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Altered cellular membrane fluidity levels and lipid peroxidation during experimental pancreas transplantation

F. A. García-Gil · C. D. Albendea · L. López-Pingarrón · P. Royo-Dachary · J. Martínez-Guillén · E. Piedrafita · M. Martínez-Díez · J. Soria · J. J. García

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Abstract Although the pathogenesis of ischemia reperfusion (IR) injury is based on complex mechanisms, free radicals play a central role. We evaluated membrane fluidity and lipid peroxidation during pancreas transplantation (PT) performed in 12 pigs (six donors and six recipients). Fluidity was measured by fluorescence spectroscopy, and malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) concentrations were used as an index of lipid oxidation. Pancreatic tissues were collected as follows: (A) donor, immediately before vascular clamping; (B) graft, following perfusion lavage with University of Wisconsin preservation fluid; (C) graft, after 16 h of cold ischemia; and (D) recipient, 30 min vascular postreperfusion. Fluidity and MDA and 4-HDA concentrations were similar in cases A, B, and C. However, there was significant membrane rigidity and increased lipid peroxidation after reperfusion (D). These findings suggest that reperfusion exaggerates oxidative damage and may account for the rigidity in the membranes of allografts during PT.

Keywords Membrane fluidity · Lipid peroxidation · Oxidative stress · Ischemia reperfusion · Pancreas transplantation

F. A. García-Gil · P. Royo-Dachary · J. Martínez-Guillén · M. Martínez-Díez
Department of Surgery, Gynaecology and Obstetrics, University of Zaragoza, Zaragoza, Spain

C. D. Albendea · E. Piedrafita · J. J. García
Department of Pharmacology and Physiology, University of Zaragoza, Zaragoza, Spain

L. López-Pingarrón
Department of Human Anatomy and Histology, University of Zaragoza, Zaragoza, Spain

J. Soria
Department of Pathological Anatomy, University of Zaragoza, Zaragoza, Spain

J. J. García (✉)
Departamento de Farmacología y Fisiología, Facultad de Medicina de Zaragoza, C/ Domingo Miral s/n, 50009 Zaragoza, Spain
e-mail: jjgarcia@unizar.es

Introduction

Biological membranes are a two-dimensional fluid of orientated lipids and proteins. According to the fluid-mosaic model proposed by Singer and Nicolson (1972), phospholipids form a fluid bilayer where they are free to diffuse laterally. The fluidity of the membrane is influenced by the concentrations of unsaturated and polyunsaturated fatty acids (PUFA) as well as cholesterol. There is considerable evidence that damage to PUFAs tends to reduce membrane fluidity, which is essential for the proper functioning of cell membranes. Thus, measurements of the membrane fluidity reflect the biophysical and biochemical characteristics of the biological membranes and are an indicator of membrane function (Li et al. 1999).

Insulin-dependent diabetes mellitus results in several complications, including neuropathy, small-vessel disease, and renal disease. In up to 70 % of diabetic patients, these complications can be prevented by insulin treatment (Mayer et al. 1999), although it is impossible to avoid complications in all patients. For patients whose diabetes is not well controlled by insulin treatment, pancreas transplantation (PT) should be regarded as a viable therapeutic modality, especially in patients with end-stage renal disease (Sollinger et al. 1988; Schulak et al. 1990; Sutherland 1997; Patil and Yerian 2010).

Despite significant improvements in PT outcomes over the past three decades (Patil and Yerian 2010), graft pancreatitis is still a major postoperative problem for these patients; it can occur in an edematous form, potentially leading to peritonitis, intraabdominal abscess formation, and, finally, graft failure (Drognitz et al. 2009). Although the pathophysiology of graft pancreatitis is not fully understood, ischemia reperfusion (IR) appears to be the main pathogenic factor (Büsing et al. 1993; Fernández-Cruz et al. 1993; Benz et al. 2002; Witzigmann et al. 2003; Drognitz et al. 2009).

Experimental animal models of warm IR (arterial occlusion and subsequent reperfusion) are useful in the study of the pathogenesis of pancreatitis. Although many models using warm ischemia were primarily designed to address problems involved in organ transplantation, it is evident that cold and warm ischemia may act differently from one another. Moreover, warm IR models reproduce few experimental paradigms, which may limit the conclusions obtained from these models compared to those obtained in the wider experimental context of organ transplantation. Further, the use of experimental PT in a large animal, such as a pig, has advantages over PT in smaller species, such as rodents, due to the closer resemblance to human physiology and pathophysiology. This allows for better clinical applications of the results (Lima-Rodríguez et al. 2008).

The plasma membrane is one of the main targets of free radicals, and alterations in membrane fluidity affect a number of cellular functions, including carrier-mediated transport, the activity of membrane-associated enzymes, cell growth, and hormone-induced signal transduction processes (Curtis et al. 1984). Hence, we used an experimental model of PT with enteric drainage of exocrine secretions and systemic venous drainage in pigs. Our aim was to analyze the impact of IR on the fluidity of cell membranes isolated from the pancreas, estimated by changes in the fluorescence polarization of 1–(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH). Since membrane fluidity essentially depends on lipid interactions, we quantified the concentrations of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA), biochemical parameters that offer an overview of the oxidative damage that IR can exert on pancreatic grafts.

Material and methods

Animals

We used 12 female Landrace pigs (six donors and six recipients) with an average weight of 40±5 kg. The pigs underwent allogenic PT with enteric drainage of exocrine secretions and systemic venous drainage. The animals were housed in the *Unidad de Investigación Mixta* of the University of Zaragoza, Spain. All animal care and experiments followed the international guidelines for the use of laboratory animals (Directive 86/609/EEC). The experimental protocol was approved by the University of Zaragoza Ethics Committee for Animal Research (PI036/09).

Experimental procedures

Prior to the surgical procedures, the pigs were fasted for 12 h with free access to water. Anesthesia and monitoring were performed as previously described by Lima-Rodríguez et al. (2008). From the donor animals, we prepared pancreatoduodenal grafts that included the whole pancreas; a segment of the duodenum that contained the pancreatic duct opening; a patch of aorta that included the celiac trunk and the superior mesenteric artery; and the venous drainage, consisting of the superior mesenteric, splenic, and portal veins. An *ex situ* perfusion lavage of the graft was performed through the celiac trunk and the superior mesenteric artery with 100–150 mL of cold University of Wisconsin (UW) preservation solution (Viaspan; Bristol-Myers Squibb SL, Madrid, Spain) at a pressure of 100 cm of water, until the portal drainage was clear. After organ recovery, the grafts were stored at 4 °C for 16 h in conventional bags that contained UW solution.

Once the recipient pig was anesthetized, a central venous catheter (Certofix; B Braun, Melsungen, Germany) was inserted into the external jugular vein and then subcutaneously tunneled to the back of the neck. This venous access was maintained throughout the postoperative follow-up period. Next, via median laparotomy, the pancreatoduodenal allograft was implanted. First, the aortic patch and portal vein were anastomosed to the recipient's infrarenal aorta and vena cava, and then the graft duodenum was anastomosed laterolateral to the ileum of the recipient, approximately 40 cm from the ileocecal valve. Prior to unclamping, 5,000 units of sodium heparin were administered intravenously. Finally, a total pancreatectomy, preserving the duodenum and its vasculature, was performed in the recipient to ensure that the allogenic pancreas graft was the only insulin source. The warm ischemia time ranged from 35 to 55 min, from the time the graft was removed from cold storage until the time of reperfusion.

Tissue samples (500 mg) were collected from the left pancreatic lobe as follows: (A) in the donor, immediately prior to vascular clamping; (B) in the graft, following the *ex situ* perfusion lavage (0 h cold ischemia); (C) in the graft, after 16 h of cold storage; and (D) in the recipient, 30 min after vascular reperfusion. In these samples, we monitored membrane fluidity levels and MDA and 4-HDA concentrations, regarded as an index of the oxidative breakdown of lipids (Janero 1990).

After transplantation, the animals were transferred to individual cages with regulated temperature and natural light. To continue analgesia, a buprenorphine transdermal patch

(35 μg/h, Transtec; Grünenthal, Mexico City, Mexico) was applied in the posterior cervical region. On postoperative day 1, the animals were allowed access to water and received 1,500 mL of Ringer's lactate solution intravenously. On day 2, access to standard commercial chow was allowed. On postoperative days 1–7, we administered 2 g/day cefoxitine intravenously. No immunosuppression was administered.

Blood samples (3 mL) were drawn from the external jugular vein under basal conditions each day following PT to measure serum concentrations of glucose. The grafts were considered functional when normoglycemia was detected for at least the first five postoperative days. When glycemia exceeded 150 mg/dL on two consecutive days, the recipients were anesthetized, the pancreatoduodenal allograft was explored via laparotomy, and samples were taken for histological analysis to diagnose and grade acute rejection according to the criteria of Drachenberg et al. (2008). The animals were sacrificed while under anesthesia.

Analytical procedures

All of the chemicals and solvents, of the highest grade available, were acquired from Sigma (Madrid, Spain). TMA-DPH was obtained from Molecular Probes (Eugene, OR, USA).

Pancreatic cellular membranes were isolated according to Meldolesi et al. (1971) with minor modifications. Briefly, the pancreas was homogenized 1:10 (w/v) in 140 mM KCl/20 mM HEPES buffer (pH 7.4) and then centrifuged at 1,000 × *g* for 10 min at 4 °C. The supernatant was centrifuged at 30,000 × *g* for 20 min at 4 °C. The pellet was resuspended in the same buffer and centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant and the buffy coat were homogenized and centrifuged at 50,000 × *g* for 20 min at 4 °C. The final pellet was resuspended 1:1 (w/v) in 50 mM Tris buffer (pH 7.4) and stored at −80 °C until assay.

Fluidity was monitored in triplicate determinations using TMA-DPH as a fluorescent probe. TMA-DPH incorporation into the membrane and the determination of membrane fluidity were carried out according to methods described in the existing literature (Yu et al. 1992). Pancreatic cellular membranes (0.5 mg protein/mL) were resuspended in 50 mM Tris (3 mL final volume) and mixed with TMA-DPH (66.7 nM). After stirring vigorously for 1 min, the preparation was incubated for 30 min at 37 °C. Fluorescence measurements were performed in a Perkin-Elmer LS-55 luminescence spectrometer equipped with a circulatory water bath to maintain the temperature at 22±0.1 °C. Excitation and emission wavelengths of 360 and 430 nm, respectively, were used. The emission intensity of vertically polarized light was detected by an analyzer oriented parallel (Iv_V) or perpendicular (Iv_H) to the excitation plane. A correction factor for the optical system, G, was used. Polarization (P) was calculated by the equation:

$$P = \frac{I_{V_V} - GI_{V_H}}{I_{V_V} + GI_{V_H}}$$

An inverse relationship exists between membrane fluidity and polarization (Yu et al. 1992). Thus, membrane fluidity is expressed as 1/P. Protein concentration was determined by the Bradford method using bovine serum albumin as the standard (Bradford 1976).

The products of lipid peroxidation, MDA + 4-HDA, were measured in the homogenates. Briefly, tissues were homogenized in 50 mM ice-cold Tris–HCl (pH 7.4) buffer and the suspensions (200 μL) were mixed with 650 μL of a methanol:acetonitrile (1:3, v:v) solution containing N-methyl-2-phenylindole. After adding methanesulfonic acid (150 μL), incubation was carried out at 45 °C for 40 min. The MDA + 4-HDA concentrations were measured with a spectrophotometer at 586 nm using 4-hydroxy-nonenal as a standard. The level of lipid peroxidation in the homogenates was expressed as mmol of MDA + 4-HDA per mg of protein.

Blood samples were centrifuged at 1,000 × *g* for 10 min in a Beckman Allegra 64R refrigerated centrifuge (Fullerton, CA, USA). Glucose concentrations in the sera were determined by hexokinase enzyme analysis.

For the histopathological examination, fragments of pancreas were fixed in 40 g/L formaldehyde buffered with phosphate solution (0.1 M, pH 7.4) at room temperature, washed in phosphate buffer, and dehydrated in graded concentrations of ethanol (70 %, 80 %, 90 %, and 100 %). The fragments were then embedded in paraffin and subsequently sectioned (thickness, 3 μm) and stained with hematoxylin and eosin for conventional optical microscopy with an Olympus BX51 light microscope.

Statistical analysis

Data are expressed as arithmetic mean and standard error (SE) values. Mean differences were determined using a paired *t*-test, with the level of significance set to $p \leq 0.05$.

Results

To determine the survival of endocrine function in the allograft, we measured blood glucose concentrations in the recipients. Immediately after transplantation, the six grafts functioned normally. On postoperative days 1–5, glucose concentrations tended to be even lower than basal levels, followed by an insidious and progressive increase in glycemia, which reached diabetic status by days 8–11 (10.3±2.7 post-operative days). Figure 1 illustrates changes in the

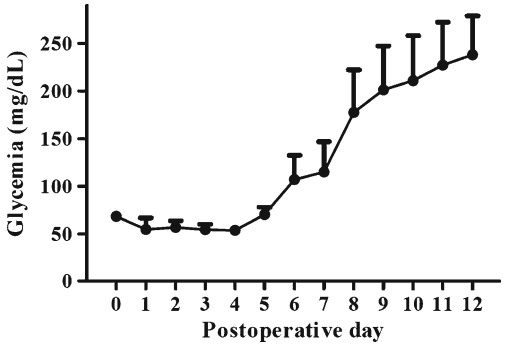

Fig. 1 Blood glucose concentrations. Six pigs received pancreas transplantations after 16 h of cold preservation and no immunosuppression. Glycemia was determined daily, beginning immediately prior to transplantation (day 0), and was continued for 12 days. Data are expressed as mean values ± SE

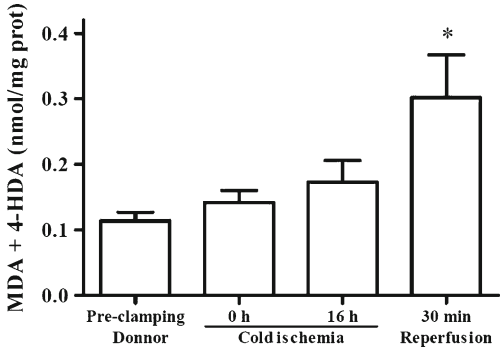

Fig. 3 Effects of ischemia reperfusion during pancreas transplantation on malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) concentrations, biomarkers of lipid peroxidation in pancreatic allografts. Maximum levels were detected in pancreas homogenates after 30 min of vascular reperfusion in the recipients. Data are expressed as mean values ± SE (n=6 pancreas transplantations). *Denotes statistical differences ($p \leq 0.05$ versus preclamping or cold ischemia)

glucose concentrations after surgery. Acute rejection was confirmed by histopathological examination. All allografts showed evidence of acute cellular rejection, mostly severe (Banff grade III) with areas of moderate rejection (grade II).

The effects of IR on membrane fluidity during PT were assessed by measurement of the fluorescence polarization of TMA-DPH. The fluidity of the regions of cell membranes in which the fluorescent probe is situated affects its rate of dynamic movements including rotation, vibration, and translation within the membrane lipid bilayer. The fluidity of cell membranes isolated from the pancreatic allografts was similar at baseline in the donor, after perfusion lavage with the UW preservation solution, and after 16 h of cold storage, but showed a significant decrease after 30 min of vascular reperfusion (Fig. 2).

Oxidative stress in the pancreatic homogenates obtained from these biopsies was monitored by measuring MDA and 4-HDA concentrations. Figure 3 shows their evolution over the course of the PT. At the beginning and after 16 h of cold ischemia,

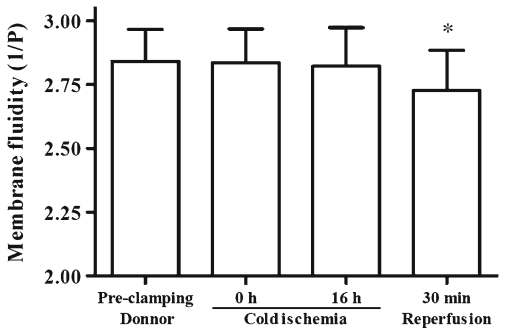

Fig. 2 Membrane fluidity levels in pancreatic biopsies. Samples were taken at baseline in the donor, immediately prior to vascular clamping, following the *ex situ* perfusion lavage of the graft with cold University of Wisconsin preservation solution (0 h, cold ischemia), after 16 h of cold storage, and, in the recipient, 30 min after vascular reperfusion. Data are expressed as mean values ± SE (n=6 pancreas transplantations). *Denotes statistical differences ($p \leq 0.05$ versus preclamping or cold ischemia)

MDA and 4-HDA concentrations in the pancreatic homogenates underwent a slight increase, although without significant differences in cases A, B, and C. However, during reperfusion, MDA and 4-HDA levels were triple those of the levels reached in the donors, indicating that vascular reperfusion had induced intense lipid peroxidation in the pancreatic allografts.

Discussion

Ischemia followed by reperfusion, which is inherent to the conditions prevailing in organ transplantation, is associated with numerous pathophysiological events, including capillary perfusion failure with subsequent endothelial damage and the release of certain proinflammatory mediators and transcription factors (Menger et al. 1992; Kurose et al. 1994; Vollmar et al. 1999; Mayer et al. 1999; Casillas-Ramírez et al. 2006). Early response to IR includes ATP degradation and glycolysis, leading to lactate production, acidosis, and calcium release from intracellular stores (Zhou et al. 2006).

Numerous experimental studies have reported exacerbated oxidative stress and lipid peroxidation as a consequence of IR. The precise mechanisms underlying the generation of free radicals in IR are not yet fully understood. Although experiments with allopurinol, an inhibitor of xanthine/xanthine oxidase, suggest that this system is the main free radical generator (Hamer et al. 1995), there are potential alternative sources of free radicals, such as defects in the electron transfer chain in mitochondria (Jaeschke and Mitchell 1989; Plin et al. 2004) and an elevated respiratory burst from phagocytes, which infiltrate the ischemic tissue due to chemotaxis (Caimi et al. 2000; Cutrin et al. 2002; Jaeschke 2003). However, most of these reports include in vivo occlusion of organ vascular support followed by declamping and organ reperfusion, thus reproducing warm ischemia.

Under the experimental conditions of our PT model, we observed that during the cold ischemia period, there were no significant modifications in the pancreatic levels of MDA and 4-HDA with respect to baseline levels. During cold ischemia, the tissue became hypoxic when stored at a low temperature in an organ preservation fluid. The purpose of this hypothermia is to decrease the enzymatic activity and the mitochondrial metabolic rate 12–13-fold from 37 to 0 °C (Belzer and Southard 1988). However, cooling alone does not allow for effective graft preservation, since it does not completely halt metabolism (Blankensteijn and Terpstra 1991). Consequently, antioxidants are frequently added to organ preservation fluids. We used UW fluid to preserve the pancreatic allografts for 16 h of cold ischemia, because it is the standard solution for the preservation of most organs in transplantation, including pancreas (Belzer and Southard 1988; Vajdova et al. 2002; Busuttil and Tanaka 2003; Southard 2004; García-Gil et al. 2011). UW preservation fluid contains millimolar levels of the antioxidants glutathione and allopurinol, lactobionic acid, a weak calcium and iron chelator, and, finally, adenosine, which may suppress free radical production by phagocytes (Sumimoto et al. 1996). Thus, the absence of lipid peroxidation during cold ischemia could be attributed to the additive effects of cooling the allograft and the presence of high concentrations of antioxidants in the UW solution.

Our data reveal a clear increase in MDA and 4-HDA concentrations in the pancreatic allografts 30 min after vascular declamping compared to preischemia and cold ischemia levels. We regard this process as beneficial, since reperfusion of the allograft with blood allows oxygen and nutrients to be reintroduced. However, there is a consensus that reintroduction of oxygen into ischemic tissue can result in an additional insult that is mediated by free radicals (Halliwel and Gutteridge 2007; Zaouali et al. 2010). The severity of the reperfusion injury depends on the ischemia time (Irazu et al. 1990) and becomes worse if the allograft contains tiny dead or dying tissue areas capable of releasing toxic agents, such as xanthine oxidase and transition-metal ions, both locally and systemically (Halliwel and Gutteridge 2007).

The endocrine pancreas is particularly vulnerable to the deleterious effects of free radicals, because it receives a higher flow of blood and oxygen compared to other tissues (Kubo et al. 1991), and because endocrine cells lack antioxidant defenses (Tiedge et al. 1997). Even so, very few reports have been published on pancreatic IR injury. Jaworek et al. (2003) reported that in rats, after clamping the inferior splenic artery for 30 min followed by 2 h of reperfusion, levels of lipid peroxidation products such as MDA were increased several-fold in the pancreas. Muñoz-Casares et al. (2006) showed that obstruction of the gastroduodenal and inferior splenic arteries induced pancreatic IR and impaired pancreatic function in male rats. Both pancreatic oxidative injuries due to IR were completely prevented by the antioxidant melatonin. Furthermore, according to a recent report by Khoury et al. (2010), who investigated whether pancreas IR induces remote organ damage using a model of isolated organs, oxidative stress due to pancreas IR promotes acute renal dysfunction, attenuated by treatment with mannitol.

Our observations of lipid peroxidation during PT are consistent with a previous study in which we showed that lipid peroxidation was only present during reperfusion (García-Gil et al. 2006). Moreover, in a separate study using the same experimental PT model, we treated both donors and recipients with melatonin as a free radical scavenger. In animals given melatonin, post-transplant normoglycemia was maintained three times longer than in animals in the control group not given melatonin; in addition, melatonin reduced accumulated levels of MDA and 4-HDA during reperfusion in the pancreatic allograft homogenates (García-Gil et al. 2011).

Membranes are known to be dynamic structures, due in large part to the fluidity of their lipids. In numerous diseases, disturbances in the levels of membrane fluidity of several organs have been implicated in aging (Reiter et al. 1999; García et al. 2011) in human patients and in animal models (Grimm et al. 2006; Clement et al. 2010; Miana-Mena et al. 2011). Among these disturbances, erythrocyte membranes from cyclosporine-treated renal transplant patients revealed increased membrane fluidity compared to healthy normal controls, which was regarded as a risk for the development of post-transplant hemolytic uremic syndrome (Vareesangthip et al. 2001). However, it is highly likely that the rigidifying effect in the erythrocyte membranes is a specific side effect of the cyclosporine treatment, because in patients treated with tacrolimus, there was no significant difference in the erythrocyte membrane fluidity compared to controls (Wrobel et al. 2003).

To our knowledge, this is the first report providing evidence that injury due to vascular reperfusion in allografts is characterized by a reduction in the fluidity of pancreatic cell membranes. The close correlation between the lipid peroxidation levels in the homogenates and the rigidity of cell membranes isolated from these pancreatic grafts suggests a possible cause/effect relationship between the two phenomena.

Descriptions have been published previously of how temporary warm ischemia causes oxidative stress and ultrastructural changes in rat liver plasma membranes (Frederiks et al. 1984), as well as loss of numerous cell functions associated with the membrane (Molitoris and Kinne 1987; Shin et al. 1989; Jourd'heuil and Meddings 2001; Benkoel et al. 2004). Lipid peroxidation is the expression of free radical damage in cell membranes. Numerous studies have reported decreases in membrane fluidity secondary to lipid peroxidation caused by oxidative damage (Irazu et al. 1990; Yu et al. 1992; García et al. 1997). These data are consistent with our results, since we observed rigidity in the pancreatic allografts when lipid

peroxidation reached the highest levels. Two structural mechanisms are suggested as the molecular causes of this rigidity. The first possible mechanism is a decrease in the polyunsaturated/saturated fatty acid ratio in the membrane, which could be related to the greater susceptibility of PUFA in membrane phospholipids to free radicals (Rice-Evans and Burdon 1993). The second possible mechanism is oxidative stress, which can induce the formation of cross-linking of lipid–lipid and lipid–protein moieties (Chen and Yu 1994).

In conclusion, the data reported here provide evidence that vascular reperfusion during PT reduces membrane fluidity and exaggerates oxidative stress in pancreatic allografts. Since lipid peroxidation is one of the main factors controlling membrane fluidity, this increased oxidative stress may account for the rigidity in membranes isolated from pancreatic allografts.

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Conflicts of interest The authors have no conflicts of interest to declare.

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