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Article

Effect of docosahexaenoic acid as a chemopreventive agent on experimentally induced hamster buccal pouch carcinogenesis

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Abstract: The purpose of the current study was directed to investigate the effectiveness of docosahexaenoic acid (DHA) as a chemopreventive agent on experimentally induced hamster buccal pouch (HBP) carcinogenesis; Material and methods: 40 Syrian male hamsters, five weeks old, were divided into 4 groups of 10 animals in each as follows, GI: Topical application of liquid paraffin alone (thrice a week for 14 weeks), GII: Topical application of 7, 12 dimethyl benz[a]anthracene (DMBA) alone (0.5 % in liquid paraffin, thrice a week for 14 weeks), GIII: Topical application of DMBA (0.5 % in liquid paraffin, thrice a week for 14 weeks) + Oral administration of DHA (125 mg/kg b.w. in 1 ml distilled water by oral gavage, thrice a week for 14 weeks on alternative days of DMBA application), GIV : Oral administration of DHA alone (125 mg/kg b.w. in 1 ml distilled water by oral gavage, thrice a week for 14 weeks); Results: Gross observations and histopathological findings revealed a-GI: normal stratified squamous epithelium b- GII: well and moderately differentiated squamous cell carcinoma (SCC) c-: GIII: showed variable results ranges from hyperkeratosis, hyperkeratosis and focal hyperplasia, mild dysplasia, and well differentiated SCC with superficial invasion of tumor cells not extended to deeper areas d: GIV: normal similar to GI. Immunohistochemical results revealed that oral DHA treatment to DMBA treated hamsters restored the normal expression of bcl-2; Conclusion: DHA has the potential to be a dietary chemopreventive agent due to its capacity to improve carcinogen detoxification and to block/suppress the initiation and promotion stages of experimentally produced HBP carcinogenesis.

Keywords: Docosahexaenoic acid; Chemoprevention; Bcl-2 family; Experimental Study; Hamster Buccal Pouch Carcinogenesis.

1. Introduction

Oral cancer is the sixth most frequent disease in the world, with roughly 700,000 new cases diagnosed each year and a 5-year survival rate of 40-50 percent [1]. The National Cancer Registry Program of Egypt (NCRPE) reported the national incidence rates of oral cancer accounts for nearly 3% of all cancer cases [2] in which the frequency of oral cancer

in 2015 was 0.9% in males and 0.75% in females [3]. Oral squamous cell carcinoma (OSCC) represent more than 90% of oral cancer [4, 5]. OSCC is a highly complex multifocal disease that requires initiation, promotion, and progression occurs when the oral epithelium is impacted by numerous genetic alterations [6, 7]. OSCC is with many risk factors involved in its development, such as tobacco smoking and alcohol consumption, human papilloma virus (HPV), nutritional deficiencies, immunosuppression, family predisposition and genetic instability [5, 8].

The 7, 12-dimethylbenz [a] anthracene (DMBA)-treated hamster model is the most extensively accepted protocol for chemical carcinogens and buccal pouch carcinogenesis as it results in various histological, neoplastic and morphological changes related to those found in human OSCCs [9]. The hamster cheek pouch is a very helpful tissue for initiating OSCCs via chemical carcinogens and for assessing chemopreventive medicines. Carcinoma is preceded by the evolution of dysplasia carcinoma and hyperplasia papilloma, similar to the leukoplakia seen in OSCC patients [10, 11].

Omega-3 polyunsaturated fatty acids (PUFAs) are considered essential since they cannot be produced by the body; hence, ingestion of omega-3-rich foods such as cold-water fish, nuts, and seed oils is required [12]. Omega-3 PUFA consumption is believed to have favorable effects on inflammation and resolution, owing to the suppression of nuclear factor kappa B (NF-B) and the creation of pro-resolution mediators such as resolvins, protectins, and maresins [13, 14].

Longer chain n-3 polyunsaturated fatty acids are formed of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [15]. Numerous in vitro investigations on cancer cell lines, as well as animal models of cancer, have demonstrated DHA and EPA's anti-proliferative, apoptotic, cytotoxic, and anti-metastatic characteristics [16, 17]. Different mechanisms for DHA and EPA's anti-cancer effects have been proposed, including the induction of reactive oxygen species (ROS) and subsequent lipid peroxidation [18], altering the composition of the plasma membrane and lipid rafts [19], altering the mitochondrial membrane potential [19] and epigenetic modification of genes involved in apoptosis [20].

Additionally, several studies have demonstrated that EPA, and particularly DHA, has the ability to affect cancer proliferation, apoptosis [21, 22] and differentiation [21], as well as, to suppress angiogenesis [14], tumor cell invasion [23] and metastasis [24]. These findings imply that DHA can both exert anticancer action and act as an adjuvant in cancer treatment, as well as alleviate some of the secondary symptoms associated with cancer, such as cachexia [25, 26].

There is evidence that DHA is effective as an anticancer adjuvant, with a particular emphasis on its ability to both increase the uptake of anticancer drugs, particularly in cells that are otherwise resistant to these drugs, and to increase the pro-oxidant and proapoptotic efficacy of some chemotherapeutic drugs Evidence exists on the efficacy of DHA as anticancer adjuvant, with particular emphasis to its capability both to enhance the uptake of anticancer drugs, especially in cells otherwise resistant to these drugs, and to increase the pro-oxidant and proapoptotic efficacy of some chemotherapies [25].

As a result, the primary goal of this study was to evaluate the effect of DHA as a chemopreventive drug on DMBA-induced HBP carcinoma. Gross inspection, histological tumor tissue alterations, and immunohistochemistry (IHC) testing with Bcl-2 antibodies were used to make the determination.

2. Materials & methods

2.1. Animals

40 Syrian male hamsters five weeks old, weighing 80-120g were obtained from the animal house, Cairo University (Cairo, Egypt). The experimental animals were kept in standard cages with sawdust bedding in a controlled environment with humidity (30-

40%), temperature (20 ± 2°C), and light (12-h light/12-h darkness). All experimental animals were given a conventional feed and free access to water.

2.2. Chemicals

DMBA (0.5%) was obtained from Sigma-Aldrich Company, dissolved in paraffin oil. DHA was obtained from Sigma-Aldrich Company.

2.3. Experimental design

The hamsters in the experiments were divided into four equal groups, each with ten animals: GI: Topical application of liquid paraffin alone (three times a week for 14 weeks), GII: Topical application of DMBA alone (the rights HPB were painted with 0.5 % DMBA in paraffin oil using a number 4 camel hair brush three times a week for 14 weeks), GIII: Topical application of DMBA (0.5 % in liquid paraffin, three times a week for 14 weeks) + Oral administration of DHA (125 mg/kg b.w. in 1ml distilled water by oral gavage [27], three times a week for 14 weeks on alternate days of DMBA application), GIV : Oral administration of DHA alone (125mg/kg b.w in 1ml distilled water by oral gavage, three times a week for 14 weeks).

After termination of the experiment, all animals were euthanized, then hamster's head were separated and fixed in 10% buffered neutral formalin solution for 24h after putting a piece of wood with suitable size, between hamster's teeth to prevent a wrinkling or sloughing of the hamster's mucosa during manipulation and preparation. Using a bard-parker scalpel, no 15, small surgical scissors and tweezers, the specimens were excised from right HPB mucosa and trimmed to 1-2 cm average size. Tissue specimens were excised, then processed for hematoxylin and eosin (H&E) and IHC staining as follow:

2.3.1. Histopathological examinations

Tissue sections of 4–5 μm thickness were cut using a rotary microtome and stained with H&E dye for microscopic examination.

2.3.2. Immunohistochemical examinations

Other tissue sections of 3 μm thickness were cut to demonstrate Bcl-2 antibody expression using the usual labelled streptavidin-biotin technique. Dewaxed paraffin-embedded tissue sections were rehydrated in distilled water after being dewaxed in graded ethanol. Incubation with 3 % H2O2 in methanol for 10 minutes blocked endogenous peroxidase. Antigen retrieval was accomplished by mixing citrate buffer solution (pH 6.0) with phosphate buffered saline and microwaving for 3 intervals of 5 minutes each at 95°C (PBS). The tissue sections were then incubated in a humid chamber at room temperature overnight at 4°C with one or two drops of primary antibody Bcl-2 in a dilution of 1:100 in Tris buffer solution. Biotinylated secondary antibody was added after washing with PBS and incubated for 30 minutes at room temperature. Following a PBS rinse, tissue slices were stained with diaminobenzidine (Sigma, USA) for 2-4 minutes to develop color. The slides were washed, counter stained with hematoxylin, and then coated with a mounting media once the color intensity was appropriate.

2.4. Staining interpretations

A light microscope was used to analyze the immunostained sections to determine the prevalence of positive cases and the location of immunostaining within the tissues. In addition, an image analysis computer system was employed to calculate the area % of

immunostaining positive cells. This was done at Al-Azhar University's Oral and Dental Pathology Department, Faculty of Dental Medicine, Boys, Cairo. The degree of positive staining for antibody was assessed using a well-established semi-quantitative scoring system that included strong staining (more than 50% stained), moderate staining (between 25 and 50% stained), weak staining (between 5% and 25% stained), and negative staining (no staining) (less than 5 % stained) [28].

2.5. Statistical analysis

The data obtained from computer image analysis were collected, revised, coded and entered to the Statistical Package for Social Science (IBM SPSS) version 20. The comparison between more than two independent groups with quantitative data was done by using One Way Analysis of Variance (ANOVA) followed by post hoc analysis using LSD test. The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the p-value was considered significant as the following: Non-significant when p-value > 0.05, significant when p-value ≤ 0.05, and highly significant when p-value ≤ 0.01.

3. Results

3.1. Gross observation Results

HBP mucosa of the GI mucosa was pink in color with a smooth surface and no observable anomalies (Figure, 1A). In GII, ulcerative regions, numerous tumor masses, and several nodular elevations were seen in the HBP mucosa. All animals were found to be depilated (Figure, 1B). In GIII, the mucosa of the HBP revealed no abnormalities other than redness at the painting site, and the animals appeared to be in good health (Figure, 1C). HBP mucosa in GIV appeared normal, and almost similar to GI mucosa (Figure, 1D).

3.2. Figure 1 (A, B, C & D)

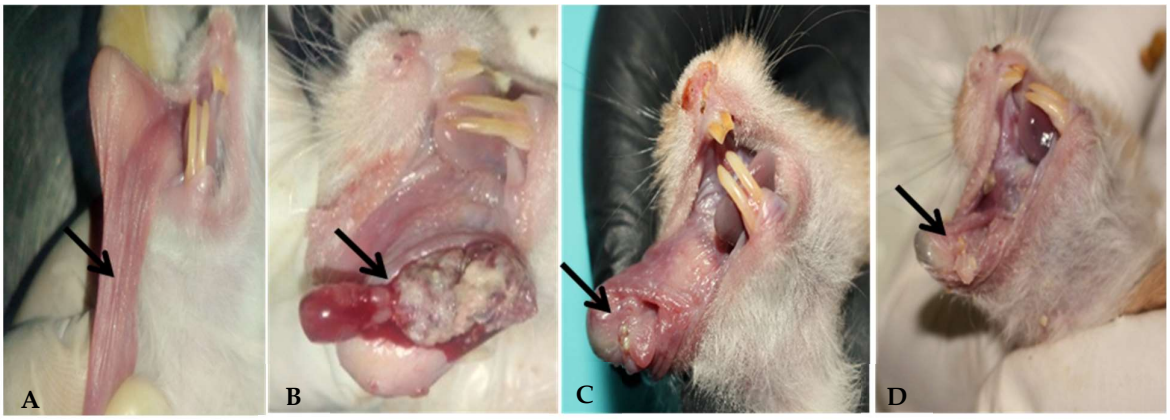

Figure 1. (A) HBP of GI showing normal buccal pouch mucosa which appeared pink in color with smooth surface (arrow); (B) HBP of GII showing large exophytic papillary tumor masses, with spontaneous bleeding (arrow); (C) HBP of GIII showing no observable abnormalities except redness at the painting site, or small exophytic tumor mass (arrow); (D) HBP of GIV showing normal mucosa almost similar to GI (arrow).

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3.3. Histopathological and immunohistochemical results

In GI, HBP mucosa, formed of thin stratified squamous epithelium, consists of basal cells, spinous cell layer, and thin keratinized cells with lacking rete ridges, was revealed in histological sections using H&E stain. The areolar layer, muscle layer, and subepithelial connective tissue (C.T) were seen (Figure, 2A). The Bcl-2 IHC labelling revealed mild (6.6%) positive expression in the basal and suprabasal layers (Figure, 2B). In GII, H&E staining revealed well to moderately differentiated SCC with deeply infiltrating islands of tumor cells into the underlying connective tissue in histological sections. The surface epithelium showed severe epithelial dysplasia. Keratin is either absent or present in trace amounts. In addition, inflammatory cells had infiltrated the underlying connective tissue (Figure, 2C). IHC staining using Bcl-2 exhibited strong positive cytoplasmic expression (mean = 69.7) throughout the tumor cells (Figure, 2D). In GIII, histological sections, using H&E stain, showed variable results ranges from hyperkeratosis (4 animals), hyperkeratosis and focal hyperplasia (2 animal), mild dysplasia (2 animal), and well differentiated SCC with superficial invasion of tumor cells not extended to deeper areas (2 animal). (Figure, 2E). IHC staining using Bcl-2 exhibited moderate positive cytoplasmic expression (mean = 23.2) throughout the epithelial layers (Figure, 2F). In GIV, histological sections using H&E stain and IHC staining using Bcl-2 and Bax expression was almost similar to that of the GI.

3.4. Figure 2 (A, B, C, D, E & F)

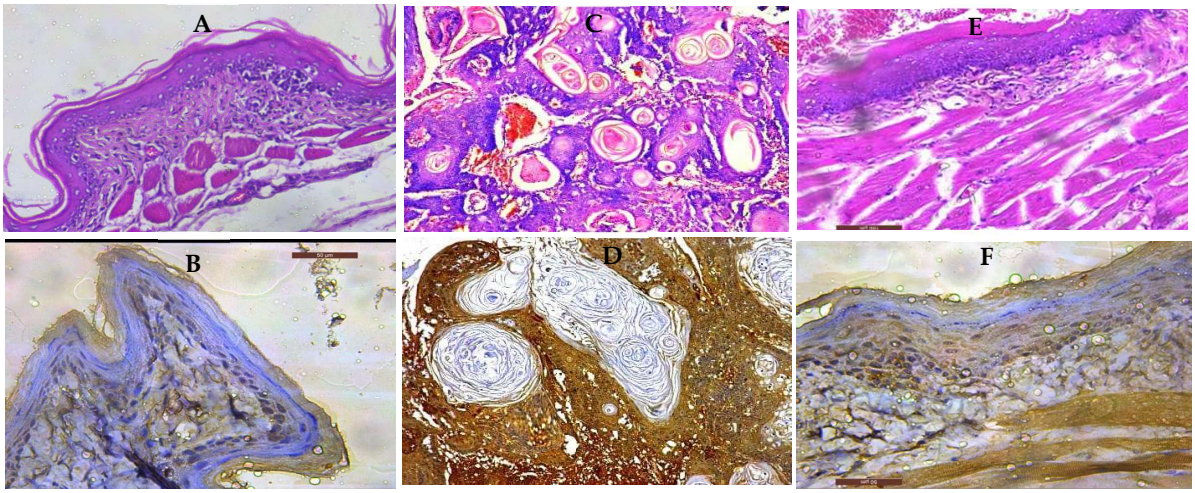

Figure 2. (A) H&E stain of GI showing keratinized stratified squamous epithelium with flattened rete ridges, sub-epithelial C.T layer and muscular layer; (B) IHC expression of Bcl-2 showing positive cytoplasmic expression in basal and suprabasal epithelial layers; (C): H&E stain of GII of HBP mucosa showing well differentiated SCC; (D) IHC expression of Bcl-2 showing positive cytoplasmic expression throughout the tumor cells; (E) H&E stain of GIII of HBP mucosa showing mild epithelial dysplasia; (F) IHC expression of Bcl-2 showing positive cytoplasmic expression throughout the epithelial layers.

3.5. Statistical analysis results

Statistical analysis results of Bcl-2 expression were obtained by comparing the area % between the groups used., GI had recorded the lowest mean area percentage (6.6%), while GII had recorded the highest mean area percentage (69.7%) and the comparison revealed that there was high significant difference between GI and GII where P value was (< 0.001), there was significant difference between GI and GIII where P value was (= 0.001) and there was high significant difference between GII and GII where P value was (< 0.001) (Table 1&2).

Table (1): comparisons between studied groups.

		Groups			*Stat. test*	*P-value*
		Group I (n = 10)	*Group II (n = 10)*	*Group III (n = 10)*		
Marker	*Mean*	*6.6*	*69.7*	*23.2*	*F = 87.2*	*< 0.001 HS*
	±SD	*2.6*	*8.6*	*4.7*		

Table (2): Post-Hoc test for multiple comparisons between studied groups.

		LSD (least significance difference)	p-value	
Group I	Group II	- 48.4	< 0.001	HS
	Group III	- 16.6	0.001	S
Group II	Group I	48.4	< 0.001	HS
	Group III	31.7	< 0.001	HS
Group III	Group I	16.6	0.001	S
	Group II	- 31.7	< 0.001	HS

4- Discussion

Oral carcinogenesis is a complicated process that occurs when the squamous epithelium is influenced by a variety of genetic and environmental factors. The main aim now is to use new chemopreventive modalities to not only treat the disease, but also to diagnose it early and possibly prevent it from progressing. The results of the Effect of DHA as a chemopreventive agent on experimentally induced HBP carcinogenesis indicated a variety of changes in the current investigation. Topical administration of DMBA to the HBP for 14 weeks caused invasive SCC, according to H&E stain results. The development of dysplastic characteristics as well as tumor cell invasion were observed in DMBA-treated animals. These findings are consistent with those of other investigators [29, 31]. The current findings, combined with those from other investigations [29, 32, 33], supported the idea that DMBA-induced HBP carcinoma go through the same alterations as in human. Many of the structural changes seen in carcinogen-treated HBP mucosa, both at the gross and light microscopic levels, are quite similar to those seen in human oral cancer development. DMBA was also chosen as the chemical carcinogen because it has the similar etiological effect in hamster SCC as alcohol and tobacco do in human OSCC [34, 35]. The current study demonstrates that the HBP carcinogenesis model is one of the best-studied animal models for studying the progressive evolution of OSCC.

In the present study, immunohistochemical results of G1 revealed weak (6.6 %) positive cytoplasmic expression of Bcl-2, in the basal and suprabasal epithelial layers. These results are in agreement with those of other investigators [36- 38], who stated that, under normal condition, the Bcl-2/Bax ratio determines whether a cell will live or die by regulating the release of cytochrome c from the mitochondria. This could be because Bcl-2 plays a role in the modulation of keratinocyte terminal differentiation by shielding their stem cells from apoptosis. In the current study, GII (DMBA painted group) at 14 weeks, gross

observation showed multiple large exophytic nodules surrounded with ulcerative and bleeding areas, some animals appeared debilitated. H&E stain, showed a development of well and moderately differentiated SCC. This is in consistence with that were shown by other studies [38, 39]. These findings could be attributed to DMBA's effect on metabolic activation; DMBA is converted to its active carcinogenic metabolite, dihydrodiol epoxide, which mediates carcinogenesis through chronic inflammation, overproduction of reactive oxygen species (ROS), activation of protooncogenes, inactivation of tumor suppressor genes, extensive DNA damage, and a reduction in DNA damage repair, reducing the ability to induce apoptosis [34, 35]. The aforementioned findings supported the idea that DMBA-induced HBP carcinoma go through the same modifications as in human, not only at a gross level but also at a light microscopic level. These findings are consistent with those of prior studies [40-42].

In the present study, the Bcl-2 immunohistochemical results at 14 weeks revealed strong (69.7%) positive cytoplasmic expression. These results are in agreement with those of other investigators [35, 38, 42- 44]. This suggests that overexpression of Bcl-2 indirectly inhibits apoptosis and hence promotes tumor progression by increasing the survival rate of neoplastic cells, allowing new genetic alterations to emerge and making them more resistant to treatment [45].

The oral injection of DHA had a robust chemopreventive impact against DMBA-induced oral carcinogenesis in this investigation, as demonstrated not only by the much lower tumor incidence but also by the normalization of the anti-apoptotic marker. When DHA chemoprevention was given continuously at the beginning and promotional stages of carcinogenesis, it greatly delayed the formation of tumors by roughly 3- 4 weeks. These findings are consistent with those of other investigators [46, 47]. This finding could be explained by the fact that DMBA can bind to DNA and cause mutagenic events that contribute to malignant transformation. DHA may protect DNA or inhibit the chemical carcinogen's mutagenesis effect. Chen et al., 2005 [48], reported that blocking agents that inhibit chemical carcinogenesis can exert their preventive effect by several mechanisms, including improving carcinogen detoxification, inhibiting cytochrome P450-mediated activation of carcinogens, scavenging free radicals, and trapping carcinogens and preventing their interaction with DNA.

In the current work, we found that hamsters treated with DMBA alone have Bcl-2 overexpression. The expression of Bcl-2 was decreased in DMBA-treated hamsters after oral dose of DHA (23.2 %). These data imply that DHA may have prevented aberrant cell proliferation during DMBA-induced oral carcinogenesis via decreasing DMBA metabolic activation or suppressing DMBA-induced Bcl-2 mutations. These findings are consistent with those of other investigators [49, 50]. In DMBA-treated hamsters, increased Bcl-2 expression indicated suppression of apoptosis by preventing the release of cytochrome C from mitochondria, as well as encouragement of carcinogenesis, which was nearly reversed by DHA chemoprevention [38, 41, 51].

There was highly statistically significant differences between the expression of Bcl-2 in GII & GIII and between GI & GII (the P value recorded (< 0.001) which mean, the increased expression of Bcl-2 in OSCC as compared to normal or oral epithelial dysplasia may be an evidence of the disease progression to carcinoma, as the results of this study suggest alterations in expression of Bcl-2 family proteins, creating a favorable environment for malignant transformation. This is in consistence with that were shown by other studies [52- 56]. The results of this investigation revealed that apoptosis inhibition is a common occurrence in oral carcinogenesis. The Bcl-2 family of proteins appears to play a

role in controlling keratinocyte terminal differentiation. Overexpression of the anti-apoptotic protein Bcl-2 protects tumor cells against apoptosis, allowing them to survive. Bcl-2 proteins are of the most prominent anti-apoptotic proteins deregulated in cancer, according to Vogler, 2014 [53- 56]. They play a role in carcinogenesis and mediate resistance to currently available anticancer drugs. Bcl-2 inhibitors are unlikely to be as effective as single medicines, but when combined with other targeted drugs, they could be quite useful. As a result, Bcl-2 inhibitors will be used in future personalized cancer treatments for cancers that express Bcl-2 protein addiction.

5. Conclusions

DHA has the potential to be an effective chemopreventive drug for a wide range of modern lifestyle disorders. The mechanisms underlying DHA's chemopreventive effect, on the other hand, need to be investigated further. With its ability to enhance carcinogen detoxification and its blocking/ suppressing effect on the initiation and promotion stages of experimentally induced HBP carcinogenesis, DHA could be considered a dietary chemopreventive agent, providing an effective dietary approach to controlling cancer incidence. As a result, our findings may be significant, implying that consuming DHA on a regular basis may lessen the incidence of OSCC in humans. Given the foregoing, omega-3 can be viewed as a rich supply of possible candidates for dietary inclusion with promising effects at pre-determined dosages to minimize toxicity.

6. Patents

Ethical Approval: Ethical approval cleared by ethical committee of Faculty of Dental Medicine (Boys- Cairo), Al-Azhar University, Egypt (Ethical Code No. 35/38-11-10-21).

Author Contributions: Conceptualization, A.M. and E.A.; methodology, A.M., A.A., K.A., M.O., and E.A.; software, A.M., M.A. K.A. and A.A.; validation, A.M. and E.A.; formal analysis, A.M. and E.A.; investigation, A.M., A.A., K.A. M.O. and E.A.; resources, A.M. and E.A.; writing-original draft preparation, A.M., A.A., K.A. and E.A.; writing-review and editing, A.M., M.A., E.A. and A.A.; supervision, A.M. and E.A.

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Data Availability Statement: The data sets used during current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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