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Histopathologic changes in dental and oral soft tissues in 2-butoxyethanol-induced hemolysis and thrombosis in rats*

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BACKGROUND: 2-Butoxyethanol (2-BE; ethylene glycol monobutyl ether) is extensively used as a solvent in surface coatings, such as lacquers, enamels, and varnishes in industrial and household cleaning products. Its major toxicity is manifested in the circulation, as it induces hemolytic anemia and thrombosis in various organs. While 2-BE has been implicated in the induction of anemia in different species, the rat has proven most sensitive, especially the female of this species. The purpose of this study was to document the effects of 2-BE on dentition, the periodontal ligament, the tongue, the salivary glands, and the oral mucosa in male and female Fischer 344 rats.
METHODS: The experiment included 40 rats divided into five groups. Four groups were exposed to 2, 3, or 4 daily doses of 2-BE, and a fifth group served as control. The rats were killed on days 2, 3, 4, and 29. The teeth and soft oral tissues were prepared for histopathologic observation.
RESULTS: The histopathologic analysis showed that the major effect of 2-BE was exerted on the odontoblasts of the incisors and on molars, with greater effect on the incisors. Foci of damaged muscle cells in the tongue were also observed. The blood vessels were dilated and congested, and a primary thrombosis was seen in the dental pulp.
CONCLUSIONS: The results of this study revealed a resemblance between the dental injuries in this rat model and those seen in sickle cell anemia in humans. This 2-BE animal model holds potential to assist in the discovery of preventive measures and/or treatment for dental injuries that occur in human diseases with hemolytic anemia.
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**This investigation is dedicated to the memory of Prof. Amiram Eldor.*
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Introduction

Thromboembolic complications in dental and non-dental tissues present serious clinical problems observed in human hemolytic disorders such as β-thalassemia and sickle cell disease (SCD) (1–5). We have recently shown that exposure of rats to the compound, 2-butoxyethanol (2-BE) results in a hemolytic anemia and widespread thrombosis and tissue infarction. 2-BE is an ethylene glycol monobutyl ether and a major environmental chemical used in the manufacture of a variety of domestic and industrial products (6).

The 2-BE-related hemolysis in rats is preceded by massive swelling, change in shape of the erythrocyte, and increased hematocrit, suggesting that 2-BE targets the membrane of these cells (7, 8). The disseminated thrombosis involves associated infarction of the coccygeal vertebrae, femur, nasal cavity, cardiac atrium, lungs, liver, and eyes and odontoblasts and pulp of the incisors (9–12). Thrombosis was always preceded by change in the erythrocytic shape 30 min post-exposure, followed by severe hemolysis 4 h later (13, 14). We showed that thrombosis and infarction developed in female rats earlier than in males exposed to the same dose, related to faster metabolic activation to yield the hematotoxic metabolite 2-BE acid (BAA) in the females.

Our mechanistic investigation suggests that interactions of several factors may generate the thrombotic crisis upon 2-BE exposure. Thrombosis may occur following an increase in plasma procoagulant factors, such as tissue factor, fibrinogen, and von Willebrand factor. Disturbed blood flow may result from alterations in rheologic properties of erythrocytes, such as increased

Table 1 Constitution of test groups

Group number	*Group size (n)*	*Treatment*	*Treatment regimen*				*Scheduled killing (day number)*
			Day 1	*Day 2*	*Day 3*	*Day 4*	
1	8 (4M and 4F)	Vehicle control	√	√	√	√	2, 3, 4, and 29[a,b]
2	8 (4M and 4F)	2-butoxyethanol (2-BE)	√	√			2[b]
3	8 (4M and 4F)		√	√	√		3[b]
4	8 (4M and 4F)		√	√	√	√	4[b]
5	8 (4M and 4F)		√	√	√	√	29

[a]Two control animals (1M and 1F) were killed on each day.
[b]Killing was commenced at 2 h after the last treatment.

adherence to the endothelium of the blood vessel wall (15, 16). We are suggesting that the thrombosis in rats may represent a cycle of events in which an initial low level of endothelial activation and/or dysfunction triggered by hemolysis and hypoxia results in additional vascular problems – thrombosis, poor blood flow, infarction, and additional hypoxia.

There are several experimental *in vitro* studies comparing the hematotoxic effects of 2-BE in rat and human erythrocytes. Udden (17) showed that human erythrocytes required exposure to a 100-fold greater concentration of BAA to develop changes in red cell deformability, osmotic fragility, and sodium content similar to those observed in rat erythrocytes. For humans, there are documented clinical cases of hemolytic effects of 2-BE but only a single report of disseminated intravascular coagulation (18, 19).

Similarities between the dental and oral pathology of implicated 2-BE-induced anemia of the rat and hemolytic diseases in human may exist. Pathologic effects of sickle cell anemia on human pulp include necrosis as a result of thrombosis, dilated blood vessels, delayed tooth eruption, and dental hypoplasia (20). Transient pain episodes in the tongue are common clinical manifestations of the disease (21). Severe gingival and periodontal infections also occur during sickle cell crisis (22). The major oral finding seen in thalassemia patients is enlargement of jaws, often expressed as maxillary hypertrophy resulting from increased bone marrow spaces; decreased bony trabeculation; increased pulpar spaces; and, sometimes, short roots (4). In this investigation, we have searched for dental and oral pathologic abnormalities in the 2-BE model that may resemble those found in humans manifesting thrombotic complications.

Various systemic disorders, including metabolic, autoimmune, infectious, and granulomatous, as well as hematologic disorders and intoxication, can affect oral soft tissues (23–25). For this reason, we also examined the effect of 2-BE exposure in submandibular glands and tongue tissue. In an effort to validate further this chemically induced hemolysis and thrombosis rat model that closely parallels human hemolytic disturbances, we followed our previous experimental protocol by exposing rats to daily doses of 2-BE and subsequently examining dental and oral histopathologic evidence of disseminated thrombosis. Thus, our objectives were to scan major dental structures, including incisors and molars, the periodontal ligaments of these teeth, and oral tissues, such as salivary glands, tongue, and oral mucosa, and to document any thrombotic or hemolytic abnormalities observed.

Materials and methods

The chemical, animals, treatments, and experimental procedures

2-Butoxyethanol of >99% purity was purchased from Sigma-Aldrich Chemical Co. (Rehovot, Israel). Fischer 344 rats of both sexes, 11–13 weeks old, were obtained from Harlan Laboratories (Jerusalem, Israel) and allowed a 3-week acclimation period to facility conditions (19–25°C, 30–70% relative humidity, and a 12-h light/dark cycle) prior to inclusion in the study. Animals were maintained on a Harlan Telkad TRM Rat/Mouse Diet; food and water were provided *ad libitum*. All procedures, care, and treatment of rats were in accordance with the principles of humane treatment outlined by the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (26). The study was conducted following the review and approval of the Committee for Ethical Conduct in the Care and Use of Laboratory Animals and after being found in compliance with the rules and regulations set forth. 2-BE dosing solutions were prepared immediately before each administration by mixing 2-BE with tap water to obtain a dose volume of 250 mg 2-BE/5 ml/kg body weight; all doses were given by gavage. At study commencement, test groups of eight animals – four males and four females per group – were dosed with 250 mg 2-BE/5 ml water/kg body weight (Table 1). An additional, equally sized group administered drinking water at a dose of 5 ml/kg served as controls. Control groups received no treatment; test groups received two to four consecutive daily treatments. The animals were observed daily for abnormal clinical signs and weighed just prior to the first dosing and at study termination. Animals were killed by CO_2 asphyxiation 2 h after the last scheduled treatment, except for group 5, which was terminated 24 days following the last dosing. Morphologic changes were evaluated by light microscopy.

Necropsy and tissue handling

Killing were conducted on days 2, 3, 4, and 29; the number of animals are detailed in Table 1. On each scheduled day, eight test animals and two controls were

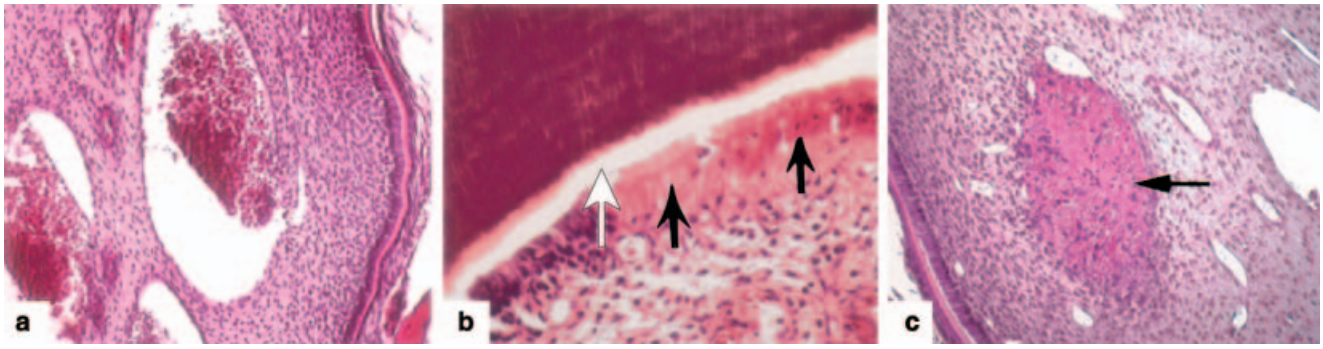

Figure 1 Photomicrographs of incisors in female rats following exposure to 250 mg 2-butoxyethanol (2-BE)/kg body weight (H&E). (a) Dilated blood vessels in the dental pulp, 3 days after exposure (×200). (b) Odontoblastic necrosis (dark arrow); note a well-defined border between necrotic and normal cell (open arrow indicates normal odondoblasts), 3 days after exposure (×400). (c) Organization of thrombi; note presence of fibrin-rich material occluding lumen of vessel (arrow); 3 days after exposure (×400).

killed by CO_2 asphyxiation. A complete necropsy and macroscopic examination were performed on all test and control animals. The upper and lower jaws, as well as the incisors and molars, were separated. The tongue, part of the buccal oral mucosa, and the mandibular salivary glands were removed and weighed. The upper jaw was dissected at three levels as follows: level 1, distal to the incisors; level 2, distal to the incisive papilla; and level 3 between the first and second molars (27). All of the samples were collected and fixed in 10% neutral-buffered formalin. The jaws containing the teeth were decalcified in ethylenediaminetetraacetic acid (EDTA) for 6–8 weeks; decalcification was verified using X-ray images. All tissues were then processed, embedded in paraffin, sectioned at 5–6 μm, and stained with hematoxylin and eosin (H&E) for microscopic examination.

Results

Microscopic changes were observed in the different tissues as described below.

Incisor teeth
Congested and dilated blood vessels and the presence of thrombi constituted the major histopathologic findings in the pulp of the incisors of the treated rats. The vascular thrombi were occlusive and consisted of amorphous fibrinoid material, which was a partly organized admixture of erythrocytes, platelets, fibrin, and other inflammatory cells. These changes showed time-dependent severity with an extensive presence at day 3. A progressive necrosis of odontoblasts was seen with a well-defined border between necrotic and vital cells (Fig. 1a–c). These severe changes were observed only in female rats, while male rats showed but moderate changes in the pulp blood vessels. The dentin exhibited normal and regular morphology of tubules and mineralized structure at all time-points, even in the vicinity of the necrotic odontoblasts. At day 29, all of these changes disappeared completely, and the tissue appearance was normal, resembling that of untreated controls.

Molar teeth
A pattern of changes similar to that observed in the incisors occurred also in molars, but with a lesser severity, including congested and dilated blood vessels and an uneven necrotic layer of odontoblastic cells (Fig. 2a–c). The latter were scarcely present in the narrow root canal of the molars (Fig. 2c). The dentin was not affected by the changes in the pulp or odontoblasts.

Periodontal ligament
Dilated blood vessels similar to those seen in the pulp were the predominant findings in the periodontal ligament of both incisors and molars (Fig. 3).

Tongue
Focal myocytic necrosis was already observed at day 2 of exposure to 2-BE increasing at day 3 with regenerative changes (Fig. 4a,b). Tongue necrosis was more severe in female rats than males, but, at day 29 post-exposure, normal tongue tissue was observed in both sexes (Fig. 4c); however, no thrombi were seen in any of the tongue sections.

Mandibular salivary glands and buccal oral mucosa
No changes in males or females, compared with the controls, were seen in these tissues at any time-point (Fig. 5).

Discussion

The most conspicuous findings revealed in this study were odontoblastic necrosis in the dental pulp of incisors and molars and muscle-cell damage in the tongue; both were probably the result of ischemic events in the blood vessels supplying these tissues rather than a direct toxic effect of 2-BE on the cells. That no changes were observed in the teeth of animals exposed for 4 days and killed 25 days following the chemical withdrawal period suggests complete regeneration of the previously infarcted tissue.

The odontoblastic cell layer is surrounded by a capillary network that penetrates these cells, reaches

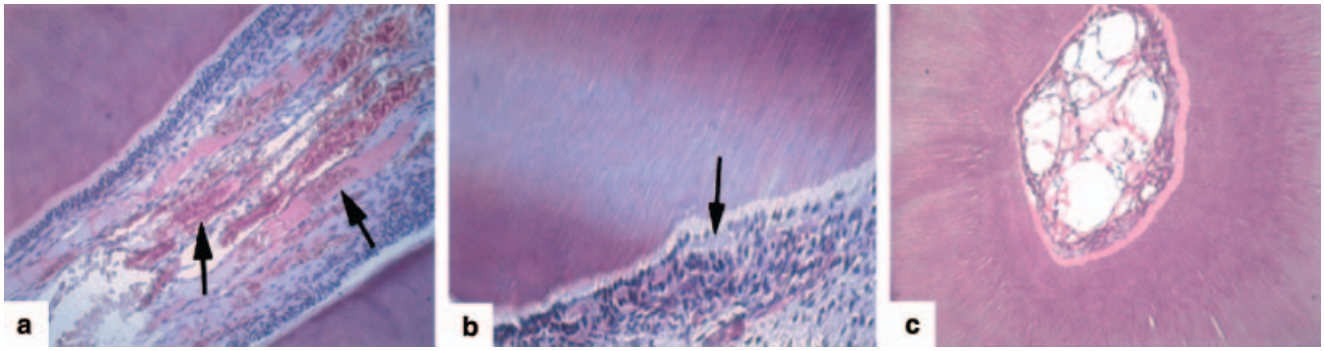

Figure 2 Photomicrographs of molars in female rats following exposure to 250 mg 2-butoxyethanol (2-BE)/kg body weight (H&E). (a) Vascular congestion and presence of fibrin (arrows) in the stroma (×400). (b) Necrotic odontoblastic cells (arrow) within intact odontoblastic cell layer; 4 days after exposure (×400). (c) Excessive vascular dilatation and total absence of odontoblastic cells either because of cell necrosis or repression by the dilated vessels; 4 days after exposure (×400).

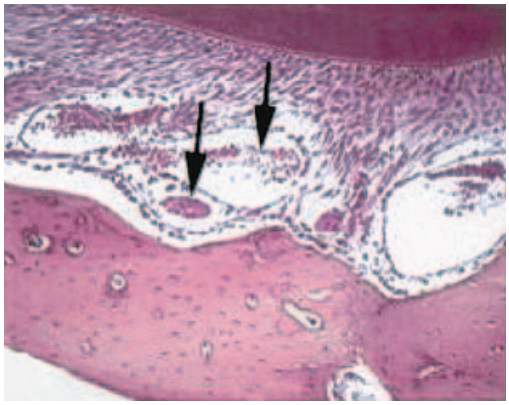

Figure 3 Photomicrograph (H&E) of periodontal ligament in female rat showing dilated blood vessels (arrows); 3 days after exposure to 250 mg 2-butoxyethanol (2-BE)/kg body weight (×100).

the pre-dentin layer, and lacks intercapillary anastomoses (28). Consequently, that thrombus formation led to capillary occlusion resulting in segmental sections of the necrotic odontoblastic layer is suggested. The thrombotic effect of 2-BE on incisor pulp in rats was previously described, although the administration of the material was via inhalation compared with gavage in this study (11). Thus, the mode of exposure to 2-BE does not change the organ affected or the severity of the cellular and tissue injuries (29).

In the present study, the effect of 2-BE on the molars was similar to that on the incisors, but to a lesser extent. This effect is assumed to be the result of the different distribution and organization of capillaries in the dental pulp of rat incisors and molars (30). In the continuously forming dentin of the incisors, the peripheral capillaries are constantly fenestrating, located within the odontoblastic cell layer, and closely related to the secretory activity of the odontoblasts. In the molars, on the other hand, which cease their formation, the capillaries withdraw from the odontoblastic cell layer and are located below it; thus, the reduced effect of 2-BE on the odontoblastic layer in the molars is explicable.

One portion of this work was the examination of oral soft tissue and salivary glands to determine their susceptibility to alterations seen in other soft tissues exposed to 2-BE, such as the nasal mucosa. The most significant changes were seen in tongue tissues of females 3 days after 2-BE exposure. Interestingly, necrosis of muscle tissue was observed with no evidence of thrombi, suggesting local infarct episodes. The tongue is highly vascular and receives a bilateral blood supply from the lingual, facial, pharyngeal, and palatine arteries. In humans, in whom tongue necrosis is a rare event, the

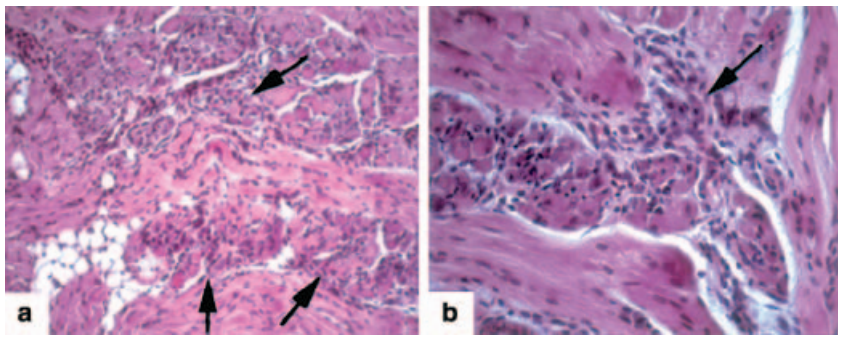

Figure 4 Photomicrographs of tongue in female rat following exposure to 250 mg 2-butoxyethanol (2-BE)/kg body weight (H&E). (a) Focal myocyte necrosis (arrows); 2 days after exposure (×200). (b) Myocyte necrosis with regenerative changes (arrow); 3 days after exposure (×400).

most frequent cause is temporal arteritis (31). The pathogenesis underlying this condition is ischemic damage because of vasculitis of the extracranial branches of the carotid artery (32). Necrosis has also been reported subsequent to the appearance of tongue lesions, tongue carcinoma, abscess of the floor of the mouth, syphilis, Hodgkin's disease, cardiac arrest, and arterial embolism (32–34).

Tongue–muscle necrosis after exposure of rats to industrial chemicals was published previously (25). 2-Methoxy-*p*-phenylanadiamine, a component of oxidative hair dyes, was shown to induce necrosis of skeletal muscle and muscles of the tongue, diaphragm, and gastrocnemius. Histopathologic changes included loss of striations, infiltration by histiocytes, and degeneration and dissolution of muscle fibers. In the case of *p*-phenylanadiamine derivatives, the myotoxicity was due to their one-electron oxidation, while the cytochrome C/cytochrome oxidase system caused disruption of mitochondrial metabolism.

Another human hemolytic anemia disorder with oral manifestations is β-thalassemia (23). In thalassemia patients, iron overload and deposition of iron have been observed in several tissues and organs, including parotid secretory serous salivary glands, resulting in impaired saliva secretion (23). This iron overload resulted from repeated blood transfusions, as well as ineffective erythropoieses and increased red blood cell breakdown. In our work, no deposition of iron or any change in salivary parenchymal tissue was seen and may due to the remarkable ability of parenchymal rat submandibular tissue to preserve blood flow even after ligation of the carotid arteries (35). This ability may be related to the decreases in the synthesis of nitric oxide and its liberation from the vascular supply to the submandibular glands. No investigation, to our knowledge, has examined histologic changes in the tongue tissue of such patients, although Abu Alhaija et al. reported smaller tongue size in thalassemic patients (24).

Odontoblastic necrosis in the dental pulp of incisors and molars and muscle-cell damage in the tongue in the 2-BE-treated rats, with the most severe changes occurring in females, bear strong resemblance to these same pathologic abnormalities observed in human thalassemia and sickle cell anemia. These similarities between the various histopathologic dental and paradental changes suggest that this unique 2-BE model may offer experimental opportunities to elucidate the mechanism(s) involved in the soft oral and dental injuries seen in human hemolytic disturbances. This model might also provide a potential means of discovery of diagnostic, preventive, and therapeutic measures for dental and oral damages that occur in hemolytic anemias in humans.

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