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Gestational Exposure to a Commercial Tooth Whitening Agent Containing Carbamide Peroxide Induces Locomotory Changes and Tissue Damage in Mice Newborns

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Author's contribution

The sole author designed, analszed, interpreted and prepared the manuscript.

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ABSTRACT

The present study evaluates the impact of gestational exposure to a commercially available tooth whitening agent in mice newborns. A total of 20 male mice and 60 female Swiss albino mice were used in the study. After mating, 30 pregnant mice were obtained which were divided into three (3) groups. Group I consisted of 6 pregnant mice which received distilled water by oral gavage from the first day of pregnancy until the 15th day after birth; Group I: included 12 pregnant mice which received 200 mg/kg body weight tooth whitening agent and Group III included 12 pregnant mice which received 500 mg/kg body weight tooth whitening agent. Locomotory behaviour, haematological parameters such as Hb, RBC and WBC counts, the antioxidant activity of brain tissue and also histopathology of spleen, liver, gastric mucosa and brain tissues of the control as well as experimental (tooth whitening agent exposed) mice newborns were studied. The locomotor activity of the tooth whitening agent exposed offspring was significantly reduced when compared to the control. Also, the exposed offspring had shown high WBC count and decreased level of

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antioxidants. The results of this study further confirm that gestational exposure to a tooth whitening agent induces oxidative stress and histopathological alterations in gastric mucosa and brain in experimental group.

Keywords: Tooth whitening agent; carbamide peroxide; tissue damage; teeth whitening; locomotor activity.

1. INTRODUCTION

Tooth colour is an important matter for esthetic dentistry professionals as well as for people who want to improve the appearance of their smiles [1]. Currently, there are numerous at home teeth whitening/bleaching systems available in the market which are regarded as safe, conservative and cost-effective esthetic procedure for the treatment of discoloured teeth. Considering the active products available for tooth whitening, carbamide peroxide and hydrogen peroxide are the most commonly used agents for different bleaching modalities which vary according to peroxide concentration, mode of application and time of exposure [2]. When compared to hydrogen peroxide, carbamide peroxide is comparatively more stable which makes the producer to assure product quality and consistency to the consumer. The whitening products available for the tooth bleaching can be applied on tooth surface at the office by a dentist or at home by the patient, under the dentist's supervision. Carbamide peroxide is a widely used and accepted agent for home-use bleaching; the gel is applied to the external surfaces of the teeth using a customised tray. Within one hour of use, approximately 50% of the tooth-bleaching agent is lost from the tray [3] and there is more chance that some of this are likely to be swallowed, which has a potential for adverse effects. In the past, 10% carbamide peroxide was considered as the standard product for the home-use bleaching technique [4]. To increase the efficiency of the whitening agents, currently higher concentrations of carbamide peroxide are used [5]. Rats given a dose of 5 g/kg body weight of proprietary preparations containing carbamide peroxide showed concentration- dependent signs of acute distress, including shallow respiration and impaired mobility [6]. A study carried out with 4 Opalescence tooth whitening products reported 10% carbamide peroxide and 20% carbamide peroxide products because of their much longer exposure period damaged enamel [7].

The increasing demand for tooth whitening has driven many researchers as well as manufacturers to develop and market more and more efficient whitening products to be used either in the dental office or at home. However, as like other artificial dental procedures, bleaching also involves risks [8]. For that reason, the present study focuses on the effect of gestational exposure to a commercially available tooth whitening agent containing 35% carbamide peroxide on the spleen, liver, gastric mucosa, brain and locomotor activity of mice newborns.

2. MATERIALS AND METHODS

2.1 Experimental Agents

The Opalescence® Tooth Whitening Agent (OTWA) containing 35% carbamide peroxide was dissolved in distilled water to a dose- dependent concentration prior to dose administration. Utmost care was given to prevent the exposure of test materials to light, which probably could decompose the peroxide in the test sample. It was administered orally to pregnant mice from day one pregnancy till the fifteenth day after delivery so that the offspring will also get exposed to the test sample indirectly by breastfeeding.200 mg/kg body weight was fixed as low dose, and 500 mg/kg was fixed as high dose based on previous reports [9].

2.2 Experimental Animals

Male and female Swiss albino mice of 8-9 weeks, weighing 25–27g were used in the present study. The animals were obtained from the animal house of the College of Pharmacy, King Saud University, Riyadh, Saudi Arabia and housed in standard cages at normal temperature and 12h light/dark cycle. The mice were given a standard diet and water *ad libitum*. All experiments and protocols were approved by the Ethics Committee for Animal Experimentation of King Saud University which complied with the *Guide for Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85–23, revised 2011).

2.3 Experimental Design and Treatments

The female mice were investigated, and three pro-oestrous female mice were housed with a

male for 12h in a mating cage. A total of 20 male mice and 60 female mice were used in the study. After mating, 30 pregnant mice were obtained and the vaginal plug wasmonitored. Its appearance was considered the first day of pregnancy and each pregnant mouse was isolated until delivery. The pregnant mice were randomly divided into 3 groups as follows:

Group I (Control): Included 6 pregnant mice which received distilled water by oral gavage from the first day of pregnancy until the 15th day after birth.

Group II (Low dose administered mice): Included 12 pregnant mice which received 200mg/ kg body weight by oral gavage from the first day of pregnancy until the 15th day after birth.

Group III (High dose administered mice): Included 12 pregnant mice which received 500 mg/kg body weight by oral gavage from the first day of pregnancy until the 15th day after birth.

Six male newborn mice from each group were sacrificed using ketamine/ xylazine anaesthesia at day 15 and day 30 after birth and samples were collected instantly. Liver, spleen, stomach, cerebrum, cerebellum and medulla oblongata were collected, washed in cold phosphate buffered saline and pieces of collected tissues were fixed in neutral buffered formalin for histological examination. For anti-oxidant activity studies, brain tissues were homogenised in cold phosphate buffered saline, centrifuged and the clear.

The supernatant was collected and stored at −20°C.

2.4 Behavioural Study

2.4.1 Locomotion activity reflex

The locomotor activity of the newborn mice was evaluated on day 25 as previously described by Ajarem and Ahmad [10]. Activities such as the number of squares crossed, duration of locomotion and time of immobility were recorded over 300s in a 30×80×80cm arena.

2.5 Haematological Studies

Blood for haematological analysis was collected in EDTA bottles. The haematological parameters studied include Hb, RBC and WBC count which was analysed using automated blood cell analyser.

2.6 Assay of Antioxidants

Glutathione (GSH) levels and activity of superoxide dismutase (SOD) were assayed in the homogenates of the brain following the method of Beutler et al., [11] and Marklund and Marklund, [12] respectively.

2.7 Histopathological Examination

The fixed liver, spleen, stomach, cerebrum, cerebellum and medulla oblongata samples were processed, and 5µm sections were cut using a microtome. The sections were stained with hematoxylin and eosin (H&E) and examined using a light microscope.

2.8 Statistical Analysis

The data were statistically analysed using IBM SPSS Statistics Version 22 and expressed as mean ± standard error of the mean (SEM). The means were compared using one-way ANOVA followed by Turkey's test *post hoc* analysis.

3. RESULTS

3.1 Locomotor Behaviour

Low and high dose OTWA exposed mice offspring showed a significant decrease in the locomotor behaviour when compared to the control mice which is quite evident from the decreased number of squares crossed, low duration of locomotion and increased time of immobility (Table 1).

Table 1. Effect of OTWA on the locomotory activity of mice newborns

Parameters	Groups	Value
No. of squares broken	Group I	175 ± 6
	Group II	164 ± 7*
	Group III	153 ± 6*
Time of movement	Group I	174 ± 5
	Group II	133 ± 15**
	Group III	134 ± 7**
Time of immobility	Group I	85 ± 4
	Group II	97 ± 8*
	Group III	125 ± 11*

* P<0.05: ** P<0.01 when compared to the control

3.2 Haematological Studies

The blood samples of the mice newborns were analysed for Hb, RBC and WBC count. The Hb and RBC count of the group I and group II mice were less than the control mice, but the differences were not significant. But the WBC count of the OTWA exposed mice newborns was significantly elevated at day 15($p<0.05$) as well as day 30 p(<0.01) (Table 2).

Table 2. Effect of OTWA on the blood parameters of mice newborns

Parameters	Days	Groups	Value
Hemoglobin	15	Group I	8.8 ± 0.2
		Group II	8.1 ± 0.5
		Group III	7.6 ± 0.6
	30	Group I	12.7 ± 0.5
		Group II	11.7 ± 0.8
		Group III	11.4 ± 0. 3
Red blood cells (RBC)	15	Group I	3.7 ± 0.4
		Group II	4 ± 0.1
		Group III	4.4 ± 0.4
	30	Group I	7 ± 0.5
		Group II	7.5 ± 0.3
		Group III	9 ± 0.7
White blood cells (WBC)	15	Group I	3.3 ± 0.1
		Group II	3.8 ± 0.2*
		Group III	5.1 ± 0.8*
	30	Group I	4.1 ± 0.09
		Group II	5.2 ± 0.5**
		Group III	6.8 ± 0.4**

* P<0.05: ** P<0.01 when compared to the control

3.3 OTWA Induces Oxidative Stress in the Brain of Mice Newborns

To analyse the OTWA induced oxidative stress in the brain, oxidative stress enzyme markers such as superoxide dismutase (SOD) and reduced glutathione peroxidase (GSH) were estimated. SOD content was significantly decreased in the brain of low-dose OTWA treated mice at day 15 ($p<0.01$) and day 30 ($p<0.05$) respectively after birth when compared with the control group (Table 3). Administration of the high-dose OTWA also induced a significant ($p<0.05$) decrease in SOD in the brain of mice at both day 15 and day 30 after birth (Table 3). Reduced glutathione level (GSH) showed significant ($p<0.001$) decreased activity in low- and high-dose OTWA administered mice as compared with the control mice at both days 15 and 30 after birth. It is interesting to note that the reduced levels of SOD and GSH in OTWA treated mice were dosedependent.

3.4 Histopathological Findings

3.4.1 Spleen

The histopathological examination of the spleen of the control group mice newborns at D30 (Fig.1 A, B) and D15 (Fig.1.C, D) showed well-demarcated zones of white pulp from the red pulp with a prominent marginal zone and marginal sinus. The central arteriole is visible with uniformly spreading Periarteriolar Lymphoid Sheaths (PALS). Red pulp shows cut sections of few arteries and erythrocytes. Inflammatory changes are absent. Section of the spleen of low dose exposed OTWA exposed mice offspring (Fig. 1. E, F, G, H) showed numerous white pulp zones in the field which are well demarcated from the red pulp with very prominent marginal zones. The central artery is noticeable, but the PALS is not distinguishable as most of the white pulp show uniform leucocytic distribution. Follicles and germinal centers are rarely encountered in white pulp. Marginal zone shows erythropoietic regions with splenic cords and lymphocytic infiltration. Sections in the spleen tissue of high dose OTWA exposed mice newborns at both D30 and D15 (Fig. 1. I, J, K, L) showed a thin capsule surrounding the splenic tissue with occasional streaks of trabeculae and prominent splenic sinusoids with granulocytes, dendritic cells and few erythropoietic zones. Red pulp showed well demarcated splenic cords with lymphocytic infiltration and occasional hemosiderin stains. Some sections at D15 noticed numerous white pulps with congested marginal zones and obliterated central artery.

Table 3. Effect of OTWA on the antioxidant activity in the brain of mice newborns

Parameters	Days	Groups	Value
Superoxide dismutase	15	Group I	9.7 ± 0.7
		Group II	4.5 ± 0.5**
		Group III	3.0 ± 0.2**
	30	Group I	11.6 ± 0.6
		Group II	10.2 ± 0.4*
		Group III	7.2 ± 0. 6*
Glutathione	15	Group I	34.3 ± 0.4
		Group II	32.4 ± 0.5***
		Group III	23.0 ± 0.7***
	30	Group I	37.6 ± 0.7
		Group II	36.3 ± 0.9***
		Group III	22.0 ± 0.9***

* P<0.05: ** P<0.01: ***P<0.001 when compared to the control

3.4.2 Liver

H&E-stained liver sections of the control mice newborns at D30 and D15 showed a central vein with a sinusoid opening into it (Fig. 2. A, B).

The sinusoids are seen lined by hepatocytes with a centrally placed large nucleus. Kupffer cells are also noticed with no inflammatory infiltration. Liver sections of Group II mice infants at D30 and D15 (Fig.2. C, D, E, F) exhibited mild leucocytic infiltration. Rows of hepatocytes subtending sinusoids, central veins and hepatic arteries were visible. Kupffer cells were also seen among the hepatocytes. At D30, hyperemic parenchyma with large venules was seen in the field. Group III mice at D30 showed large venules with uniformly staining parenchyma. Hepatic sinusoids were visible, but the tissue shows some degree of congestion obliterating the sinusoids. Also, a portal triad with a hepatic vein and biliary duct along with its connective tissue were visible (Fig. 2. G, H). In group III newborns at D15 liver sections were seen with scattered central veins). Hepatocytes with a large nucleus and Kupffer cells were also observed (Fig. 2.I, J).

3.4.3 Gastric mucosa

Gastric mucosa of the mice offspring in the control group at D30 and D15 showed intact epithelium with numerous gastric pits lined with mucus- secreting cells, parietal cells and acinar glands. No inflammatory infiltration was seen. Some sections at D30 showed villi lined with columnar epithelium having intervening abundant mucus- secreting cells (Fig. 3. A, B, C, D). Histological examination of the gastric mucosa sections of Group II newborns at D30 and D15 showed larger gastric pits lined with epithelium and large globular mucus- secreting cells (Fig. 3. E, F, G, H). Parietal cells and chief cells were also distributed among the mucosal layer. All the layers of the stomach are well detailed in the slide. At D30, a compactly arranged epithelium around the basement membrane which places over the smooth muscle layer of muscularis mucosae was also noticed. Gastric mucosa sections of Group III mice newborns at D30 showed muscularis mucosae with cut sections of blood vessels containing abundant erythrocytes. Gastric pits were also seen running the entire length of the mucosa with fewer mucosal cells. Parietal cells with eosinophilic cytoplasm and well- defined nucleus were also observed (Fig. 3. I, J). At D15 epithelial cells lining the gastric pits showed large globose mucous cells and parietal and chief cells were seen distributed towards the base of the epithelium. Smooth muscle fibres of the muscularis mucosae, the submucosa were clearly visible (Fig. 3. K, L). Also, the histological evaluation of gastric damage shows a very high losing of epithelial cells,,oedema in the upper mucosa, hemorrhagic damage and presence of inflammatory cells. So, these effects combiningly maximise the cell damage of stomach after exposure.

3.4.4 Brain

3.4.4.1 Cerebral cortex

Histological examination of the sections of the cerebral cortex of group I mice at D30 and D15 showed pyramidal cell bodies with granular neuroplasm with centrally placed nuclei. Glial cells like oligodendrocytes, asterocytes and few microglia were noted in the connective tissue. No leukocytic infiltration was seen (Fig. 4. A, D). Group II mice newborns exhibited degenerative changes in the form of pyknotic cells and also neurocyte chromatolysis was also well evident among the neurons (Fig. 5. A, D). Group III mice offspring showed multipolar neurons with their cell bodies and axons. Few cells with pyknosis and neurocyte chromatolysis were also seen (Fig. G, J).

3.4.4.2 Cerebellum

Control mice group at D30 and D15 showed well demarcated molecular layer and granular layers with the intervening row of Purkinje cells with large flask- shaped cell bodies (Fig. 4. B, E). Molecular layer shows cell bodies of the neurons, few blood vessels and granular layers shows abundant glial cells with no leukocytic infiltration. Group II (Fig. 5. B, E) and Group III mice at D30 and D15 (Fig. 5. H, K) showed few visible pyknotic changes. Granular layer, molecular layer and the intervening Purkinje cells were seen. Oligodendrocytes, asterocytes and microglial cells were visible.

3.4.4.3 Medulla oblongata

Histological examination of Group I mice at D30 and D15 showed abundant medullary neurons and associated glial cells, predominantly oligodendrocytes. The connective tissue was uniformly stained with no infiltration of inflammatory cells (Fig. C, F). In Group II mice newborns at D30 and D15, large multipolar medullary neurons with long axons were seen. Glial cells were noted with few pyknotic cells. Neurocyte chromatolysis was also noticed (Fig. 5.C, F). Group III mice offspring showed numerous multipolar neuronal cells with numerous oligodendrocytes, asterocytes and

sparingly few microglial cells at D30 and D15. Apoptotic changes in the form of neurocyte chromatolysis were also evident among neurons (Fig. 5. I, L).

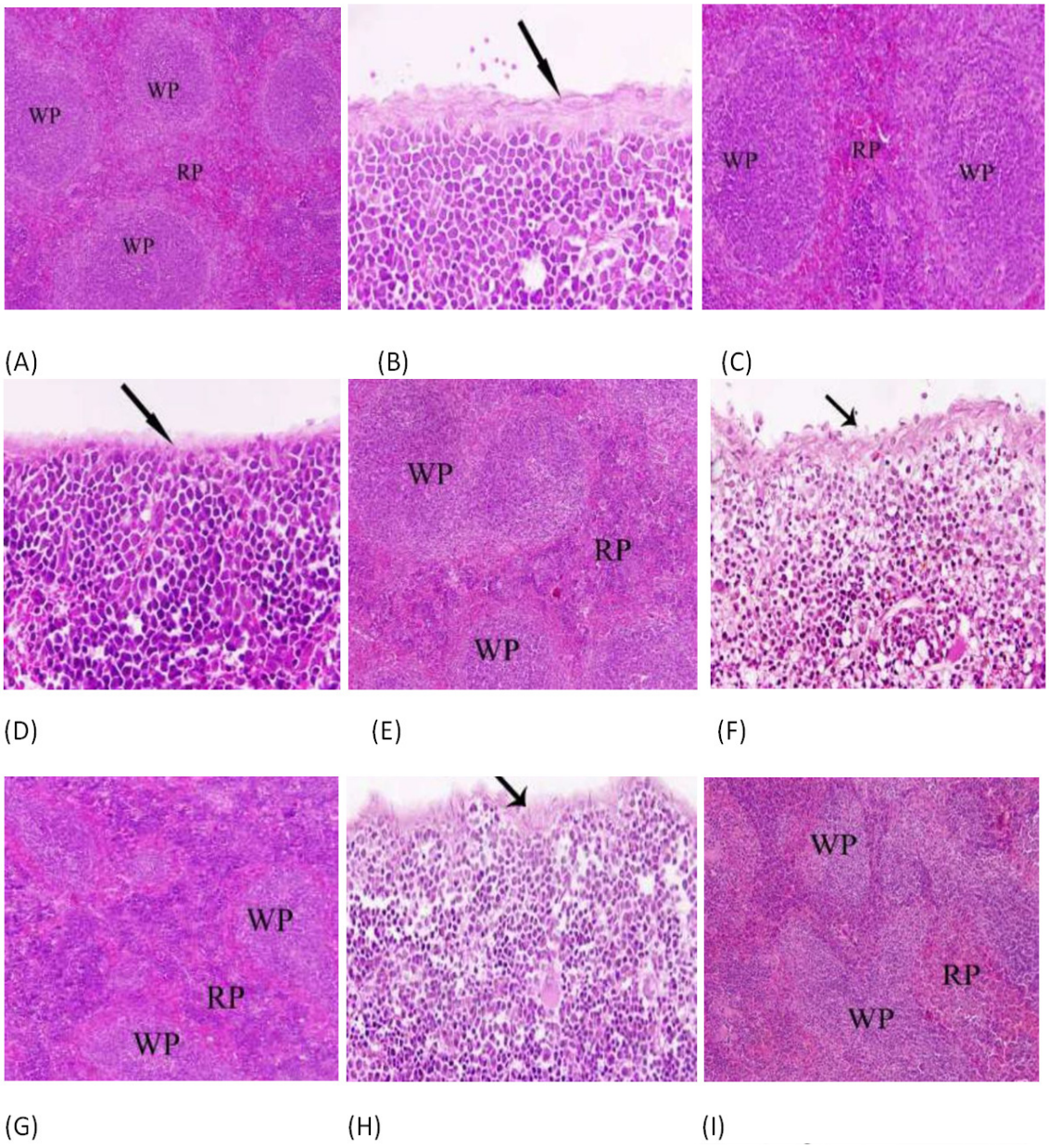
4. DISCUSSION

The concern regarding the safety issues of tooth bleaching was initially raised with increased growth of at-home bleaching. The effects of perinatal exposure to teeth whitening agents have not been previously studied. Hence the present study aims to analyse the impact of perinatal exposure to a commercially available tooth whitening agent containing 35% carbamide peroxide on mice newborns. Carbamide peroxide is the main ingredient in the commercial tooth whitening agents, and it dissociates into urea and hydrogen peroxide in the presence of moisture in the oral cavity [4,13]. The teeth whitening property is mainly attributed to the released hydrogen peroxide. The primary source of the safety concerns with tooth bleaching originated from the known toxicity of H_2O_2, especially its capability to produce free radicals, including hydroxyl radicals [14].

To study the ability of the animals to cope with a new situation can be assessed by examining the changes in their behavioural activities, such as locomotion, immobility etc. [15]. Results reveal that mice newborns exposed to OTWA markedly differ from the control group of mice in the locomotory behaviour since they showed a reduced number of squares crossed, less duration of locomotion and more immobility duration. This might possibly due to the affection of neuronal pathways in cerebellum and cerebral cortex. In support of this, histopathological analysis of OTWA exposed mice offspring exhibited pyknosis and chromatolysis in both cerebellum and cerebral cortex. The study also focused on the impact of OTWA on blood parameters such as Hb, RBC and WBC count of newborn mice. It is already reported that haematological parameters, such as a count of haemoglobin, erythrocytes and white blood cells can be used as indicators of toxicity and have a broad potential application in environmental and occupational monitoring [16,17]. Results showed that perinatal exposure to OTWA decreased haemoglobin count and RBC count which was non-significant and significantly increased the WBC count in mice offspring The increase in WBC count is probably because of the OTWA mediated inflammatory response in various tissues of the newborns which would have caused recruitment of inflammatory cells to these sites thereby causing an initial decrease in the level of circulatory WBC [18]. This initial decrease in the level of circulatory WBC would have probably activated the marrow cells to produce more myeloid precursors which in turn would have brought about the increase in WBC count in the peripheral blood. The inflammatory response was more in the mice group exposed to a higher dose of OTWA which is evident from the increased WBC counts of that group.

In this study, the effect of OTWA on the level of SOD and GSH in the brain tissues of 15 days old and 30 days old mice newborns were assessed and compared to the control group mice infants. It is well known that the safety concerns about peroxide containing whitening agents are mainly associated with potential biological effects of free radicals [19]. It was found that the level of antioxidants in the OTWA administered mice was significantly decreased than the control group. But the levels of SOD and GSH in D30 mice newborns were elevated in comparison with D15 mice newborns. This implies that pregnant mice exposed to OTWA would have caused inflammatory brain changes in the newborns which would have led to the consumption of antioxidants like SOD and GSH thereby decreasing their levels. The relative increase in the SOD and GSH levels in the 30 days old mice newborns suggest that the inflammatory insult caused to the newborn brain would have been minimal and that the inflammatory damage would have decreased by 30 days. Previous studies indicate that oxidative reactions of free radicals with proteins, lipids and nucleic acids, with the consequential potential pathological damage, may be associated with ageing, stroke and other degenerative diseases [20]. The results of this study confirm the role of OTWA in inducing oxidative stress and histopathological alterations in gastric mucosa and brain. Hydrogen peroxide had shown the corrosive effect on the gastric mucosa leading to erosions in the gastric epithelium in previous studies. Apart from certain inflammatory changes, OTWA does not produce any major impact on the spleen and liver tissues of exposed newborns. Most of the histopathological changes shown by OTWA exposed newborns are self-limiting and reversible. Even though, it is evident that OTWA holds the potential of transplacental barrier disruption. Though 15 day sample

showed more inflammatory changes to the glandular tissue, two weeks' time is sufficient enough to heal all those injuries. Previous studies also show that gastric mucosal injuries started to heal by 24 hours itself in adult rats. Being an unstable compound by itself hydrogen peroxide loses its potential as a toxicity inducing agent in organs like liver where centrilobular necrosis was absent even on administration for thirty days. Mild hemolysis may have occurred from hydrogen peroxide toxicity as observed by the increased presence of RBCs and deposition of hemosiderin from worn out erythrocyte metabolism. In neuronal tissue also hydrogen peroxide has shown the potential to cross the blood- brain barrier as was evident by the pyknotic cells and the presence of neurocyte chromatolysis.



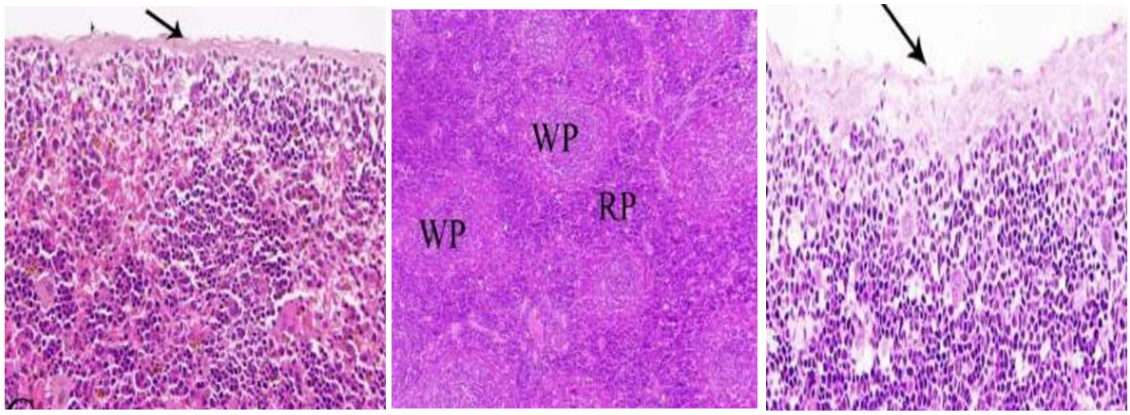
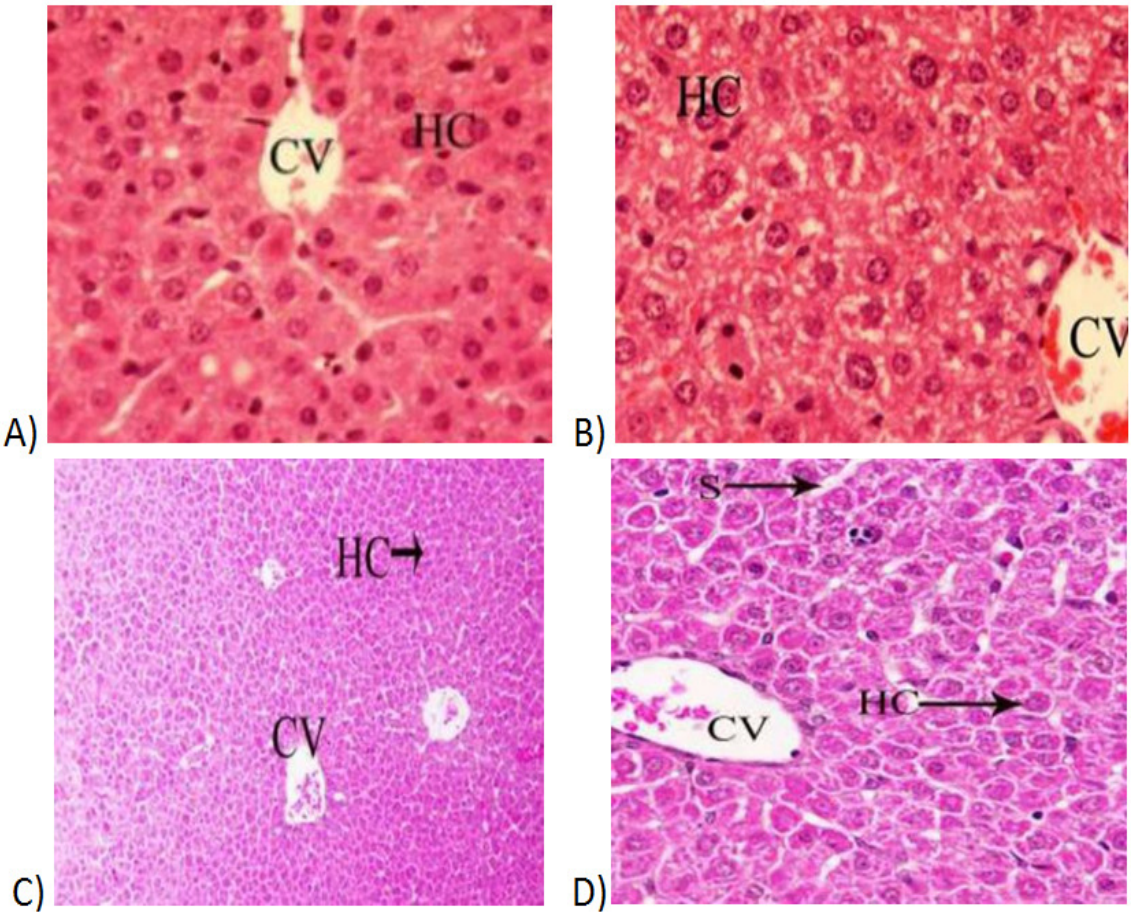


Fig. 1. Histological sections of Spleen: A, B - Group I (30 days); C, D - Group I (15 days); E,F - Group II (30 days); G, H Group II - (15 days); I, J Group III (30 days); K, L Group III (15 days) WP: white pulp, RP: Red pulp: Arrow: Capsule of spleen



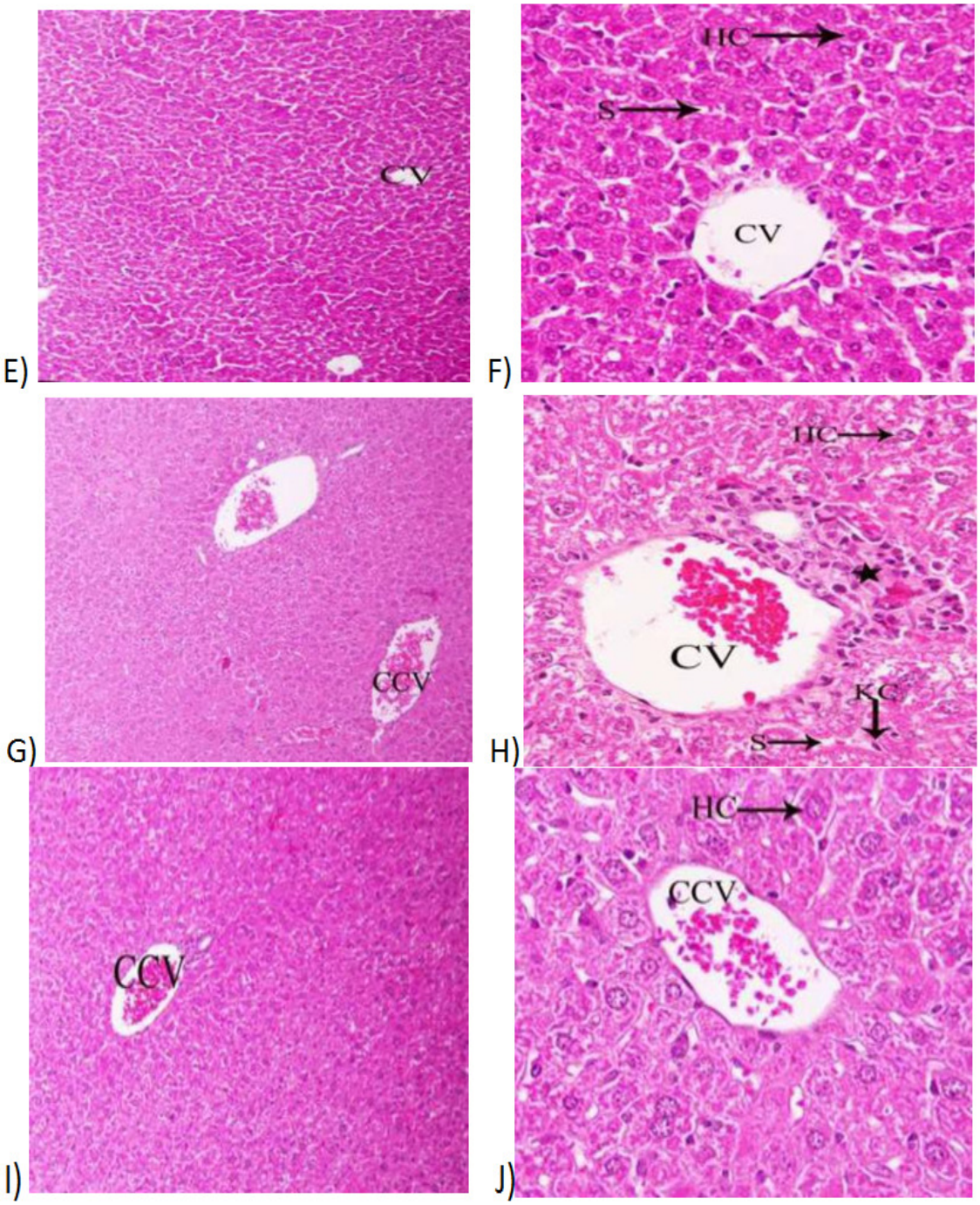


Fig. 2. Histological sections of Liver: A- Group I (30 days); B - Group I (15 days); C, D - Group II (30 days); E, F - Group II (15 days); G, H - Group III (30 days); I, J - Group III (15 days) CV: central vein, CCV: congested central vein, HC: hepatocyte, *: inflammatory cells.

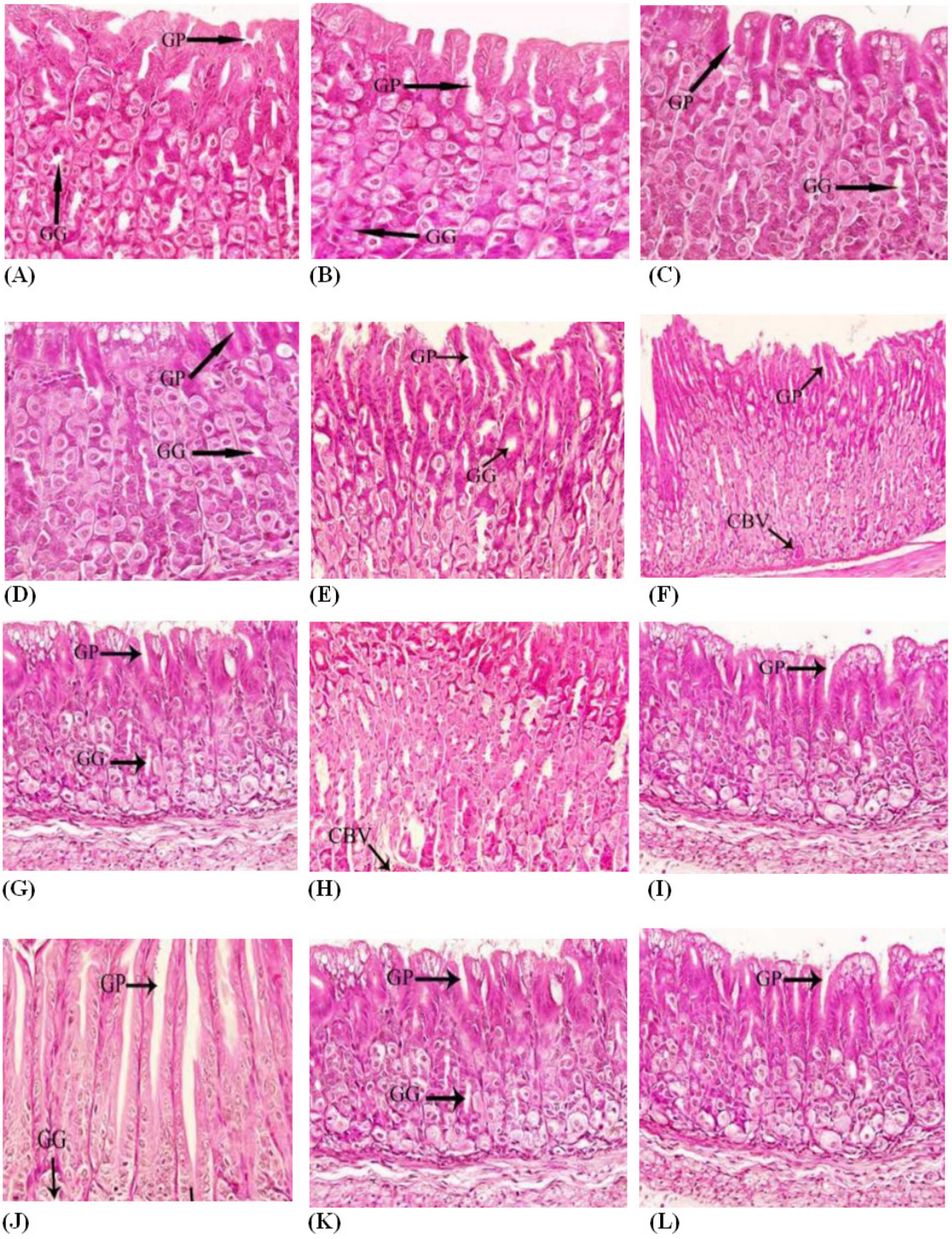


Fig. 3. Histological sections of Gastric mucosa: A, B - Group I (30 days); C, D - Group I (15 days) ; E,F - Group II (30 days); G, H Group II - (15 days); I, J Group III (30 days); K, L Group III (15 days)
GP: Gastric pits, GG: Gastric gland, CBV: Congested blood vessels

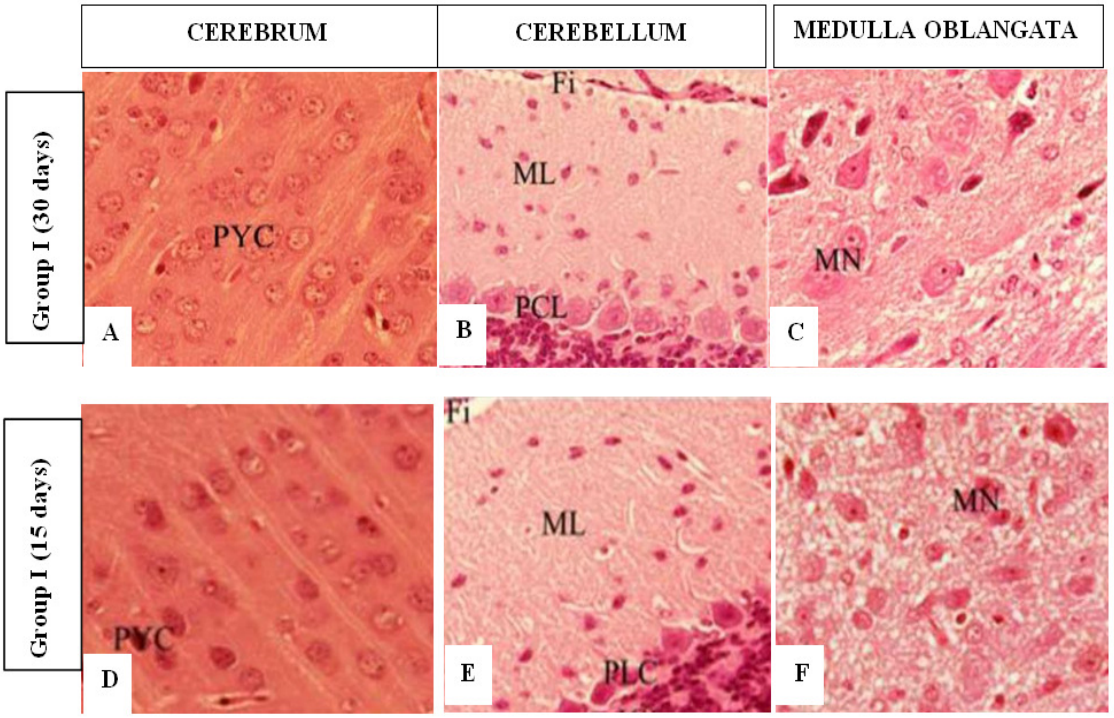
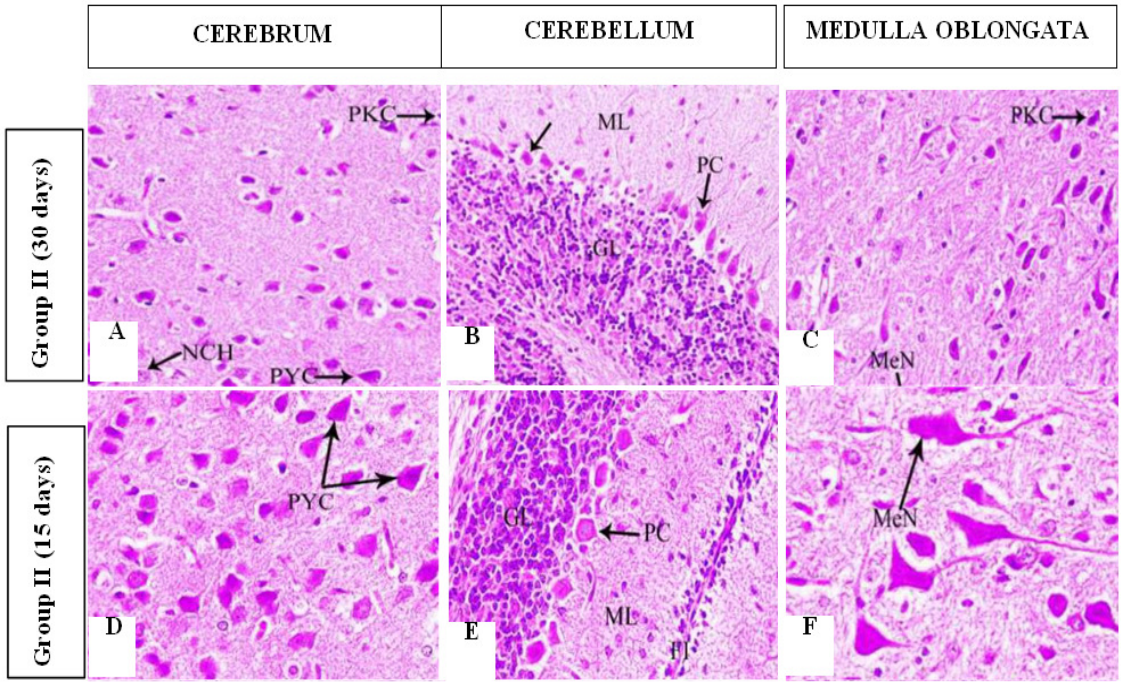


Fig. 4. Histological examination of brain tissues of Control mice
A, B, C - Group I (30 days); D, E, F - Group I (15 days), PYC: Pyramidal cell, FI: fissure, GL: Granular layer, ML: Molecular layer, MN: Medulla neurons



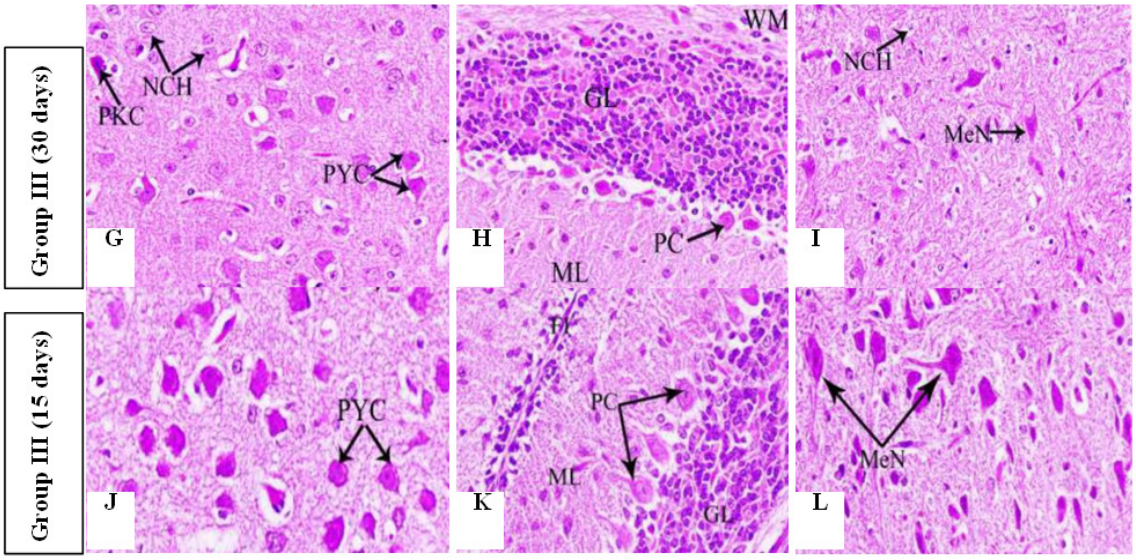


Fig. 5. Histological examination of brain tissues of experimental mice: A, B, C - Group II (30 days); D, E, F - Group II (15 days); G, H, I - Group III (30 days); J, K, L - Group III (15 days PYC: Pyramidal cell, NCH: Neurocytechromatolysis, PKC: Pyknosis, PC: Purkinje cell, FI: fissure, GL: Granular layer, ML: Molecular layer, WM: White matter, MeN: Medulla neurons

5. CONCLUSION

The results of this study further confirm that gestational exposure to a tooth whitening agent induces oxidative stress and histopathological alterations in gastric mucosa and brain of exposed mice offsprings. Finally, the study recommends that limited use of any material should be practiced during the perinatal period because it is a very critical stage and to avoid any negative impact on the new offspring.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the authors.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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