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Acute consumption of a high-fat diet prior to ischemia-reperfusion results in cardioprotection through NF-κB-dependent regulation of autophagic pathways

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WITH OBESITY AND RELATED CARDIAC pathologies on the rise worldwide (12, 17, 29), there is a strong focus on the interdependence of diet and myocardial health. Chronic consumption of fat has traditionally been associated with arteriosclerotic cardiovascular diseases, including atherosclerosis, stroke, and myocardial infarction (MI) (3, 21, 30, 33). This year, ~620,000 Americans will experience a new coronary event (defined as first hospitalized MI or coronary heart disease-related death), and ~295,000 will experience a recurrent event (20). Although the cardiovascular effects of long-term consumption of a high-fat diet (HFD) have been well studied, these studies often overlook the influences of short-term consumption of fats. Precedents for beneficial effects of high-fat consumption are observed in long-term retrospective data supporting an “obesity paradox” (9, 13, 16, 33), wherein under certain conditions, an elevated BMI positively correlates with improved prognosis or decreased risk of cardiovascular pathology (2, 9, 15, 21, 29, 30, 33). Short-term consumption of omega-3 fatty acids is also implicated in protection against cardiac dysfunction, though the mechanism for this is still uncertain (3). Current data suggest that postischemia, short-term (subchronic) HFD improves cardiac contractility (4, 5), and exposure to a high-fat diet has been shown to alter the murine heart’s response to infarct-induced heart failure (7). However, there are no existing data for an acute effect of HFD on infarct size after MI. Herein, we demonstrate such an effect in a murine model.

A potential mechanism for the cardioprotective effect of acute HFD is metabolic regulation of autophagy, which influences the extent of post-MI injury. Autophagy is an evolutionarily conserved process utilized by eukaryotic cells to regulate the turnover of persistent proteins and damaged organelles (23, 31). In mammalian biology, autophagy is divided into three categories: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy involves a formation of a double-membrane structure that engulfys cytoplasmic components, then docks and fuses with a lysosomal compartment to form an autolysosome. The contents of this compartment are then degraded via hydrolytic reactions that lead to the recycling of the contents utilizing macromolecular synthesis and ATP generation (23). At basal levels, macroautophagy is thought to be a homeostatic mechanism, but under conditions of stress, it may act as an adaptive catabolic process. The upregulation of the initiating protein in autophagosome formation (beclin 1, Atg 5) is involved in ischemic preconditioning and is hypothesized to inhibit apoptotic caspase activity (caspase-8, caspase-3) and promote a protective phenotype (23, 31, 46).

A known mediator of the balance between autophagy and apoptosis is NF-κB (11, 22). As a key protein complex that regulates gene transcription, NF-κB is expressed in almost all animal cell types and is involved in a variety of cellular stress responses (43). It is known to underlie transcription of the gene programs responsible for late-phase preconditioning (6, 8, 14),...
as well as regulate the expression of beclin-1 in ischemic preconditioning (35, 47, 48, 50) and postconditioning (46). This mechanism is thought to be part of a priming effect that lends to postischemic increases in autophagy and decreases in apoptosis (48, 50).

Our study focused on the effects of short-term HFD upon MI to determine whether it is possible to separate the chronic adverse effects of high-fat exposure from a potential protective effect against cell death. Herein, we investigate the hypothesis that short-term high-fat feeding prior to infarction protects the heart against ischemic injury through a NF-κB-dependent regulation of cell death pathways. Our results show that acute HFD mediates a strong cardioprotection against ischemia-reperfusion (I/R) injury that is lost at 6 wk of HFD. This effect is dependent upon NF-κB signaling and is associated with NF-κB-dependent increases in beclin-1, LC-3, autophagy, and reduced apoptosis. The physiological response to acute HFD is an independent and separable phenomenon from the pathophysiological response seen with obesity and metabolic syndrome due to chronic (6 wk or longer) feeding on a HFD. Whether the loss of acute HFD-induced cardioprotection is related functionally to the onset of glucose resistance and obesity is a question of potentially high clinical impact. Understanding this phenomenon could lead to the development of new therapies, which may be utilized to protect patients against ischemic injury.

MATERIALS AND METHODS

Animal protocols. All mice were maintained in accordance with institutional guidelines and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, revised 2011), and the University of Cincinnati Institutional Animal Care and Use Committee approved this study. Transgenic IκBα dominant-negative mice (2M) have been previously characterized. Because NF-κB is not required during normal cardiac function, but induced primarily by stress, these animals have normal function and no deficits in cardiac form or function (8, 14). All studies were performed using strain-matched male mice (C57BL/6J or 2M transgenic mice and nontransgenic, sibling controls). Male animals were chosen due to the estrogenic effects seen in female animals in a long-term diet-induced obesity studies, on which this modified protocol is based (38).

I/R surgical model. The in vivo I/R surgical model was performed via reversible occlusion of the left anterior descending coronary (LAD) artery (8, 43). Occlusion of the LAD was performed for 30 min and followed by full recovery of the animal. Euthanasia of animals took place at 24 h of reperfusion unless otherwise indicated. For tissue isolation, heart tissue was extracted, and left ventricle (LV) was tissue dissected away (ischemic section isolated by sight of blanching when necessary), rinsed in sterile PBS, and flash frozen in liquid nitrogen required during normal cardiac function, but induced primarily by stress, these animals have normal function and no deficits in cardiac form or function (8, 14). All studies were performed using strain-matched male mice (C57BL/6J or 2M transgenic mice and nontransgenic, sibling controls). Male animals were chosen due to the estrogenic effects seen in female animals in a long-term diet-induced obesity studies, on which this modified protocol is based (38).

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High-fat feeding. Animals were fed a purified high-fat diet (HFD) with 60% kcal/fat (Research Diets D2409) or on a control diet (CFD); 11% kcal/fat (7922 NIH-07 mouse diet; Harlan Laboratories, Indianapolis, IN). In-house normal chow was compared with purified control (Research Diets D12450Bi), and we found that animals on these have no significant differences in metabolic parameters (data not shown). Thus, we employed the in-house chow as a control (designated CFD) for all studies. Animals were fed ad libitum prior to euthanasia at an age indicated for I/R surgery (12–14 wk of age). No significant changes in body weight or consumption were observed in short-term feeding studies unless indicated in results.

Serum analysis. Serum from HFD and CFD animals was extracted via cardiac puncture into an EDTA-charged collection tube. Animals were euthanized for immediate sample collection. Five-hundred microliters of whole blood was spun down at 5,000 rpm in an EDTA-charged tube, and red blood cell/white blood cell fractions were discarded. Determinations of substrates in plasma/serum/lymph were made using specific colorimetric assays. Reactions were run in microtiter plates and analyzed via a plate reader.

Dietary analyses of serum lipid levels were done with assistance from the Cincinnati Mouse Metabolic Phenotyping Center.

Stool fat analysis. Fecal fat content was analyzed in CFD mice and HFD animals. Mice were transferred to individual cages. Food intake was measured, and feces were collected every 3 h over a 24-h time interval. Fecal fat content was determined gravimetrically, as previously described (18, 34). Briefly, stool pellets were dried at 65°C and weighed. Fat content was determined semiquantitatively by organic extraction with chloroform:methanol:5 M HCl (1:1:2). Lipids were weighed after organic extraction and solvent evaporation. Lipid mass was normalized to the amount of fecal material put into the extraction to determine percent fat absorption.

Twenty-four-hour metabolic assessment. A food reservoir was placed on a sensitive balance and introduced to the animal through specially designed cages that prevent defecating/urinating into or spillage of food. Data were generated at intervals of 1 min to 23.99 h and 59 min. Meal patterns were analyzed through systematic measurements of intake frequency and duration of each meal. Both total energy expenditure and relative rates of carbohydrate vs. fat oxidation were determined via indirect calorimetry (18).

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Western blot analysis. Total protein lysate was prepared from flash-frozen hearts, and blotting was performed, as previously described (28). Aliquots of total protein (20–30 µg) were separated on Novex gels (Life Technologies, Grand Island, NY), transferred to nitrocellulose membrane (Life Technologies), and blocked for 1 h at room temperature with 5% milk-TBS-Tween. Primary antibodies for beclin-1, LC-3 (Cell Signaling, Danvers, MA), and GAPDH (Sigma, St. Louis, MO) were added per the manufacturers’ instructions, and secondary antibodies (anti-mouse, GE Healthcare; anti-rabbit, Santa Cruz Biotechnology, Santa Cruz, CA) were added for 1–2 h at room temperature. Proteins bands were visualized using Western Lightning reagents (Perkin Elmer, Waltham, MA) and the FluorChemE (ProteinSimple, Santa Clara, CA). The densitometry of the bands was determined using AlphaEase software (ProteinSimple) and normalized to GAPDH for loading control.

Quantitative real-time PCR. Synthesis of cDNA from isolated total RNA was performed using an RNA-to-cDNA kit (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. A starting amount of 1.0 µg of total RNA was used for cDNA synthesis, and optical density was used to determine quantity and quality of product. Quantitative real-time PCR (qRT-PCR) was done in 20-µl total reaction volume using a Stratagene MX3000P machine using a SYBR Green 2X qRT-PCR master mix (Applied Biosystems).
All reactions were performed in triplicate on each plate with a minimum of three independent experimental replicates. Gene expression values were calculated using the difference in target gene expression relative to 18S mRNA using a delta Ct method. Values are normalized to control expression relative to 18S and SE from delta Ct of target to 18S values is shown as a measure of population distribution.

Data analysis. Statistical analysis was performed by ANOVA using a single factor within-subjects design. Where significance was indicated, post hoc testing was performed using the Holm-Sidak method for comparing individual means and correcting for family-wise error (SigmaPlot v.11.0, Systat Software, San Jose, CA). Data are presented as means ± SE, and differences were regarded as significant at \( P \leq 0.05 \). Alpha values were set at 0.05 prior to experiment, and on the basis of observed data, power analysis was used to determine group size.

RESULTS

Short-term high-fat diet protects against ischemia-reperfusion injury. To investigate the role of HFD on cardioprotection, animals were put on a HFD prior to infarction. A significant decrease was observed in infarct size (normalized to area at risk) after 24 h, 1 wk, or 2 wk of feeding relative to control-fed (CFD) animals (11.36% ± 1.66, 6.98% ± 0.38, and 13.1% ± 1.68, respectively, vs. 25.78% ± 2.08, \( P \leq 0.05 \)). After 6 wk of HFD, infarct size was not significantly different from CFD animals (25.94% ± 6.52 and 25.78% ± 2.40, respectively, \( P > 0.05 \)) (Fig. 1, A and B).

To investigate the duration of this protection after discontinuation of the HFD, mice were fed high-fat chow for 24 h and then returned to a CFD for 24, 48, or 72 h to assess the potential for sustained protection against I/R injury at time points corresponding with a traditional late phase of cardioprotection (6) (Fig. 1C). When animals were removed from the HFD for 24 h, the cardioprotection was sustained, and infarct size significantly decreased relative to CFD mice (13.72% ± 1.79 vs. 26.70% ± 4.28, \( P \leq 0.05 \)). This protective effect and decreased infarct size were not apparent at 48 h and 72 h after high-fat feeding (Fig. 1D).

Influence of acute high-fat feeding on serum lipid profiles and metabolism. The association of high-fat feeding with improved patient outcomes via the obesity paradox is often associated with dyslipidemia and the development of metabolic syndrome (28). To investigate the parameters often seen with long-term high-fat feeding, serum triglycerides, free fatty acids, and cholesterol were analyzed via ELISA assay performed on serum extracted from mice at the time points relevant to the observation of the cardioprotective effect due to a high-fat diet and compared with serum isolated from CFD animals (Fig. 2). Upon 24 h of HFD, no significant change was observed in total cholesterol or circulating nonesterified free fatty acids relative to CFD animals (Fig. 2, A and B). Serum triglycerides (Fig. 2C) were elevated, as expected, but were lower compared with values reported in chronic feeding studies and not outside of the physiological range (29). Subsequent experiments defining the cardioprotective high-fat effect were focused upon the 24-h timepoint to study the mechanism of the initiation of the cardioprotective effect.

On the basis of the elevated triglyceride levels, further inquiry was made of lipid absorption and energetics. Absorption of dietary lipid was assessed via intestinal assay, and the

\[\text{Infarct Size} \times 100\% \text{ (of area at risk)}\]

Fig. 1. Acute high-fat feeding mediates cardioprotection. A: representative histological sections from triphenyltetrazolium chloride (TTC) and blue dye counterstained left ventricle (LV) tissue with control-fed diet (CFD), 24 h high-fat diet (HFD), and 6-wk HFD. B: infarct size in LV postinfarct with CFD and HFD + 24 h CFD and HFD + 24 h CFD + 24 h CFD. C: infarct size with 24-h HFD and return to CFD for 24 h, 48 h, and 72 h (n > 6, *\( P \leq 0.05 \) vs. CFD).

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nonabsorbed lipid fraction was assessed via analysis of fecal content (Fig. 2D). Duodenal sections were isolated from 24-h HFD and CFD-fed animals and demonstrated an increase in lipid accumulation in villi at 24 h (Fig. 2E). Animals were fed on a HFD for 24 h, and stool samples were collected at 3-h increments over one light-dark cycle. Lipid analysis from fecal samples indicates comparable amounts of lipid excretion from mice on CFD and HFD (Fig. 2D), confirming absorption of fat into the system.

Assessment of mice over 24 h on a HFD showed a slightly significant increase in V\textsubscript{O2}, with no change in V\textsubscript{CO2} (Fig. 3A). No changes were observed in body weight (Fig. 3B), but there was a small significant increase in energy expenditure ($P \leq 0.05$) (Fig. 3C) between the HFD and the CFD over a 24-h time period. Feeding patterns were consistent between the groups with food intake slightly higher (data not shown), but not statistically different in high-fat animals consistent with previously reported finding (1).

Effect of cardiac NF-\kappaB blockade upon infarct size. To determine whether this new phenomenon of decreased infarct injury after short-term HFD is dependent upon NF-\kappaB activity, as is seen in other forms of preconditioning that protect against I/R injury (39), we employed the 2M mouse model with cardiac specific blockade of NF-\kappaB activation (37, 43, 47, 48). Results showed that 2M transgenic animals (2M Tg) abrogated the cardioprotection after 24-h HFD [35.91% ± 6.03 (2M Tg) vs. 11.36% ± 1.66 (WT), $P \leq 0.05$], while the nontransgenic 2M-sibling control (2M nonTg) showed no difference relative to WT (Fig. 4) [16.38% ± 0.70 (2M nonTg) vs. 11.36% ± 1.66 (WT), $P > 0.05$]. Abrogation of protection was confirmed by direct comparison between 24-h HFD 2M Tg animals (35.91% ± 6.03) and 2M nonTg animals (16.38% ± 0.70; $P \leq 0.05$). Infarct data for 2M Tg sham animals on a control diet were previously described for this model by Tranter et al. (43).

High-fat-mediated cardioprotection alters autophagic and cardioprotective profiles in the myocardium. The balance between autophagy and apoptosis is important in determining cell fate (14) and appears to be modified after ischemic preconditioning (23, 35, 48). We hypothesized that HFD-induced cardioprotection was mediated, at least in part, by modifying this balance. To test this hypothesis, we measured changes in the autophagic proteins, beclin-1 and LC-3 (23), under the conditions of HFD-mediated cardioprotection in WT and 2M mice. We observed that 24 h of high-fat feeding in wild-type animals resulted in a significant increase in beclin-1 and LC-3 levels [total expression (I and II)] relative to control-fed counterparts (Fig. 5A) with mRNA levels increasing at 3 h and returned to baseline before 24 h (Fig. 5B). Levels of mRNA for HSP70.3,
which has been shown to have NF-κB-dependent cardioprotective properties in ischemic preconditioning (42, 43), were also shown to increase 5.6-fold in 24-h HFD animals relative to control. (Fig. 5D; \( P \leq 0.05 \)).

To determine the role of autophagy in high-fat-mediated cardioprotection, studies were performed with chloroquine, as described by Perry and colleagues (27, 36). Wild-type animals were injected with saline or chloroquine with control and 24 h of high-fat feeding (four groups). Animals on a high-fat diet with saline injection showed cardioprotection relative to control-fed animals with intraperitoneal injection of saline (30.40% ± 4.68 vs. 23.31% ± 4.65, \( P \leq 0.05 \)). Animals on a high-fat diet with chloroquine showed no protection relative to control-fed animals injected with chloroquine (31.32% ± 8.32 vs. 36.47 ± 5.50, \( P \leq 0.05 \)) (Fig. 6).

DISCUSSION

This study demonstrates that acute exposure to HFD (prior to injury) protects against damage from MI via an NF-κB-dependent mechanism. The initial animal studies indicated that short-term feeding prior to the injury (24 h–2 wk) significantly decreased infarct size (Fig. 1) and resulted in preservation of cardiac function (as confirmed by the preservation of ejection fraction by echocardiography) (data not shown) in wild-type HFD animals. We interpret this to mean that protection of the myocardium against infarction leads to preserved function directly related to enhanced myocardial salvage after I/R, as is seen for many forms of cardioprotection. To determine whether or not there is a direct protective effect upon function, one would have to extend ischemic times to equalize infarct size and compare function post-MI; this is beyond the scope of the present study. In our model, the protective effect of a HFD is manifest after only 24 h, maintained to 2 wk of HFD, and lost by 6 wk. Previous studies support that HFD improved cardiac function during control of ischemia-reperfusion-induced physiological dysfunction (22) and identified cardioprotective gene profile changes in animals fed an 8-wk saturated-fat diet (4, 12, 19). However, these studies failed to control for initial infarct size, and so it is unclear to what extent functional improvement was due to myocardial salvage. Furthermore, this study was performed after longer-term HFD, at timepoints where insulin resistance, obesity, and related pathophysiological processes would confuse analysis of signaling and gene expression.
To differentiate the short term from the chronic (pathological) effects of high-fat feeding, we assessed absorption of the high-fat component, as well as the changes to circulating lipids. We found that fat was, in fact, absorbed from the feces and the GI tract, with no significant changes to circulating nonesterified free fatty acids (Fig. 2A) and total serum cholesterol (Fig. 2B). Triglycerides, on the other hand, were increased, indicating uptake of fat (Fig. 2C), although, their levels were still within a physiological range that is established to be nonpathological (60–161 mg/dl) (4, 5).

Even though the changes to serum lipids were minimal, it is well established that increased caloric availability alters the cellular energy production pathways (38). We assessed for basal increases in respiration and found increased V˙O$_2$ independent of V˙CO$_2$. Basal increases in V˙O$_2$ suggest an increase in respiration, classically associated with Krebs cycle utilization of fatty acid substrates (1, 3, 40), and a thermogenic shift in energy expenditure was associated with exposure to lipid levels. This evidence suggests that the lipids are processed in the system and do not pathologically alter any homeostatic process, again distinguishing our short-term model from long-term HFD (10, 32, 45). The data above, together with the finding that the cardioprotection dissipates by 6 wk—the beginning of development of glucose resistance and weight gain in this model (40)—supports the hypothesis that the initiator of the HFD-induced cardioprotection is unlikely to be associated with the pathological phase of the chronic high-fat diet model.

That the protection is sustained after an additional 24 h on control diet (after initial 24-h HFD; Fig. 1D) supports a role for long-term signaling and/or gene expression as a potential mechanism of HFD-induced cardioprotection against MI. We hypothesized that the effect involves stimulation of gene programs associated with NF-κB, as is true for other precondi-
tioning stimuli that protect against I/R injury (44). Our data demonstrate that short-term, high-fat feeding is dependent upon NF-κB (as the effect was not seen in 2M transgenic mice; Fig. 4), not unlike results presented by Tranter et al. (43) and Zeng et al. (50), which demonstrated a similar dependency of preconditioning upon this pathway. As a side note, we observed that the infarct size after 24 h HFD was significantly larger in 2M transgenic mice relative to WT (C57BL/6j) mice on CFD, a fact that may be related to the genetic background of our 2M model, which was obtained by backcrossing the transgene from SviI/129 into the C57 background (43); thus, these mice represent a unique background that may not be exactly the same as the wild type. However, we note that whereas the 24-h HFD results in a 55% decrease in infarct size in wild-type animals (cardioprotection), abrogation of NF-κB results in a 54% increase in infarct size compared with non-transgenic littermates fed a 24-h HFD. Thus, the extent of cardioprotection in WT mice is similar to the extent of infarct increase in 2M transgenic relative to WT mice on the HFD. Because we have already shown that 2M transgenic mice on CFD diets have very small infarcts, due to antithetical proinjury signaling of NF-κB without a prior protective stimulus [Tranter et al. (43)], we did not include this group, but focused upon the comparison discussed above, as we previously did for ischemic preconditioning (IPC) (8, 43). We conclude that NF-κB is necessary for high-fat diet-induced cardioprotection.

On the basis of the NF-κB dependence observed in high-fat-mediated cardioprotection and IPC, we hypothesized that regulation of cell survival and autophagic pathways, known to require NF-κB and to be active in IPC, may be involved. In IPC, beclin-1 is implicated in the inhibition of excessive autophagy in the reperfusion phase and cooperates with antiapoptotic pathways to diminish the cell death induced by I/R injury (35, 49). Beclin-1 is a protein that is integral to the formation of the autophagosome and signifies initiation, while LC-3 is cleaved on the external part of the autophagosome and signifies activation of autophagy (23). Thus, taken together, these two markers were used to determine the activation and progression of autophagy. In this study, we confirmed that autophagy is initiated with an increase in beclin-1 mRNA levels within 3 h of high-fat feeding and at the protein level within 24 h. Moreover, the increases in beclin-1 and LC-3 protein levels are NF-κB-dependent. Along with a rise in LC-3 protein levels after 24 h of HFD, these increases in expression indicate that acute HFD triggers NF-κB-dependent autophagy. These data and similar results in studies of late preconditioning (24, 35, 48, 50) support the concept that “autophagic priming” of the autophagic machinery serves to reduce injury after I/R.

To determine the role of autophagy in high-fat-mediated cardioprotection, experiments with a known autophagic inhibitor were employed. Chloroquine is a lysosomal inhibitor used in assays to inhibit autophagy (25, 36). In this study, mice injected with chloroquine, 22 h into a 24-h HFD showed no decrease in infarct size relative to saline-injected controls. We concluded that inhibition of autophagic lysosomal activity abrogates cardioprotection (27). Taken with the NF-κB-dependent priming effect described by the protein elevation of Beclin-1 and LC-3, we concluded that high-fat-mediated protection is dependent, at least in part, upon initiation of the autophagic process.

In conclusion, the findings of this study show that short-term, high-fat feeding initiates a cardioprotective effect against I/R injury. This effect is absent in long-term (6 wk) feeding. The 24-h HFD, which produces a 50% infarct reduction, is shown to be independent of pathological changes in serum lipid levels, lipid absorption, or metabolic energy parameters. The protection is shown to be dependent on activity of NF-κB in cardiomyocytes. The increase in autophagy is NF-κB-dependent, and likely includes direct regulation of beclin-1 mRNA and protein levels; evidence that beclin-1 is NF-κB-regulated has been previously published (22). This suggests that the increase in autophagy primes the heart, such that apoptosis is reduced upon infarction, as supported in models of preconditioning (26, 41, 50). Further studies are necessary to delineate the detailed mechanism of how an acute high-fat diet activates NF-κB and NF-κB-dependent gene regulation, leading to initiation of cardioprotection.

This work opens a new perspective on the acute effects of a high-fat diet. Future work will determine whether these effects are linked to the obesity paradox and whether studying the mechanism can identify therapeutic targets for cardioprotection. Furthermore, understanding the mechanism of acute high-fat diet-mediated cardioprotection will allow us to determine why this effect diminishes coincident with the onset of glucose resistance in this model. Does a similar mechanism affect endogenous cardioprotective pathways in the obese? Given the increasing numbers of obese and metabolic syndrome diagnoses in established and developing countries, understanding the relationship between fat intake and myocardial health is critically important.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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