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Original Article

EFFECT OF NARINGENIN ON LIPIDS, LIPOPROTEINS AND LIPID METABOLISING ENZYMES IN 7,12-DIMETHYL BENZ(A)ANTHRACENE-INDUCED MAMMARY CARCINOGENESIS IN SD RATS

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ABSTRACT

Objective: Mammary carcinoma is one of the most common and prominent cause of cancer-related death in women worldwide. The role of fatty acid synthesis and altered lipid metabolism in cancer progression was well established. Cancer cells undergo enhanced *de-novo* lipogenesis and liver uptake. The magnitudes of bioflavonoids in lipid management against various cancers have been established. In the present study, we evaluated the efficacy of a bioflavonoid Naringenin (NGN) to re-establish the lipid metabolic alterations in 7, 12-dimethyl benz (a) anthracene (DMBA)-induced mammary carcinoma in Sprague Dawley (SD) rats.

Methods: DMBA-induced mammary carcinoma was developed using air pouch technique (20 mg in 0.5 ml olive oil) following a 118 d experimental protocol. NGN (40, 80 mg/kg b. w.; i. p.) was given for 28 d after promotional stage (90 d) of carcinoma bearing animals. The changes in body weight (b. w.), lipids, lipoproteins and lipid metabolising enzymes (LME) level were estimated and correlated with anticancer potentials of NGN.

Results: The results indicated a dose dependant significant ($p<0.05$) restorative effect of NGN on body weight of cancer-bearing animals. The changes in lipid level in plasma and liver tissue were re-established after treatment. Lipoproteins and LME were also changed in cancer-bearing animals with that of NGN treatment significantly ($p<0.01$).

Conclusion: NGN potentially reverted the changes in body weight, lipid profile, LME that config. its anticancer potential against mammary carcinogenesis.

Keywords: Mammary carcinoma, Naringenin, Lipids, Lipoproteins, Lipid metabolism.

INTRODUCTION

Breast cancer is a malignant tumour characterised by uncontrolled proliferation, invasion and metastasis [1]. Malignant mammary carcinoma is one of the most common hormonal cancers among women and is the second most prominent cause of cancer-related death in females worldwide. According to the American Cancer Society, 3.1 million women in US bear invasive breast cancer till Jan 2014 [2]. Early detection and prevention of some of the cancer progression could be possible as up to a certain stage it is a slowly progressive disease that takes many years to become invasive. Various factors responsible for cancer progression were always studied and targeted for the curative approaches. An association has reported between lipid and cancer in various epidemiological studies [3-5]. It has well established that the neoplastic cells able to synthesize lipids like embryonic tissue [6].

The role of fatty acid synthesis in tumour progression has established by many researchers in mammary and prostate cancer where increased expression of fatty acid synthase was diagnosed [7, 8]. Cancer cells showed specific metabolic alterations and enhanced synthesis of proteins, nucleic acids and lipids for their rapid proliferation and progression [9, 10]. Besides this increased lipid consumption and biogenesis by cancer cells supported by a huge literature [11, 12]. This represents the necessity of lipid by carcinoma cells for various structural functionality like cholesterol-rich membrane rafts for plasma membrane functions, the functionality of membranous organelles like mitochondria and ER, to increase membrane lipid saturation for the protection of cancer cells from oxidative damage by reducing lipid peroxidation [10, 13, 14].

The magnitude of bioflavonoids in disease management is far and wide. The scientific substantiation consistently revealed an association between dietary intake of naturally occurring bioactive molecules and cancer biology.

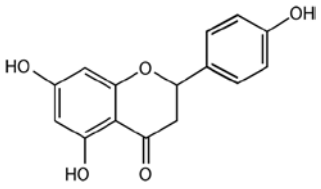

Fig. 1: Structure of NGN

Naringenin (NGN, 5, 7-dihydroxy-2-(4-hydroxyphenyl) chroman-4-one) is a flavanone bioflavonoid that is abundant in citrus fruits and tomatoes [15]. It has been pharmacologically evaluated as a potential anticancer agent in various cancers and also is effective on MCF-7 breast cancer cell line [16]. Although NGN has been reported to have lipolytic potential in various fat induced atherosclerosis models, its effect on DMBA-induced cholesterol metabolism regulation imbalance and lipid management in cancer-bearing experimental animals has not studied extensively.

In the present study, our group has evaluated the efficacy of NGN on modification in levels of lipids (triglycerides (TG), phospholipids (PL), total cholesterol (TC), free cholesterol (FC) and free fatty acids (FFA)), lipoproteins (low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c) and very low density lipoprotein cholesterol (VLDL-c)) and LME (lecithin cholesterol acyltransferase (LCAT), lipoprotein lipase (LL) and total lipases (TL), cholesterol ester hydrolase (CEH), cholesterol ester synthase (CES)) in DMBA-induced mammary carcinoma animals.

MATERIALS AND METHODS

Experimental animals and drug preparations

Female SD rats (50–55 d old) in each group were used in this study. Animals have obtained from the central animal facility, Birla

Institute of Technology Mesra, Ranchi Jharkhand, India (Registration No. 621/02/ac/CPCSEA). All animals were kept in polyacrylic cages and maintained under standard conditions (room temperature 24-27 ºC and humidity 60-65 % with 12:12 light: dark cycles). The food was provided in the form of pellets (Hindustan animals feed, Gujrat, India) and water *ad-libitum*. A week before the beginning of experiment acclimatization to the laboratory environment has given to the animals. All experiments involving animals complies with the ethical standards of animals handling, and IAEC approved the study protocol (protocol approval no. BIT/PH/IAEC/24/2013). NGN (Sigma-Aldrich, India) was suspended in 1% (w/v) carboxymethyl cellulose (CDH (P) Ltd, New Delhi, India).

Cancer induction and treatment protocol

Mammary tumours were induced by DMBA using the "air pouch technique" with some modifications as required [17, 18]. Briefly, about 2–3 ml of sterile air was injected subcutaneously just beneath the mammary fat pad region of the second and third mammary glands. The air inside was stabilized for 24 h to form a pouch. A single dose of 20 mg DMBA in 0.5 ml of olive oil was vortexed to obtain a uniform suspension and was injected into the air pouch.

Four experimental groups of six animals in each group used for the study as follows:

Group I: Control, served with 0.5 ml olive oil in air pouch once at day 1.

Group II: Induced Control, DMBA (20 mg in 0.5 ml olive oil once at day1).

Group III & IV: NGN treatment groups at 40 mg/kg b.w. and 80 mg/kg b.w. doses respectively for 28 d.

All rats were checked by palpation every week to monitor tumorigenesis. When a tumour was first palpated, the tumour location was recorded. NGN treatment was started after attaining the promotional stage of tumorigenesis (90 d) from the time of carcinogen administration. Adenocarcinomas were confirmed by histological examinations in cancer-bearing animals after 118 d. From day 1, the variations in the body weight during the study period were recorded for normal, induced and treatment groups at regular interval of 7 d till the experiment was finished (day 118).

Estimation of lipids, lipoproteins and LME

Lipid was extorted from the tissue by the technique given by Folch *et al.* [19]. Using the Folch aliquots for tissue and plasma samples all the lipid components were estimated. TG was estimated by the method of Van Handle [20] with small modifications done by Rice *et al.* [21]. PL was estimated according to the method given by Rouser *et al.* [22]. TC was determined according to the method given by Parekh and Jung [23]. FC was determined by enzymic scheme given by Leffler and Mc Dougald [24]. FFA was estimated by the technique given by Horn and Monahan [25]. Plasma lipoproteins were fractionated by dual precipitation technique [26]. The addition of heparin–magnesium to plasma precipitated VLDL-c, LDL-c and HDL-c which were left in the supernatant and subsequently, the content was measured in the fraction. LL was estimated in plasma and tissue samples according to the method is given by Schmidt [27], TL was estimated in both plasma and tissue samples according to the method of Bier [28], Estimation was done by the method of Kothari *et al.* [29], the activity of LCAT was assayed using the method of [30] with modifications from the method of [31].

Statistical analysis

All experimentally collected data were analyzed through one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests with identical sample size using Graph Pad Prism (version 5.0). The variation was considered significant with $p<0.05$. All the values were expressed as mean±standard error mean (SEM).

RESULTS

Body weight assessment

Change in b.w. during 118 d studies was recorded weekly and represented graphically in fig 2. The control rat revealed a normal increase in b.w. with no signs of abnormality. DMBA-induced rats showed a normal increase in b.w. in the early proliferation period of 6 w. A significant ($p<0.01$) decrease in b.w. was recorded which further accelerated after the 11th week. A significant ($p<0.001$) decrease in b.w. were recorded as compared to control at last. Similar variations were recorded in induction phase as that of Induced control and in animals treated with NGN (40 mg/kg b.w.). These rats showed a significant ($p<0.05$) decrease in b.w. when compared to control Group I animals after 118 d of study protocol whereas a increased level of protection ($p<0.01$) was recorded in animals with the higher dose (80 mg/kg b.w.) of NGN as compared to control. Body weights of all NGN treated animals were significantly ($p<0.05$) decreased than that of induced control.

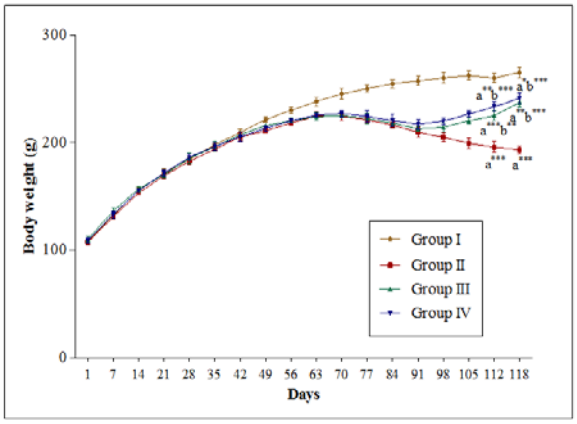

Fig. 2: Effect of DMBA and NGN treatment on experimental animals

Each value shows mean±SEM; n=6. where, a-Group II, III, IV compared with Group I, b-Group III, IV compared with Group II,***-p<0.001, **-p<0.0, *-p<0.05; Group I-normal control; Group II-Induced Control; Group III-DMBA+NGN(40 mg/kg b.w.); Group IV-DMBA+NGN (80 mg/kg b.w.)

Effect of NGN on lipids and lipoproteins levels in plasma and liver of all experimental animals

The level of TG, PL, TC, FC and FFA in plasma and liver tissue samples for all group animals estimated which are depicted in table 1. Induced group animals illustrate a significant ($p<0.001$) raise in all lipids level in both samples as compared to control group I animals. In plasma samples, NGN treated animals at 40 mg/kg b.w. (Group III), showed a significant decrease in lipids which are at a level of $p<0.05$ for PL, TC, FC and of $p<0.01$ for TG and FFA when compared with induced control animals. Group IV (80 mg/kg b.w.) animals, recorded to have a significant ($p<0.001$) decline in all lipids level as compared to cancer-bearing group II animals.

In liver samples, a greater protection level of NGN treatment on lipids level can be seen. In group III animals a significant decrease at a level of $p<0.01$ was recorded for TG and PL which was at a level of $p<0.001$ for TC, FC and FFA as compared with induced control group animals. At higher doses the levels were significantly ($p<0.001$) decreased for all as compared with induced control. In both samples, NGN treatment showed a decrease in all lipids near normalcy when compared with control animals in a dose dependent manner as depicted in table 1.

The plasma lipoproteins (HDL-c, LDL-c and VLDL-c) profile for all experimental group animals was depicted in fig. 3. DMBA-induced untreated group II animals recorded to have significantly ($p<0.001$) declined HDL-c and significant ($p<0.001$) increase in LDL-c and VLDL-c levels as compared to Group I control animals. When treated with NGN, Group III animals revealed an insignificant ($p>0.05$) but an raise in HDL-c level and a significant ($p<0.01$) decline in LDL-c and VLDL-c lipoproteins level as compared to cancer-bearing untreated animals. Whereas, when treated with high dose (80 mg/kg

b.w.) Group IV animals revealed a significant (p<0.001) decline in HDL-c and significant increase in LDL-c and VLDL-c at a level of p<0.001 as compared with induced control group II animals. NGN treatment decreased LDL-c and VLDL-c plasma level near normalcy at a level of p<0.01 and p<0.05 respectively when compared to control group animals.

Table 1: Estimation of lipid levels in plasma and liver of all experimental group animals

Groups	Triglycerides (mg/dl)	Phospholipids (mg/dl)	Total cholesterol (mg/dl)	Free cholesterol (mg/dl)	Free fatty acid (mg/dl)
Lipid levels in Plasma samples of experimental animals					
Group I	40.10±1.16	91.88±1.62	123.85±2.16	93.36±1.14	15.16±0.76
Group II	68.27±1.92a***b**	157.06±1.23a***	145.94±1.89a***	147.93±1.67a***	36.17±1.70a***
Group III	59.98±1.12a***b***	105.36±1.12a***b***	139.67±1.71a**b^{ns}	132.83±1.51a***b***	25.47±1.11a***b***
Group IV	45.89±1.39a^{ns}b***	98.84±1.46a*b***	135.31±1.88a*b**	99.75±0.94a*b***	19.88±0.76a^{ns}b***
Lipid levels in Liver samples of experimental animals					
Group I	9.01±0.11	8.04±0.09	4.41±0.06	2.24±0.07	6.75±0.18
Group II	15.99±0.10a***	11.67±0.08a***	9.14±0.04a***	4.90±0.08a***	8.48±0.08a***
Group III	11.20±0.11a***b***	9.48±0.06a*** b***	5.69±0.08a*** b***	3.81±0.07a*** b***	7.69±0.09a*** b***
Group IV	9.65±0.11a**b***	8.72±0.07a*** b***	5.05±0.05a*** b***	2.67±0.07a** b***	7.19±0.08a^{ns} b***

Each value shows mean±SEM; n=6. where, a-Group II, III, IV compared with Group I, b-Group III, IV compared with Group II,***-p<0.001, **-p<0.0, *-p<0.05, ^{ns}-p>0.05; Group I-normal control; Group II-Induced Control; Group III-DMBA+NGN(40 mg/kg b.w.); Group IV-DMBA+NGN (80 mg/kg b.w.).

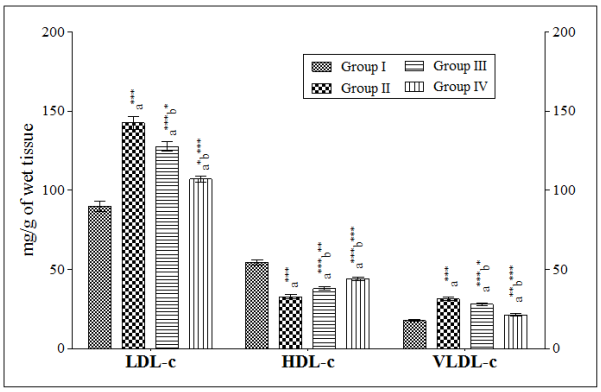

Fig. 3: Lipoproteins level in all experimental group animals

Each value shows mean±SEM; n=6. where, a-Group II, III, IV compared with Group I, b-Group III, IV compared with Group II,***-p<0.001, **-p<0.0, *-p<0.05; Group I-normal control; Group II-Induced Control; Group III-DMBA+NGN(40 mg/kg b.w.); Group IV-DMBA+NGN (80 mg/kg b.w.). LDL-c: Low-density lipoprotein cholesterol, HDL-c: High-density lipoprotein cholesterol, VLDL-c: Very low density lipoprotein cholesterol

Effect of NGN treatment on LME of all experimental group animals

The modification in levels of lipid metabolising enzymes (LME) were estimated in plasma (LCAT, LL, TL) and liver tissue (LCAT, LL, TL, CEH, CES) samples of all experimental group animals in fig. 4 and fig. 5 respectively. DMBA-induced mammary carcinoma bearing untreated animals bears a significant (p<0.001) decrease in LCAT and LL but a significant (p<0.001) increase in TL in plasma as well as liver samples and a significant (p<0.001) increase in CEH and CES in liver samples as compared to control group I animals. In plasma samples, group III animals showed an insignificant (p>0.05) changes in all LME as compared to cancer-bearing group II animals. When treated with 80 mg/kg b.w., group IV animals showed a significant increase in LCAT and LL at a level of p<0.01 and p<0.05 respectively and significantly (p<0.01) decrease TL when compared with induced control group II animals. But, a significant (p<0.01) change was observed in group III animals that increased with dose (Group IV) towards normalization when compared with control group I animals. In liver samples, NGN treated group III animals showed a significant (p<0.05) decrease in LCAT and LL whereas a significant (p<0.05) increase in CEH and CES level with having an insignificant increase in TL as compared to tumour bearing animals. On high dose NGN treatment, LCAT and LL enzyme levels were significantly (p<0.001) decreased and TL, CEH and CES levels were significantly (p<0.001) increased in group IV animals as compared to induced control group II animals. Normalization was recorded on high dose (80 mg/kg b.w.) treatment only when compared with control group I animals.

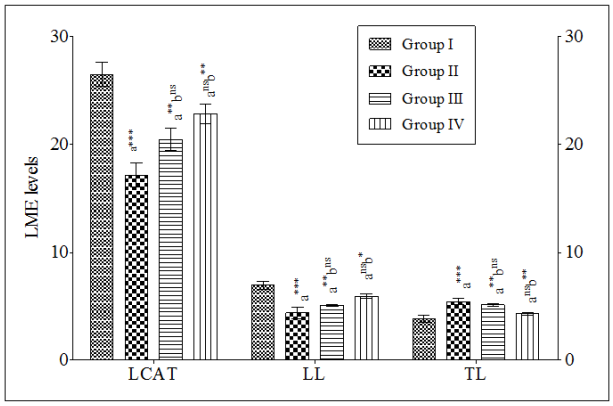

Fig. 4: LME level in plasma of all experimental group animals

Each value shows mean±SEM; n=6. where, a-Group II, III, IV compared with Group I, b-Group III, IV compared with Group II,***-p<0.001, **-p<0.0, *-p<0.05; Group I-normal control; Group II-Induced Control; Group III-DMBA+NGN(40 mg/kg b.w.); Group IV-DMBA+NGN (80 mg/kg b.w.). Units: Lecithin Cholesterol-acyl Transferase (LCAT) (mol of cholesterol esterified/h/mg protein), Lipoprotein Lipase (LL) (mol of free fatty acid liberated/h/mg protein), Total Lipase (TL) (mol of p-nitrophenol liberated/h/mg protein)

DISCUSSION

Preclinical DMBA-induced mammary carcinoma model in SD-rats resembles clinically developed ductal carcinoma using various histological and biochemical changes. Anatomical changes like b.w. can reflect the abnormality and physiological changes. Cancer incidences significantly show consequent weight loss after certain duration [32]. In carcinogenesis, irregularity of various pathways brings activation or overexpression of several genes involved in lipid and lipoprotein synthesis [9]. Various studies reported that neoplastic cells/tissues can synthesize lipids or reactivate lipid synthesis [6, 7]. Mammary carcinoma exhibits increased expression of lipid synthesizing enzymes and machinery that may play a role in cancer pathogenesis [8]. But the relation behind increased lipid and lipoprotein levels and cancer progression are not defined precisely. Previous studies reported that lipid and lipoproteins (LDL/VLDL) induce phenotypic changes that result in proliferation and migration of mammary carcinoma [33-35].

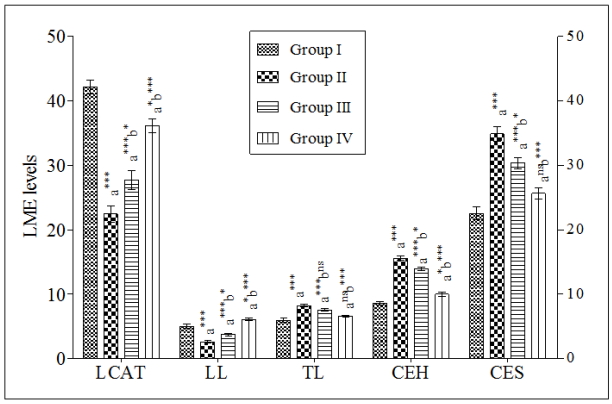

Fig. 5: LME levels in liver tissues of all experimental group animals

Each value shows mean±SEM; n=6. where, a-Group II, III, IV compared with Group I, b-Group III, IV compared with Group II,***-p<0.001, **p<0.0, *-p<0.05; Group I-normal control; Group II-Induced Control; Group III-DMBA+NGN(40 mg/kg b.w.); Group IV-DMBA+NGN (80 mg/kg b.w.). Units: Lecithin Cholesterol-acyl Transferase (LCAT) (mol of cholesterol esterified/h/mg protein), Lipoprotein Lipase (LL) (mol of free fatty acid liberated/h/mg protein), Total Lipase (TL) (mol of p-nitrophenol liberated/h/mg protein), Cholesterol Ester Hydrolase (CEH) (mol of cholesterol liberated/h/mg protein), Cholesterol Ester Synthetase (CES) (mol of cholesterol esterified/h/mg protein).

In our study, increased proliferation and progression of carcinogenesis may be correlated with increased total cholesterol and triglycerides. Cholesterol and phospholipid content are responsible for the fluidity of lipid membrane. Carcinoma increases level of cholesterol and phospholipids that may contribute to the further progression of cancer [36]. The liver is the major organ involved in lipid metabolism and distribution. The alteration in the level of lipids, lipoproteins in plasma as well as liver during carcinogenesis and tumour progression has been observed previously [37]. The changes that occur during cancer progression in liver mediated metabolism and lipoprotein-mediated transportation brings the plasma lipid level change. The apolipoprotein-B mediated modulation of low-density lipoprotein receptors results in LDL-c rush into the plasma [22]. The increased synthesis of LDL-c and VLDL-c ultimately elevate total cholesterol and triglycerides respectively in mammary carcinoma bearing animals. The reduced level of HDL-c is mainly compensated by LDL-c and vice versa hence in cancer-bearing animals the increased level of LDL-c and VLDL-c can be justified [25]. The changes in lipid metabolising enzymes can be correlated with progressive carcinogenesis. LCAT and LL control the clearing of free cholesterol and triglycerides from the plasma by etherification and cleavage respectively [22].

Hence increased the level of triglycerides, total cholesterol and free cholesterol in untreated cancer bearing animals was recorded due to reduced activities of these fat-splitting enzymes. Increased lipogenesis in cancer-bearing animals was mediated by large production of lipid peroxides. Hyperlipidemia could reflect the increased activity of TL during progressive carcinogenesis [18]. The elevation in total body fat was associated with increased total lipase activity in mammary carcinoma bearing subjects when compared with normal one was reported [38]. Low level of plasma cholesterol was maintained by LCAT by increasing incursion of ester cholesterol into the cells. In mammary carcinoma bearing animals, low LCAT levels result in increased plasma cholesterol contents. Also, LCAT mediates formation of HDL-c which is reduced due to reduced activity of the enzyme. Lipoprotein activity was inhibited by HDL by competing with the enzyme substrate in cancer-bearing animals. This inhibition of LCAT makes avail more lipids to the cell contributing further proliferation of carcinoma cells. CEH and CES enzymes maintain cholesterol balance inside the cell. The disproportion in the level of cholesterol may result due to the hyperlipidemia conditions. The subsistence of a causal association between a elevated lipid content and superior cancer risk has reported as one of the mechanisms of cellular abnormality by interaction with various signalling pathways that leads to transformed gene expression, effects on liberated oestrogenic concentration (in case of mammary carcinoma) and anatomical & physiological alterations in cell membranes resulting in modification in hormone and growth factor receptors [18]. Hence, NGN potentially quashes the alterations in lipid metabolism and plasma and tissue levels of lipids in DMBA-induced mammary carcinoma bearing animals when compared with normal levels of control group animals.

CONCLUSION

From the above results, we can conclude that NGN treatment to DMBA-induced cancer-bearing female SD-rats in their promotional stage of multistage carcinogenesis brings restorative changes in the altered lipid, lipoproteins and LME levels. It also normalizes the decrease in the body weight (like untreated animals) when compared with normally growing control group animals. Herbal compounds could exhibit multiple mechanisms behind their anticancer activity. Although the underlying mechanisms were not precisely known, but from this part of the study, we can conclude that re-establishment of body weight, lipid metabolism alterations and underlying enzymic changes, could be one of the prospective for the anticancer potential of NGN against mammary carcinoma condition.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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