal lamina; NGC: Necrotic germ cell.

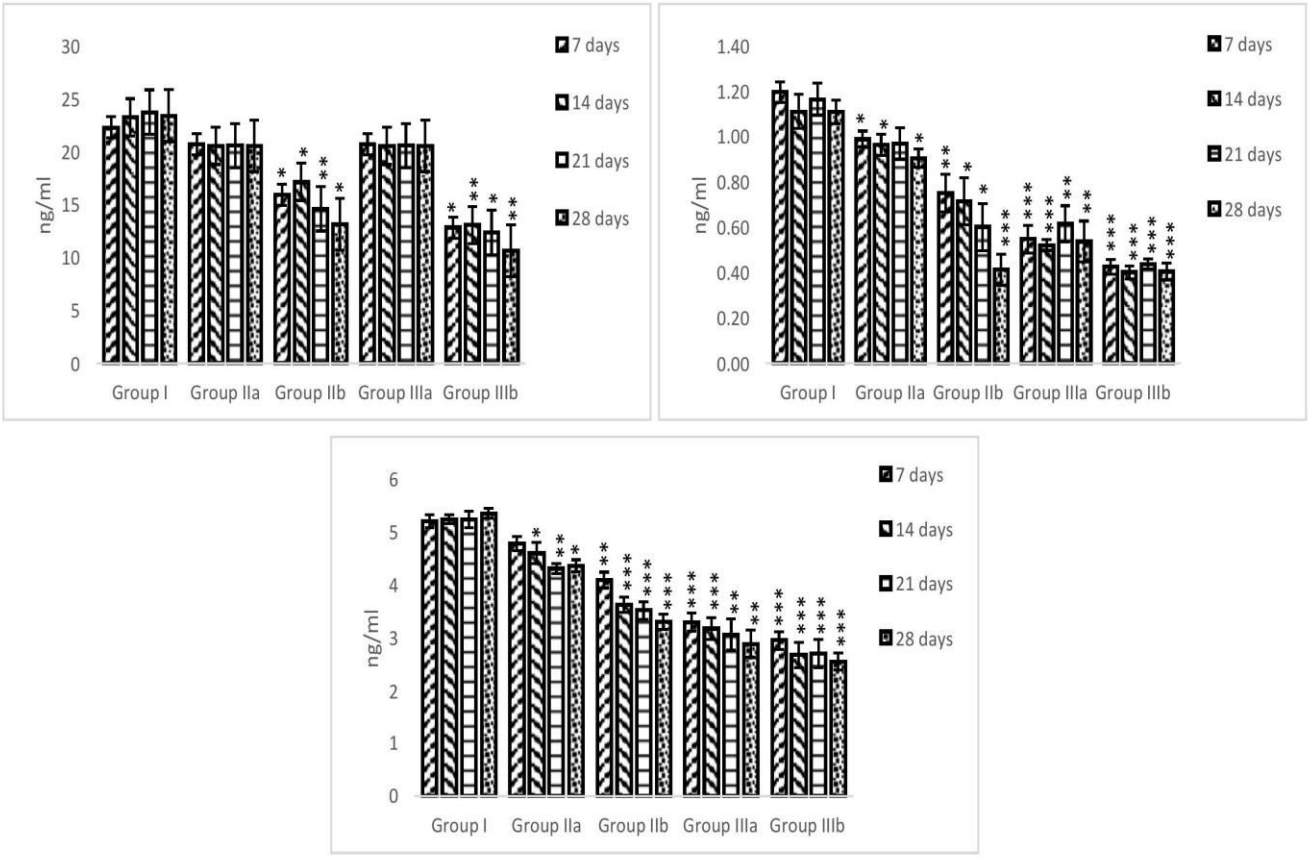


Fig. 6: Level of testosterone and gonadotropins (A: FSH; B: LH; C: Testosterone) in mice administered with Azorubine and Amaranth at periodic intervals. *P<0.05, **P<0.01, ***P<0.001 against Group I.

administration, which remained until 14 days (Fig. 6C).

Discussion

Food colourants are in use since ancient times. During early days less people were interested in how these small amount additives could damage health. However, recently consumers are being more aware of ingredients that are being served by food industries. Many restaurants and food packages are now highlighting ingredients that are potentially allergic to consumers. This awareness has led to a wide range of research on food additives those have major purpose to enhance taste and visual characteristics. Azo dyes are the largest groups of synthetic dyes. Application of these dyes are broad, ranging from printing colours to textile and from cosmetics to food products (Benkhaya *et al.*, 2020). Azo dyes pose grave threat to human health, their cleaved products such as; benzidine induce tumours, p-phenylenediamine is an allergen (Chung, 2016). There are many other known and unknown by-products that have various implications on human health.

In the present study two azo dyes of distinct classification-based health hazard were used to examine its role in reproductive health of male. Where azorubine is a well-established food colourant with no serious side-effects and has clearance from Codex alimentarius, amaranth has been associated with cancer (Omaye, 2004). At the same time some countries allow amaranth as food additives with special provisions. With these two azo dyes, this study intended to scale series of damages that may occur in case it was consumed on daily basis. The present study indicated gross toxicity by both dyes as body weight gain of animals significantly declined during period of investigation. However, $^{1/4}LD_{50}$ dose of both dyes

have relatively lower deleterious effect on body weight comparing to $^{1/2}LD_{50}$ dose. This assumption was in accordance with the study carried out by Holmes *et al.* (1978), who have reported significant decline in body weight gain following administration of azorubine for nine weeks with doses >2000 mg/kg in Sprague-Dawley rats.

This study indicated significant decline in testicular weight. It is to be noted that testicular weight normally remains constant in mature rodents and least affected comparing to other organs under dietary constraints (Greaves, 2007). However, age, malnutrition and toxicity cause independent effects on testicular size and weight (Handelsman and Staraj, 1985). Thus, specific decline in testicular weight of those animals administered with azo dyes is indicative of malnutrition and/or toxicity. A dose dependent decline in testicular wight indicate direct role of dyes in causing independent impact on testicular growth. Previous studies have reported association of azo dyes with intestinal cancers (Gung *et al.*, 1978). Another study by Gray and Ostby (1993) indicated that prenatal exposure of azo dyes can significantly reduce testis weight in mice. Therefore, in this study reduction in testicular weight may have been resulted from multiple factors that includes both malnutrition and testicular toxicity. It was also apparent from this study that reduction in testicular weight was significantly higher in those animals administered with amaranth. It appeared that amaranth impose higher testicular toxicity comparing to azorubine. Although there is no report on testicular toxicity by amaranth but previous studies do claim genotoxic and cytotoxic effects on other organs (Poul *et al.*, 2009). It is possible that it may have specific targeted effect on testicular tissue or other reproductive organs.

Cauda epididymis act as transient storage house for sperms. It is located on the dorsal pole of each testis, any impact on sperm production or change in epididymal milieu can cause significant impact on fertility ability of sperm (Johnson *et al.*, 2010). Although sperm density was mostly unaltered in animals treated with azorubine and early days of exposure of amaranth, it appeared that 28 days investigation could not impact storage pool of sperm. However, on 28^{th} day significant alteration was observed in animals administered with azorubine ($^{1/2}LD_{50}$ dose) and amaranth. Percentage motility in cauda epididymal sperm indicated significant decline following administration of amaranth. This decline in motility was present at all dose levels specifically on days 14, 21, and 28. It appeard that effect of amaranth possibly takes more than week to arrive at cauda epididymis, or damages occurred in sperms during spermatogenesis may predominantly occupy cauda epididymis by second week of exposure. Similarly, azorubine also indicated significant reduction in motility by third week of exposure. Its reduction in motility could be due to damages in tail and/or mid-piece of sperm. Previous studies have noted role of reactive oxygen species in mitochondrial dysfunction, those are present in large numbers at the mid-piece of newly formed sperm (Sanocka and Kurpisz, 2004; Bedard and Krause, 2007). It appears that reduction in motility of sperm in azo dyes administered animals is a result of excessive targeted oxidative stress, however, more studies are required to ascertain this claim.

Abnormality in sperms present in cauda epididymal sperm is normal, these deformities include head tail separation, coiled tail, bend tail and deformities in mid-piece (Bernard, 1985). Nonetheless, disproportionate or predominant number of abnormal sperm indicate specific dysfunction of associated organs, primarily testis and epididymis. The present study indicated extremely high percentage of abnormal sperms in cauda epididymis of animals administered with higher dose of amaranth. Similarly, number of abnormal sperms were also high in animals exposed to azorubine, however, percentage of abnormal sperms were significantly lower comparing to amaranth. Progressive increase in the number of abnormal sperm by both dyes indicated dose- duration of exposure dependent

effect. Without doubt amaranth showed extreme impact on percentage of abnormalities in sperms present incauda epididymis. It was also evident by sperm morphological analysis where damages such as cytoplasmic droplets, head less sperms, coiled tail and un-uniformed plasma membrane was common. It also confirmed earlier assumption that motility in amaranth exposed animals reduced significantly. Most damages were in tail sections of the sperm. Sperm tail malformation is mostly associated with male infertility. Primary ciliary dyskinesia is considered major reason behind malformation of tail structure in oxidative stress (Kobayashi and Takeda, 2012; Linck *et al*., 2016). Besides this genetic autosomal recessive disorder, oxidative stress is another main cause of tail deformities in sperm (sabeti *et al.*, 2016). Previous studies have noted oxidative damages in vital organs of experimental animals exposed to azo dyes. Thus, it may be possible that azorubine and amaranth pose oxidative stress in testicular and epididymal tissues, where effect of amaranth is significantly belligerent comparing to azorubine.

Histological observation of testis indicated distinct pattern of damages in azorubine and amaranth treated animals. Azorubine treated animals mostly showed disorganization of germ cells and interference in spermatogenesis. Amaranth exhibited vacuolization in seminiferous tubules and necrotic germ cells. Observation of damages found in azorubine treated animals resembled damages found in animals administered with herbal contraceptives, such as-- disoriented spermatogonia, disorganization of germ cells, partially filled lumen of seminiferous tubules (Sinha, 1990; Kusemiju *et al*., 2010; Xiong *et al.*, 2011). Histological pattern of seminiferous tubules showing abundance of vacuolization is indicative of cellular toxicity (Shubin *et al.*, 2016). Similarly, presence of necrotic germ cells in the seminiferous tubules is also indicative of toxic effects (Niknafs *et al.*, 2015). It appears that azorubine has more of the obstructive interference in the testicular functions rather than destructive interference of amaranth. Role of period of exposure also played significantly for both dyes in extending interferences during spermatogenesis, leading to substantial reduction in newly formed sperms.

Pituitary gonadotropins regulate steroidogenesis and spermatogenesis by stimulating Leydig cells and Sertoli cells (Schulz *et al.*, 2001). LH regulates steroidogenesis in Leydig cells and FSH maintain spermatogenesis through Sertoli cells. The present study observed significant decline in the level of FSH in animals treated with azorubine and amaranth. Higher dose of azorubine ($^{1/2}LD_{50}$) indicated highly suppressed expression of LH, this could be due to dysfunction of Leydig cells. Histological observation indicated abnormal localization of Leydig cells. In contrast, animals exposed to amaranth showed complete disappearance of Leydig cells which corroborated with level of LH that appeared independent from dose variability. Reduction in level of FSH was dose dependent in both groups of animals (azorubine and amaranth administered), apparently the effect of azo dyes was indirect. There are many studies that associate role of pituitary with azo dyes induced adversities. It is possible that regulation of FSH in exposed animals were linked to other cascade of metabolic interventions.

Testosterone is the main anabolic steroid that is required for development of testes. Absence of testosterone blocks spermatogenesis beyond meiotic stage, thus, plays essential role in male fertility (Haywood *et al*., 2003; De Gendt *et al.*, 2004). The current study noted steady decline in testosterone based on dose and duration of exposure by both dyes. However, decline in groups administered with amaranth were extremely significant comparing to control. It is important to note that testosterone is primarily synthesized in Leydig cells (Oh, 2014), which were absent in most amaranth treated animals. It also explained that lower LH level was directly related to lowering of testosterone. Previous studies have noticed regulation of testosterone biosynthesis by LH, as it stimulates Leydig cells by production of cyclic AMP (Stocco, 2000). Although there was

significant alteration in hormonal homeostasis required for normal spermatogenesis and steroidogenesis, animals exposed to both dyes revealed distinct pattern between each other. Effects of azorubine was substantially lower comparing to amaranth. Based on histopathological study of testis and hormonal analysis, effects of amaranth were most likely irreversible, whereas, damages induced in amaranth treated animals were likely to resume upon withdrwal from treatment.

Conclusion

Based on the observations of this study it is concluded that amaranth pose serious damages to the testicular function and integrity of sperm stored in cauda epididymis. Although azorubine also induced serious disorganization of germ cells and deformities in cauda epididymal sperms, its resumption upon withdrawal was likely. Damages observed in histological architecture of testis indicated were related to oxidative stress and cytotoxicity. Distinctively, amaranth was more cytotoxic comparing to azorubine, thus, damages in testicular tissues were excessive in animals administered with amaranth. Predominant presence of head less sperms and cytoplasmic droplets in sperm tails in amaranth treated animals could be linked to reduction in male fertility rate.

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